ELSEVIER

European Journal of Pharmacology 312 (1996) 257–261

ejp

The human neuroblastoma cell line, IMR-32, expresses functional corticotropin-releasing factor receptors

Joanne E. Hogg *, Jan Myers, Peter H. Hutson

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR, UK

Received 14 March 1996; revised 13 June 1996; accepted 14 June 1996

Abstract

Corticotropin-releasing factor (CRF) receptors in IMR-32 human neuroblastoma cells were characterized after differentiation with 2.5 μM 5′-bromo-2′-deoxyuridine for 10 days. Scatchard analysis of [^{125}I-Tyr^{0}]ovine CRF binding revealed a high affinity binding site with a dissociation constant of 0.59 nM and a maximum binding capacity of 142 fmol/mg, the affinity of which was decreased by guanosine 5′-*o*-(3-thiotriphosphate). This binding was displaced in the following order of potency: human/rat CRF > ovine CRF > urotensin I > sauvagine > bovine CRF > [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) > α-helical CRF-(9–41), indicative of the CRF$_1$ receptor subtype. Functional coupling of this receptor was confirmed by CRF-induced increases in cyclic AMP, which were antagonised by α-helical CRF-(9–41) and [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41).

Keywords: CRF receptor; IMR-32 human neuroblastoma cell line; Differentiation; Radioligand binding; Adenylate cyclase

1. Introduction

The 41-amino acid hypothalamic hormone, corticotropin-releasing factor (CRF, Vale et al., 1981), activates the pituitary-adrenal axis to release adrenocorticotropin but also has diverse effects on the reproductive, cardiovascular, gastrointestinal, immune and nervous systems (for a review, see Owens and Nemeroff, 1991). CRF has a broad distribution in the central nervous system and has been attributed a neuromodulatory role in the mediation and coordination of behavioural, physiological and neuroendocrine responses to stress, as well as being implicated in stress-related disorders such as depression and anxiety (for a review, see Dunn and Berridge, 1990).

Recent cloning studies have identified two subtypes of the CRF receptor in the central nervous system and periphery, which belong to the seven transmembrane domain, G protein-coupled receptor family, including, amongst others, secretin and calcitonin. The CRF$_1$ receptor (Chen et al., 1993; Vita et al., 1993) is expressed primarily in pituitary and brain, particularly cerebellum, cerebral cortex and olfactory bulb (Chalmers et al., 1995; De Souza, 1987; for a review, see De Souza and Kuhar, 1986), while the CRF$_2$ receptor has a distinct pharmacology and distribution, being expressed mainly in heart and lung, as well as in brain, and having a higher affinity for the structurally and functionally related peptides, sauvagine (frog) and urotensin I (fish) than for the native ligand, CRF (Lovenberg et al., 1995b; Stenzel et al., 1995). The CRF$_2$ receptor exists in two alternatively spliced forms, CRF$_{2\alpha}$ and CRF$_{2\beta}$, which also exhibit differential distributions, conceivably with distinct functions, the CRF$_{2\beta}$ subtype being the peripheral receptor (Lovenberg et al., 1995a).

Functional CRF receptors have been described in various tissues including cell lines arising from the pituitary gland and peripheral organs such as lung (e.g. De Souza, 1987; Moody et al., 1994; Vita et al., 1993). The present study describes the pharmacological characterization of functionally coupled CRF receptors in a human neuronal cell line, IMR-32.

2. Materials and methods

2.1. Cell preparation

IMR-32 human neuroblastoma cells (American Type Culture Collection) were grown to confluency in Dulbecco's Modified Eagles Medium containing 2% L-glutamine, 1% penicillin/streptomycin solution (Hyclone), 10%

* Corresponding author. Tel.: 44 279 440454; fax: 44 279 440712.

PII S0014-2999(96)00470-0

foetal calf serum and 1% Minimun Essential Medium amino acid solution (Gibco) in 800 ml flasks incubated with 5% CO_2 at 37°C. Differentiation of IMR-32 cells, manifested by neurite growth, was achieved by the addition of 2.5 μM 5′-bromo-2′-deoxyuridine (Sigma) to the culture medium for 10 days, replenished every 3 days (Sher et al., 1989), after which time cells were harvested by scraping and centrifugation.

Preparation of cell membranes involved two homogenisation (30 volumes of 5 mM Tris HCl, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.2) and centrifugation (48 000 × *g* for 20 min at 4°C) steps, in between which the cell suspension was left on ice for 30 min to aid lysis. Final homogenisation was in 30 volumes of 50 mM Tris HCl, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.2.

2.2. Radioligand binding

[^{125}I-Tyr^0]ovine CRF (specific activity 2200 Ci/mmol, DuPont NEN) receptor binding to IMR-32 cell membranes was performed in 500 μl 50 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 2 mM EGTA, 0.2% bovine serum albumin, 0.1 mM bacitracin, 100 kIU/ml aprotinin (n.b. 1 trypsin inhibitor unit/TIU ≈ 1300 kallikrein inhibitor units/kIU) (Sigma) with approximately 100 μg of membrane protein (De Souza, 1987), assessed using bovine serum albumin as standard (Bio-Rad assay). Radioligand binding was terminated after 2 h at room temperature by rapid filtration through GFC filters (Whatman), presoaked for at least one week in 1% polyethylenimine (Sigma), with three 5 ml washes of cold buffer, followed by 1272 CliniGamma counting (Wallac). Non-specific binding was defined using a final concentration of 2.5 μM ovine CRF (Peninsula).

Saturation of [^{125}I-Tyr^0]ovine CRF receptor binding to IMR-32 cell membranes was investigated at eight concentrations of radioligand in triplicate (0.01–1 nM, $n = 3$) in the presence and absence of the stable GTP analogue, guanosine 5′-*o*-(3-thiotriphosphate) (GTP-γ-S, Sigma) at a concentration of 100 μM.

Eight point displacement curves (0.1–1000 nM, $n = 3$–4) were performed at 0.05 nM radioligand in duplicate for the analogous species variants of CRF, ovine CRF, bovine CRF and human/rat CRF (Peninsula), the CRF-related non-mammalian peptides, sauvagine (Peninsula) and urotensin I (Bachem), and the CRF receptor antagonists, α-helical CRF-(9–41) (Peninsula) and [D-Phe^{12}, Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) (Bachem) (Curtis et al., 1994). A selection of peptides (human), the receptors for which belong to the same family as the CRF receptor, were also examined at a concentration of 1 μM only: thyrocalcitonin (calcitonin), growth hormone releasing factor (GRF), secretin, vasoactive intestinal peptide (VIP), glucagon (Sigma) and alpha calcitonin gene-related peptide (CGRPα, custom synthesis at Cambridge University). All peptides were reconstituted in 0.2% bovine serum albumin and stored in aliquots at −70°C.

2.3. Functional studies

Adenylate cyclase activity was determined by measuring cyclic AMP (cAMP) concentrations in fresh IMR-32 cell membranes (approximately 5 μg protein) in 250 μl of 50 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 2 mM EGTA, 0.2% bovine serum albumin, 0.1 mM bacitracin, 100 kIU/ml aprotinin, 1 mM isobutyl-1-methyl xanthine, 0.5 mM ATP, 1 μM GTP, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase (Sigma). The accumulation of cAMP was terminated after incubation at 37°C for 10 min by immersion in boiling water for 30 s. Samples were diluted by 100 and acetylated before measurement of cAMP concentration (pmol/mg) in quadruplicate using the Amersham [^{125}I]cAMP scintillation proximity assay kit.

The CRF receptor agonists, ovine CRF, bovine CRF, human/rat CRF, sauvagine and urotensin I were assessed in the cAMP assay at a single concentration of 1 μM, while the putative antagonists, α-helical CRF-(9–41) and [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41), were tested at 100 μM ($n = 3$). Both antagonists were also assayed at the same concentration in the presence of either ovine CRF or sauvagine at 1 μM.

2.4. Data analysis

Saturation binding data was subjected to Scatchard analysis to obtain values for K_D (dissociation constant) and B_{max} (maximum binding capacity per mg of protein). Binding inhibition data was analysed using the dose response curve analysis software provided by RS1 (BBN Corporation), the best fit being determined by partial *F*-test and affinity being expressed as K_i values (calculated from the IC_{50} using the Cheng-Prusoff equation, Cheng and Prusoff, 1973). All values quoted are arithmetic mean ± S.E.M., except K_i and K_D values, which are quoted as

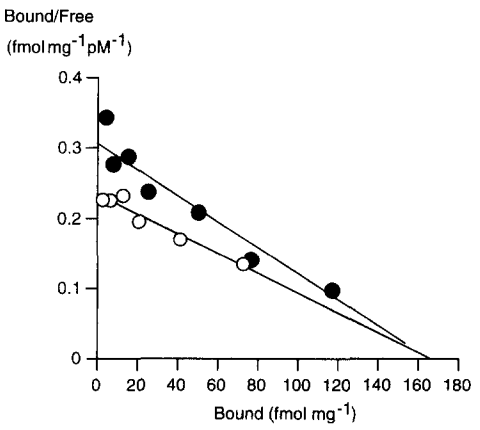

Fig. 1. Scatchard analysis of [^{125}I-Tyr^0]ovine CRF binding to IMR-32 cell membranes in the absence (●, correlation coefficient, $r = 0.96$) and presence (○, $r = 0.97$) of 100 μM GTP-γ-S (data shown are from a typical experiment performed in triplicate).

geometric mean (mean − S.E.M., mean + S.E.M.). Statistical significance, where quoted, was determined using Student's t-test (1-tailed) at $P < 0.05$.

3. Results

Specific binding of [^{125}I-Tyr^{0}]ovine CRF to IMR-32 cell membranes represented 32.5 ± 1.3% ($n = 11$) of total binding at approximately 0.05 nM radioligand. Scatchard analysis of the saturation binding data generated linear plots, indicating the presence of a single high affinity binding site ($K_D = 0.59$ (0.50, 0.69) nM, $B_{max} = 142 \pm 22$ fmol/mg, $n = 3$), the affinity of which was significantly decreased ($P < 0.05$) in the presence of 100 μM GTP-γ-S, while the maximum number of binding sites was unchanged ($K_D = 0.94$ (0.81, 1.09) nM, $B_{max} = 167 \pm 6$ fmol/mg, $n = 3$). A representative experiment is illustrated in Fig. 1.

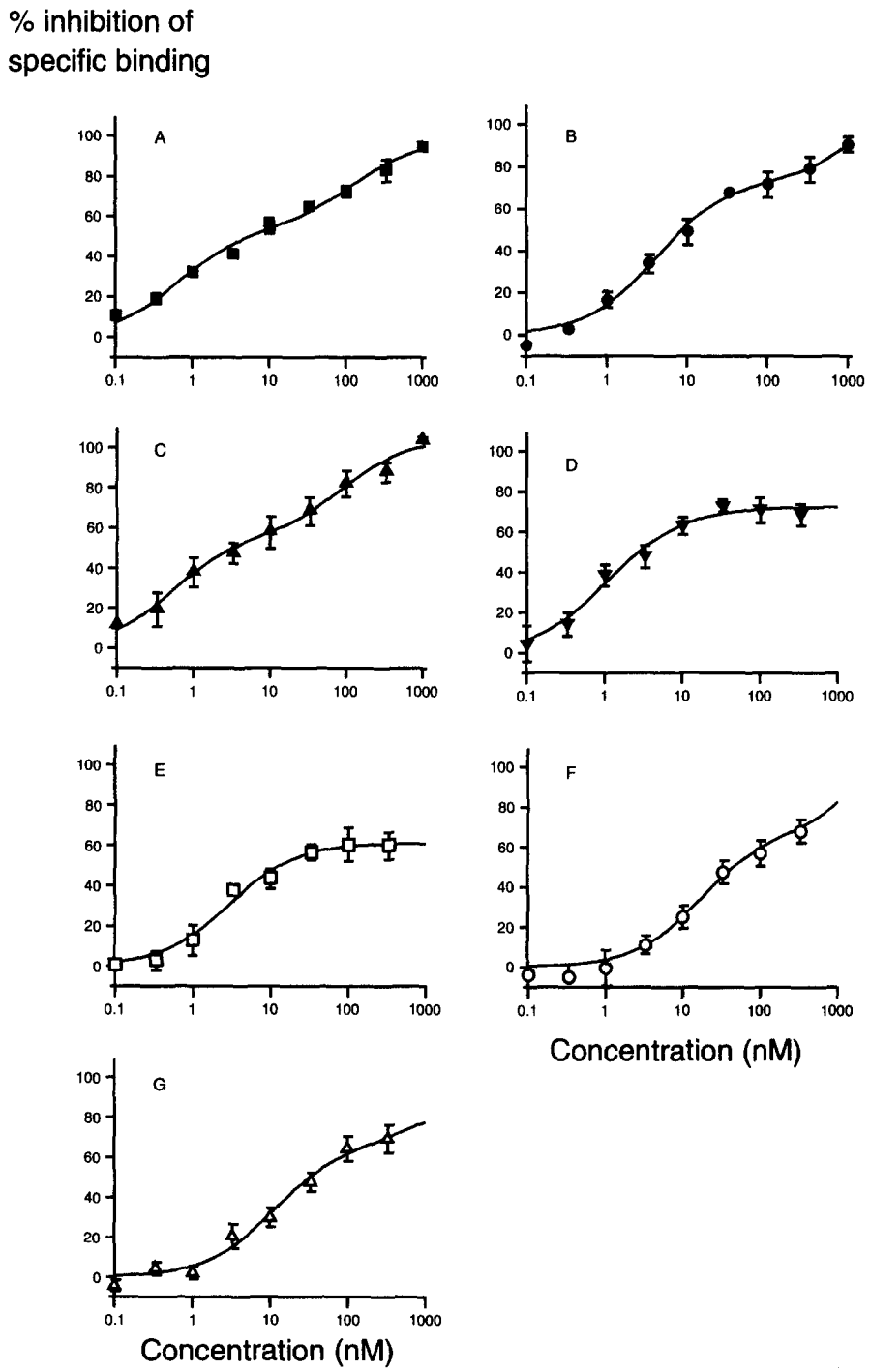

Fig. 2. Displacement of [^{125}I-Tyr^{0}]ovine CRF specific binding to IMR-32 cell membranes by ovine CRF (A), bovine CRF (B), human/rat CRF (C), urotensin I (D), sauvagine (E), α-helical CRF-(9–41) (F) and [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) (G) (mean ± S.E.M., $n = 3$–4).

Table 1
Inhibition of [^{125}I-Tyr^{0}]ovine CRF specific binding to IMR-32 cell membranes. K_i values represent the geometric mean (mean − S.E.M., mean + S.E.M., $n = 3$–4) of either the high affinity (K_i1) or the low affinity (K_i2) site (NA = not applicable, NS = insufficient data)

Peptide	K_i1 (nM)	K_i2 (nM)
Ovine CRF	0.65 (0.58, 0.72)	157 (87, 283)
Bovine CRF	3.52 (2.71, 4.58)	351 (164, 755)
Human/rat CRF	0.46 (0.32, 0.66)	56 (34, 93)
Urotensin I	0.80 (0.56, 1.14)	NA
Sauvagine	2.26 (1.59, 3.22)	NA
α-Helical CRF-(9–41)	16.6 (14.1, 19.6)	NS
[D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}] human/rat CRF-(12–41)	7.80 (4.61, 13.2)	NS

Dose response curves for the displacement of [^{125}I-Tyr^{0}]ovine CRF specific binding by CRF analogues in IMR-32 cell membranes were best fitted to a two-site equation, except those for urotensin I and sauvagine, which both produced one-site curves that maximally displaced approximately 60% of specific binding (Fig. 2). Values for the K_i of the high affinity site in each case (and the low affinity site where appropriate) are given in Table 1. Calcitonin, GRF, secretin, VIP, glucagon and CGRP(all elicited < 10% inhibition of [^{125}I-Tyr^{0}]ovine CRF specific binding at concentrations of 1 μM ($n = 3$).

The basal concentration of cAMP detected in IMR-32 cell membranes was 463 ± 52 pmol/mg ($n = 3$), a value which was significantly increased (by 40–50%, $P < 0.05$) in the presence of either ovine CRF, bovine CRF, human/rat CRF, urotensin I or sauvagine at 1 μM (Fig. 3), concentrations which effected maximal displacement of [^{125}I-Tyr^{0}]ovine CRF binding. Neither α-helical CRF-(9–41) nor [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) significantly altered basal cAMP levels in IMR-32 cell membranes at 100 μM, but, at the same concentration, both peptides significantly antagonized the stimulatory effects of either 1 μM ovine CRF or 1 μM sauvagine, reducing cAMP concentration to levels which were not significantly different to basal (Fig. 3).

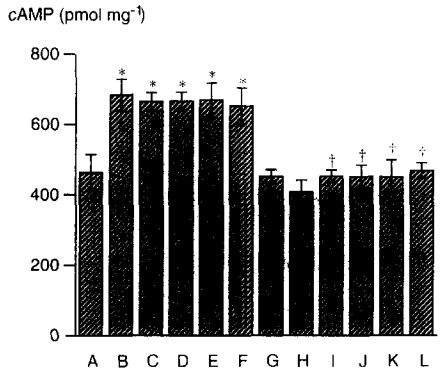

Fig. 3. Cyclic AMP concentration in IMR-32 cell membranes under basal conditions (A) and in the presence of 1 μM: B: ovine CRF, C: bovine CRF, D: human/rat CRF, E: urotensin I, F: sauvagine, G: 100 μM α-helical CRF-(9–41), H: 100 μM [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41), I: α-helical CRF-(9–41) plus ovine CRF (concentrations as above), J: α-helical CRF-(9–41) plus sauvagine, K: [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) plus ovine CRF, L: [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) plus sauvagine (mean ± S.E.M., $n = 3$; * significant difference from basal, + significant difference from agonist alone, $P < 0.05$).

4. Discussion

Differentiated IMR-32 cells appear to express saturable, high affinity CRF receptors, labelled by [^{125}I-Tyr^{0}]ovine CRF, the K_D for which (0.59 nM) corresponded well with that reported by Vita et al. (1993) for the cloned human CRF_1 receptor expressed in COS-7 cells (1.6 nM) and by De Souza and Kuhar (1986) for the rat pituitary receptor (2.0 nM using [Nle^{21},^{125}I-Tyr^{32}]ovine CRF). Undifferentiated IMR-32 cells exhibited a lower expression of this CRF receptor, as indicated by decreased [^{125}I-Tyr^{0}]ovine CRF specific binding (data not shown). The stable GTP analogue, GTP-γ-S, evoked a decrease in ligand affinity without altering the number of [^{125}I-Tyr^{0}]ovine CRF binding sites, suggesting that this CRF receptor is linked to a G protein in the IMR-32 cell line. Over the range of [^{125}I-Tyr^{0}]ovine CRF concentrations (0.01–1 nM) used in saturation experiments, the radioligand appeared to bind to a single population of binding sites, as indicated by the linear Scatchard plots. However, the higher concentrations of cold ovine CRF (0.1–1000 nM) used in displacement experiments revealed a two-site curve, suggesting the presence of an additional low affinity binding site with a K_D of 157 nM. A similar finding was reported by De Souza (1987) in rat olfactory bulb using [^{125}I-Tyr^{0}]human/rat CRF as a radioligand (K_D values of 0.2 and 20 nM) and by De Souza and Kuhar (1986) in rat striatum using [Nle^{21},^{125}I-Tyr^{32}]ovine CRF (K_D values of 1.8 and 100 nM), although the nature of this low affinity binding site remains unclear.

Pharmacological characteristics of the high affinity [^{125}I-Tyr^{0}]ovine CRF binding site were investigated by determining the affinities of various CRF-related peptides in displacing 0.05 nM radioligand binding from IMR-32 cell membranes and were comparable to those previously described for the CRF receptor in rat brain (Curtis et al., 1994; De Souza, 1987; De Souza and Kuhar, 1986) and for the cloned human CRF_1 receptor expressed in COS cells (Chen et al., 1993; Vita et al., 1993), with reported K_i values for ovine CRF and human/rat CRF between 1 and 4 nM. The K_i value for ovine CRF in the present study (0.65 nM) corresponded closely to the apparent K_D for radiolabelled ovine CRF of 0.59 nM. The presence of two [^{125}I-Tyr^{0}]ovine CRF binding sites in IMR-32 cell membranes was confirmed by the displacement curves for analogues of CRF, but not for the homologous peptides, urotensin I and sauvagine, which displaced binding from the high affinity site only, representing approximately 60% of the total binding. The two CRF receptor antagonists exhibited slightly lower affinity than the agonists. The rank

order of affinity for these peptides (human/rat CRF > ovine CRF > urotensin I > sauvagine > bovine CRF > [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) > α-helical CRF-(9–41)) is consistent with the CRF_1 receptor subtype, rather than the CRF_2 receptor subtype (sauvagine > urotensin I ≥ human/rat CRF > ovine CRF), which shares approximately 70% homology with the CRF_1 receptor (Chalmers et al., 1995; Lovenberg et al., 1995a,b). The specificity of the [^{125}I-Tyr^{0}]ovine CRF binding site was reinforced by the lack of displacement with calcitonin, GRF, secretin, VIP, glucagon and CGRPα, the receptors for which share approximately 30% homology with the CRF receptor (Chen et al., 1993).

The functional coupling via G protein linkage of the [^{125}I-Tyr^{0}]ovine CRF receptor in IMR-32 cell membranes was confirmed by an increase in production of cAMP in the presence of CRF and its related agonists, via stimulation of the enzyme adenylate cyclase. The relatively small increases in cAMP observed could be explained by high background levels of cAMP, possibly due to other G protein-linked receptors present in this cell line, in combination with a comparatively low expression of the CRF receptor. In agreement with previous studies (Chen et al., 1993; Curtis et al., 1994; Vita et al., 1993), the functional assay verified α-helical CRF-(9–41) and [D-Phe^{12}, Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) similarly as antagonists at this CRF receptor, abolishing the stimulation of cAMP evoked by both ovine CRF and sauvagine. It has been noted here and elsewhere (Curtis et al., 1994) that these two CRF receptor antagonists have similar affinities in radioligand binding assays, but [D-Phe^{12},Nle^{21,38},C$^{\alpha}$-MeLeu^{37}]human/rat CRF-(12–41) has been reported to be more potent than α-helical CRF-(9–41) in vivo (Curtis et al., 1994), possibly due to differential distribution or affinity at the CRF-binding protein.

To conclude, the present study described functionally coupled, high affinity CRF receptors in a human neuronal cell line, IMR-32, the pharmacology of which indicated similarity to the CRF_1 receptor subtype.

References

Chalmers, D.T., T.W. Lovenberg and E.B. De Souza, 1995, Localization of novel corticotropin-releasing factor receptor (CRF_2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF_1 receptor mRNA expression, J. Neurosci. 15 (10), 6340.

Chen, R., K.A. Lewis, M.H. Perrin and W.W. Vale, 1993, Expression cloning of a human corticotropin-releasing-factor receptor, Proc. Natl. Acad. Sci. USA 90, 8967.

Cheng, K.-C. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Curtis, A.L., D.E. Grigoriadis, M.E. Page, J. Rivier and R.J. Valentino, 1994, Pharmacological comparison of two corticotropin-releasing factor antagonists: in vivo and in vitro studies, J. Pharmacol. Exp. Ther. 268 (1), 359.

De Souza, E.B., 1987, Corticotropin-releasing factor receptors in the rat central nervous system: characterization and regional distribution, J. Neurosci. 7 (1), 88.

De Souza, E.B. and M.J. Kuhar, 1986, Corticotropin-releasing factor receptors in the pituitary gland and central nervous system: methods and overview, Meth. Enzymol. 124, 560.

Dunn, A.J. and C.W. Berridge, 1990, Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses?, Brain Res. Rev. 15, 71.

Lovenberg, T.W., D.T. Chalmers, C. Liu and E.B. De Souza, 1995a, $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues, Endocrinology 136 (9), 4139.

Lovenberg, T.W., C.W. Liaw, D.E. Grigoriadis, W. Clevenger, D.T. Chalmers, E.B. De Souza and T. Oltersdorf, 1995b, Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain, Proc. Natl. Acad. Sci. USA 92, 836.

Moody, T.W., F. Zia, R. Venugopal, L.Y. Korman, A.L. Goldstein and M. Fagarasan, 1994, Corticotropin-releasing factor stimulates cyclic AMP, arachidonic acid release, and growth of lung cancer cells, Peptides 15 (2), 281.

Owens, M.J. and C.B. Nemeroff, 1991, Physiology and pharmacology of corticotropin-releasing factor, Pharmacol. Rev. 43 (4), 425.

Sher, E., S. Denis-Donini, A. Zanini, C. Bisiani and F. Clementi, 1989, Human neuroblastoma cells acquire regulated secretory properties and different sensitivity to Ca^{2+} and α-latrotoxin after exposure to differentiating agents, J. Cell Biol. 108, 2291.

Stenzel, P., R. Kesterson, W. Yeung, R.D. Cone, M.B. Rittenberg and M.P. Stenzel-Poore, 1995, Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart, Mol. Endocrinol. 9, 637.

Vale, W., J. Spiess, C. Rivier and J. Rivier, 1981, Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin, Science 213, 1394.

Vita, N., P. Laurent, S. Lefort, P. Chalon, J.-M. Lelias, M. Kaghad, G. Le Fur, D. Caput and P. Ferrara, 1993, Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors, FEBS Lett. 335 (1), 1.