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Characterisation using microphysiometry of CRF receptor pharmacology

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Abstract

We have assessed the utility of the Cytosensor microphysiometer for studying the pharmacology of recombinant CRF receptors. Chinese hamster ovary cells stably expressing the human CRF_1 or CRF_2 receptor were perfused in the Cytosensor with bicarbonate-free Hams F12 (pH 7.4) containing 0.2% bovine serum albumin. The rank order of potencies of agonist peptides were CRF = sauvagine = urocortin = urotensin at CRF_1 (pEC_{50} values 11.16 ± 0.17, 11.37 ± 0.14, 11.43 ± 0.09 and 11.46 ± 0.13; $n = 4$), and urocortin = sauvagine > urotensin > CRF at CRF_2 (pEC_{50} values 10.88 ± 0.12, 10.44 ± 0.05, 9.36 ± 0.12 and 8.53 ± 0.07; $n = 7–9$). α-Helical CRF (9–41) was a competitive antagonist at the CRF_2 receptor ($pK_B = 6.99 \pm 0.08$, $n = 4$), but was a partial agonist at the CRF_1 receptor ($pEC_{50} = 6.85 \pm 0.08$, $E_{max} = 33\%$, $n = 3$). CP 154,526 was a competitive antagonist at the CRF_1 receptor ($pK_B = 8.17 \pm 0.05$, $n = 6$), but was inactive at the CRF_2 receptor. These data are consistent with established CRF receptor pharmacology and show that the Cytosensor is a viable method for assessing the functional activity of CRF-receptor agonists and antagonists.

Keywords: Cytosensor microphysiometer; CRF (corticotropin-releasing factor); Stress; Urocortin; α-Helical CRF (9–41); CP 154,526

1. Introduction

1.1. Corticotropin-releasing factor receptor pharmacology

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide that regulates the secretion of adrenocorticotropin (Vale et al., 1981), and plays an important role in the coordination of the stress response (Owens and Nemeroff, 1991; Orth, 1992). In addition to this neuroendocrine role, CRF acts as a neurotransmitter in the brain (De Souza, 1995; Chalmers et al., 1996), as well as having immunological actions (Owens and Nemeroff, 1991). Hypersecretion of CRF in the brain may contribute to depression, anxiety disorders and anorexia nervosa, whilst deficits in CRF are associated with neurodegenerative diseases such as Alzheimer's (Orth, 1992; De Souza, 1995).

Two human CRF receptors, CRF_1 and CRF_2, with distinct anatomical and pharmacological profiles have been cloned (Chen et al., 1993; Liaw et al., 1996; for review, see De Souza, 1995). Similarly, two CRF receptors have been cloned in the rat (Chang et al., 1993; Perrin et al., 1993, 1995; Chalmers et al., 1995), although there are two splice variants of the rat CRF_2 receptor, $CRF_{2\alpha}$ and $CRF_{2\beta}$ (Kishimoto et al., 1995), that have differential distributions (Lovenberg et al., 1995a). The human CRF_2 receptor corresponds to the rat $CRF_{2\alpha}$ splice variant (Liaw et al., 1996). Both CRF_1 and CRF_2 couple via G_s to a stimulation of cAMP formation (De Souza, 1995; Nabhan et al., 1995), although the coupling efficiency of the CRF_2 receptor is lower (Donaldson et al., 1996).

In addition to CRF, the CRF peptide family consists of the fish peptide urotensin I, the frog peptide sauvagine and their mammalian equivalent urocortin (Vaughan et al., 1995; Donaldson et al., 1996). In contrast to CRF, these peptides have similarly high potencies at both the CRF_1 and CRF_2 receptors (Lovenberg et al., 1995b; Donaldson et al., 1996).

The main CRF receptor antagonist used to date has been the peptide α-helical CRF (9–41), which acts at both receptor subtypes (Owens and Nemeroff, 1991; Dieterich and De Souza, 1996), although there is some evidence that suggests a degree of selectivity for the CRF_2 receptor

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(Fisher et al., 1991; Kishimoto et al., 1995). Recently, however, a non-peptide CRF_1 receptor-specific antagonist, CP 154,526, has been described (Lundkvist et al., 1996; Schulz et al., 1996; Mansbach et al., 1997).

1.2. The Cytosensor microphysiometer

The Cytosensor microphysiometer (Molecular Devices, UK) is a device that quantifies cellular metabolic activity by measuring the changes in the extracellular acidification rate, as a reliable index of the integrated functional response to receptor activation (Owicki et al., 1990; Smart et al., 1997b; Jordan et al., 1998). The acidification rate is measured as the change in potential across a silicon light-addressable sensor, caused by the accumulation of hydrogen ions during periods of cessation of the flow of medium (McConnell et al., 1992). In the current investigation, the utility of the Cytosensor for studying CRF receptor pharmacology has been confirmed. Firstly, the rank order of potencies for the agonists CRF, sauvagine, urocortin and urotensin I at the various receptor subtypes are consistent with the established pharmacology of these receptors. Secondly, the pK_B values for α-helical CRF (9–41) and CP 154,526 are, once again, consistent with the published affinities for these CRF receptor antagonists. Furthermore, once validated, this assay was used to assess the functional affinity of several novel compounds, and showed that these were CRF_1-specific antagonists with moderate affinity.

Part of this work has previously been communicated to the British Pharmacological Society (Smart et al., 1997a).

2. Methods

2.1. Cloning and expression of the CRF receptors in Chinese hamster ovary cells

The human CRF_1 and CRF_2, as well as the rat CRF_1 and $CRF_{2\alpha}$, receptors were constructed as described previously (Suman-Chauhan et al., 1999). The full length gene for each receptor was then sub-cloned into the mammalian expression vector pCr/CMV and transfected into Chinese hamster ovary-pro5 cells.

2.2. Culture of Chinese hamster ovary cells

Chinese hamster ovary-pro5 cells were routinely grown as monolayers in Minimum Eagle's Medium-Alpha medium supplemented with 10% foetal calf serum (GIBCO, UK), and maintained under 5% CO_2 at 37°C. Cells were passaged every 4–5 days, and the highest passage number used was 16. Prior to use in the Cytosensor, the cells were seeded on the polycarbonate microporous membranes of the capsule cups (Molecular Devices) to achieve the density of ~0.6 million cells per cup on the day of the experiment.

2.3. Cellular acidification measurements using the Cytosensor microphysiometer

A Cytosensor microphysiometer (Molecular Devices) was used to measure the extracellular acidification rate, as

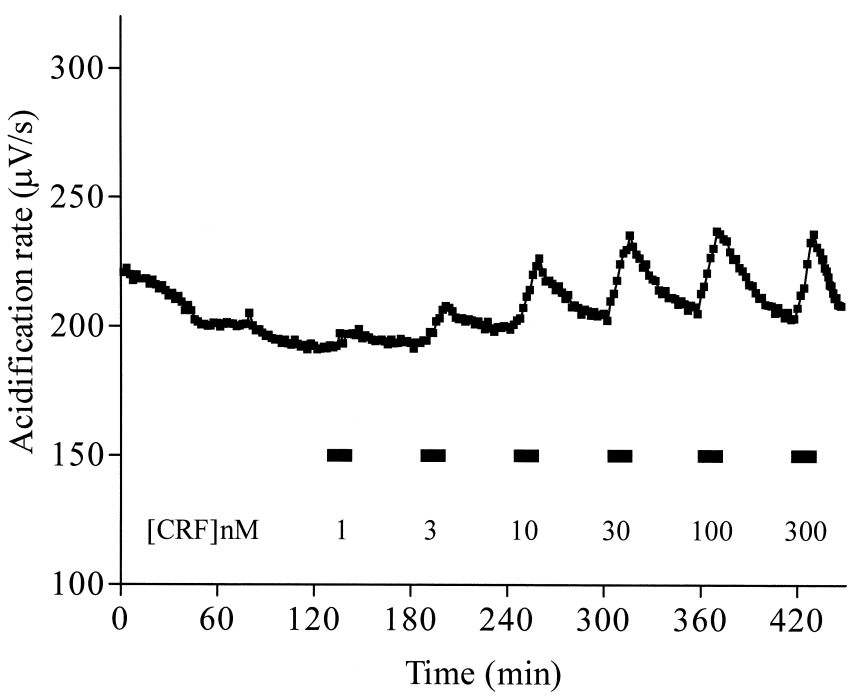

Fig. 1. A typical trace of the CRF-induced acidification response in CHO-human CRF_2 cells. CHO-human CRF_2 cells were perfused in a Cytosensor and serially challenged (10 min exposure every 45 min) with increasing concentrations (1–300 nM) of CRF, as indicated by the black bars. The acidification rate was measured every 2 min.

described previously (McConnell et al., 1992; Jordan et al., 1998). CHO cells expressing recombinant human CRF_1 or CRF_2, or rat CRF_1 or $CRF_{2\alpha}$, receptors were seeded into Cytosensor capsule cups (see above) and placed in sensor chambers at 37°C inside the Cytosensor, and maintained by a flow (120 μl/min) of bicarbonate-free Hams F12 containing 0.2% bovine serum albumin (pH 7.4). The flow was halted for 22 s at the end of each 2 min pump cycle, and the rate of acidification (μV/s) measured for 15 s during that period. CRF, sauvagine, urocortin or urotensin (0.1 pM–300 nM) were introduced sequentially in the perfusate for 10 min at 45 min intervals. In some studies, α-helical CRF (9–41) (30–300 nM), CP 154,526 (10–100 nM) or one of several novel compounds (PD 173307, PD 173602, PD 173713 and PD 174239; 10 nM–1 μM) (Wustrow et al., 1998) was included in the perfusate throughout.

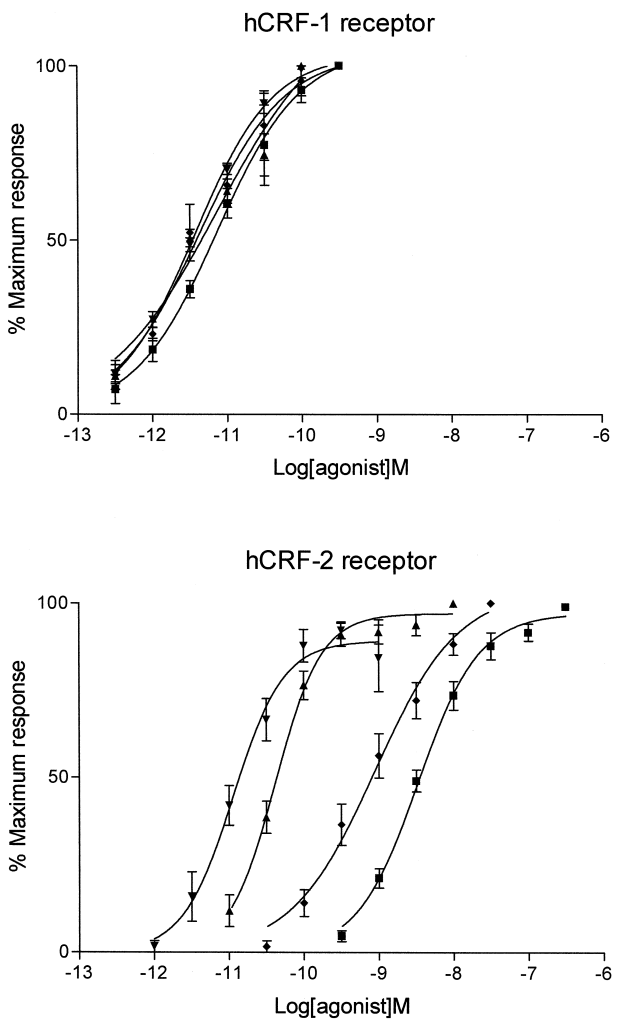

Fig. 2. Concentration–response curves for CRF-like ligands in CHO-human CRF_1 and CHO-human CRF_2 cells. The cells were challenged with CRF (■; 0.3 pM–300 nM), urocortin (▼; 0.3 pM–10 nM), sauvagine (▲; 0.3 pM–10 nM) or urotensin I (◆; 0.3 pM–30 nM) and the acidification rate measured. Data are normalised to a maximal CRF response, and are given as mean ± S.E.M., $n = 4–9$.

Table 1
Agonist potencies at CRF receptors stably expressed in CHO cells
Data are pEC_{50} values, given as mean ± S.E.M., $n = 4–9$.

Ligand	Human CRF_1	Human CRF_2	Rat $CRF_{2\alpha}$
CRF	11.16 ± 0.17	8.53 ± 0.07	8.70 ± 0.12
Sauvagine	11.37 ± 0.14	10.44 ± 0.05	10.64 ± 0.03
Urocortin	11.43 ± 0.09	10.88 ± 0.12	10.67 ± 0.09
Urotensin I	11.46 ± 0.13	9.36 ± 0.12	9.85 ± 0.05

2.4. Data analysis

Agonist effects were quantitatively determined as the increase in the acidification rate response (peak minus basal), and expressed as μV/s. For clarity of presentation, some pooled data are expressed as a percentage of the maximal response to CRF within the individual chamber, in order to minimise the day to day variation in the magnitude of the acidification response resulting from cell culture variables. Data are presented as mean ± S.E.M unless otherwise stated. Curve fitting and parameter estimation were carried out using GraphpadPrism 2.00. Apparent pK_B values were calculated using the Gaddum equation:

$$pK_B = \log(\text{concentration ratio} - 1) - \log(\text{antagonist concentration}).$$

All mean pK_B and EC_{50} values are calculated from individual curves.

2.5. Materials

PD 173307 (Cyclopropylmethyl-[3-(2,4-dichloro-phenyl)-2,5-dimethyl-pyrazolo[1,5 -a]pyrimidin-7-yl]-propyl-amine), PD 173602 (3-(2,4-Dichloro-phenyl)-2,5-di-

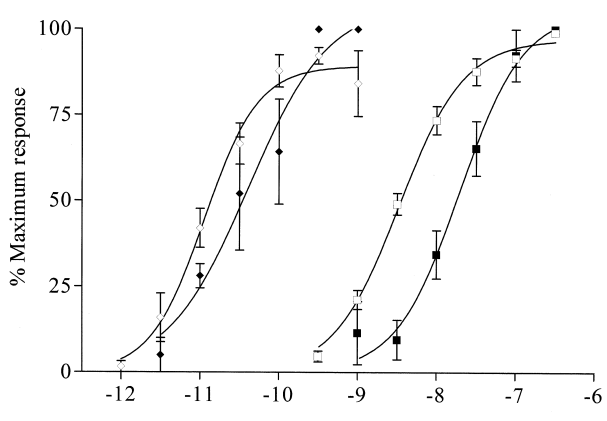

Fig. 3. α-Helical CRF (9–41) antagonises the urocortin- and CRF-induced acidification response in CHO-human CRF_2 cells. Cells were challenged with urocortin (diamonds; 1 pM–1 nM) or CRF (squares; 0.3–300 nM) in the presence (filled symbols) or absence (open symbols) of 300 nM α-helical CRF (9–41). Data were normalised to a maximal CRF response, and are given as mean ± S.E.M., $n = 4$.

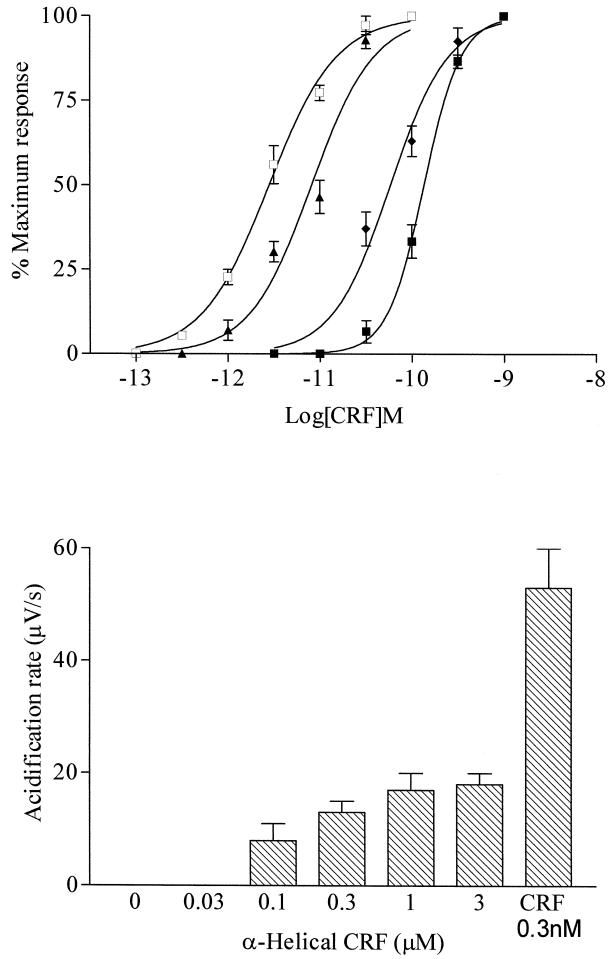

Fig. 4. The effects of α-helical CRF (9–41) at the human CRF_1 receptor. Upper panel: cells were challenged with CRF (; 0.1 pM–1 nM), in the presence of 30 (▲), 100 (♦) or 300 (■) nM α-helical CRF (9–41). Data were normalised to a maximal CRF response, and are given as mean ± S.E.M., $n = 6$. Lower panel: cells were challenged with CRF (30 nM) or α-helical CRF (9–41) (30 nM–3 μM) and the acidification rate measured. Data are mean ± S.E.M., $n = 4$.

methyl-7(2-propyl-piperidin-1-yl)-pyrazolo[1,5-a]pyrimidine), PD 173713 ([3-(2,4-Dichloro-phenyl)-2,5-dimethyl-yrazolo[1,5-a]pyrimidin-7-yl]-dipropyl-amine), PD 174239 ([3-(2,4-Dichloro-phenyl)-2,5-dimethyl-yrazolo[1,5-a]pyrimidin-7-yl]-ethyl-(2-methoxy-ethyl)-amine)(Wustrow et al., 1998) and CP 154,526 were synthesised at Parke-Davis, whilst all the peptides were purchased from Bachem (UK).

3. Results

Chinese hamster ovary cells stably expressing recombinant human CRF_1 or CRF_2 receptors maintained stable basal acidification rates of 150–300 μV/s within 45 min of the start of perfusion (Fig. 1). CRF increased the acidification rate in these cells, characterised at higher concentrations as a broad monophasic response that peaked 10–12 min after the onset of agonist perfusion and then returned slowly to baseline (Fig. 1). This acidification response to CRF was concentration-dependent (Figs. 1 and 2), as were the acidification responses to the peptide agonists sauvagine, urocortin and urotensin (Fig. 2). The rank order of potencies obtained for human CRF_1 and human CRF_2 receptors were CRF = sauvagine = urocortin = urotensin and urocortin = sauvagine > urotensin > CRF, respectively (Table 1), consistent with the established pharmacology of these receptors, and the concurrent radioligand binding data (Suman-Chauhan et al., 1999). Likewise, the rank order of potencies for the rat $CRF_{2\alpha}$ receptor was urocortin = sauvagine > urotensin > CRF (Table 1), again consistent with the accepted pharmacology of this receptor.

The CRF receptor peptide antagonist, α-helical CRF (9–41), caused a parallel rightward shift of both the urocortin and CRF concentration–response curves in Chinese hamster ovary cells expressing the human CRF_2 receptor (Fig. 3), indicating that this peptide was a competitive antagonist, with an apparent pK_B of 6.99 ± 0.08 ($n = 4$). Similarly, α-helical CRF (9–41) caused a concentration-related parallel rightward shift of the concentration–response curve to CRF in Chinese hamster ovary cells expressing the human CRF_1 receptor (Fig. 4), with an apparent pK_B of 7.97 ± 0.15 ($n = 6$). However, α-helical CRF (9–41) also acted as a partial agonist (E_{max} of 33%, compared to CRF) at the human CRF_1 receptor, with an pEC_{50} of 6.85 ± 0.08 ($n = 3$). The non-peptide CRF antagonist, CP 154,526, caused a concentration-dependent parallel rightward shift of the CRF concentration–response curve at the human CRF_1 receptor (Fig. 5), with an apparent pK_B of 8.17 ± 0.05 ($n = 6$). A similar effect was found at the rat CRF_1 receptor (apparent pK_B of 8.23, $n = 2$), but CP 154,526 had no effect at either the rat or human CRF_2 receptor (data not shown). Moreover, CP 154,526 had no agonist activity at any of the CRF receptors tested (data

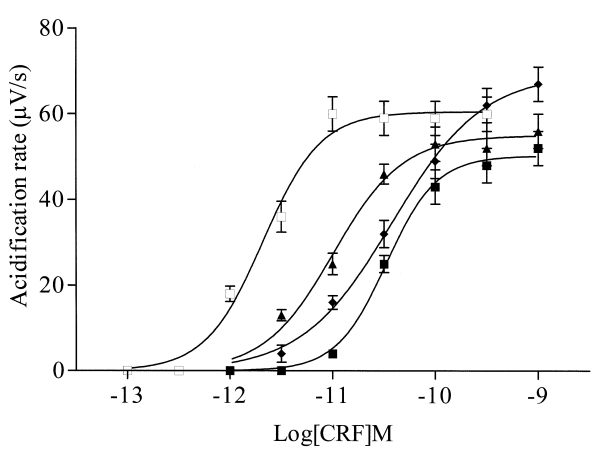

Fig. 5. CP 154,526 is a competitive antagonist at the human CRF_1 receptor. Cells were challenged with CRF (□; 0.1 pM–1 nM) in the presence of 30(▲), 100 (♦) or 300 (■) nM CP 154,526. Data are mean ± S.E.M., $n = 5$.

not shown). Taken collectively, these data confirm that CP 154,526 is a CRF_1 receptor-specific antagonist.

The novel compounds PD 173307, PD 173602, PD 173713 and PD 174239 all acted as competitive antagonists at the human CRF_1 receptor, with pA_2 values of 8.06, 8.08, 7.44, and 7.07, respectively (data not shown). However, these compounds were inactive at the CRF_2 receptor, consistent with the concurrent radioligand binding studies (Wustrow et al., 1998; Suman-Chauhan et al., 1999).

4. Discussion

The Cytosensor microphysiometer has been used previously to study the effects of activation of cloned receptors transfected in host cells, and those constitutively expressed in immortal cell lines (Denyer et al., 1993, 1994; Smart et al., 1997b). The current investigation has shown that the Cytosensor is a valid method for studying CRF receptor pharmacology. Firstly, the rank order of potencies for the four main members of the CRF peptide family at CRF_1 and CRF_2 receptors are consistent with the established pharmacology of these receptors (Donaldson et al., 1996). Secondly, the affinities identified using microphysiometry for α-helical CRF (9–41) and CP 154,526 are again consistent with the affinities previously reported for these antagonists (Kishimoto et al., 1995; Lundkvist et al., 1996). Moreover, the Cytosensor was able to detect in vitro the partial agonist effects of α-helical CRF (9–41), that have previously only been reported in vivo (Menzaghi et al., 1994).

CRF caused a concentration-dependent increase in the acidification rate when perfused onto Chinese hamster ovary cells expressing the recombinant human CRF_1 or CRF_2 receptor, indicative of increased metabolic activity due to the activation of various signal transduction pathways, as reported for other receptor types (Owicki et al., 1990; Smart et al., 1997b; Jordan et al., 1998). Other members of the CRF peptide family also increased the acidification rate, and the rank order of potency for these agonists of CRF = urocortin = sauvagine = urotensin and urocortin = sauvagine > urotensin > CRF, at the human CRF_1 and CRF_2 receptors respectively, were consistent with the accepted pharmacology of these receptors (Chen et al., 1993; Donaldson et al., 1996; Liaw et al., 1996). Likewise, the rank order of potency at the rat $CRF_{2\alpha}$ receptor (urocortin = sauvagine > urotensin > CRF) was consistent with the established pharmacology for this receptor (Lovenberg et al., 1995b; Vaughan et al., 1995; Chalmers et al., 1996). Furthermore, the rank order of potency, as well as the actual potencies, obtained using microphysiometry for the rat $CRF_{2\alpha}$ and human CRF_2 receptors are virtually identical, providing further evidence that these two receptors are the equivalent entities in their respective species (Liaw et al., 1996; but see Valdenaire et al., 1997).

Previous studies have shown that the CRF_2 receptor is poorly coupled to cAMP formation, relative to the CRF_1 receptor (Chalmers et al., 1996; Donaldson et al., 1996). Indeed, in the present study, CRF was ~100-fold more potent at the CRF_1, compared to the CRF_2 receptor. However, urocortin and sauvagine had similar potencies at the CRF_1 and CRF_2 receptors in the Cytosensor, and equivalent findings from cAMP studies have been reported (Donaldson et al., 1996), indicating that the poor coupling efficiency of the CRF_2 receptor is only with respect to CRF. Our data provide further support for the view that urocortin is the endogenous ligand for the CRF_2 receptor (Vaughan et al., 1995). It is also worth noting that the actual potencies obtained for all the agonists at each receptor in the present study are considerably higher than those reported from radioligand and cAMP studies (Vaughan et al., 1995; Chalmers et al., 1996), probably reflecting the greater degree of amplification possible with microphysiometry (Owicki et al., 1990; McConnell et al., 1992). These apparent differences do not result from the use of different recombinant cell lines in the present study as parallel studies have shown that these cells express the expected binding affinities (Suman-Chauhan et al., 1999). An earlier, much more limited, Cytosensor study in human embryonic kidney 293 cells expressing the human CRF_2 receptor found concentrations of urocortin in the nanomolar range were needed to elicit a response (Valdenaire et al., 1997). This discrepancy could reflect the relative activity of the receptor in different host cells, but more probably is an artefact of the methodology used. We have found that exclusion of the 0.2% bovine serum albumin from the medium causes a ~1000-fold rightward shift of the CRF concentration response curve (data not shown) and the medium used in this earlier Cytosensor study did not contain bovine serum albumin (Valdenaire et al., 1997).

In the current investigation, α-helical CRF (9–41) was a competitive antagonist at the CRF_2 receptor, with a K_B of ~100 nM, consistent with its published affinity at this receptor (Grigoriadis et al., 1996a,b). α-Helical CRF (9–41) also antagonised the acidification response to CRF at the CRF_1 receptor, with a K_B of ~10 nM, again consistent with previous reports (Perrin et al., 1993; Gulyas et al., 1995; Dieterich and De Souza, 1996). These data suggest that α-helical CRF (9–41) has an ~10-fold selectivity for the CRF_1 receptor, yet others have reported CRF_2 selectivity (Kishimoto et al., 1995). Indeed, α-helical CRF (9–41) antagonises the cardiovascular actions of CRF with a higher affinity than it does the release of adrenocorticotropin in vivo (Fisher et al., 1991), and this has been cited as further evidence for CRF_2 receptor selectivity (Kishimoto et al., 1995; Turnbull et al., 1996). Nevertheless, others have reported that, in vitro, α-helical CRF (9–41) inhibits cAMP formation with similar affinities at both receptor subtypes (Grigoriadis et al., 1996b). These conflicting data indicate that attempts at the identification of the CRF receptor type involved in effects in vivo based

on antagonism with α-helical CRF (9–41) should be viewed with caution.

It is worth noting that α-helical CRF (9–41) also acted as a partial agonist at the CRF_1 receptor in the Cytosensor, eliciting a concentration-dependent acidification response with an EC_{50} of 140 nM and an E_{max} of 33% compared to CRF. These data are of particular interest as α-helical CRF (9–41) has been reported to be a partial agonist in vivo (Menzaghi et al., 1994), but does not stimulate cAMP formation in vitro (De Souza, 1995; Chalmers et al., 1996). The detection of this partial agonism in the Cytosensor is probably due to the relative sensitivity of microphysiometry compared to traditional assays, because of the high degree of signal amplification, as discussed above.

CP 154,526 was a competitive antagonist at the human CRF_1 receptor in the Cytosensor, with a K_B of 6.8 nM, consistent with its published affinity at this receptor (Lundkvist et al., 1996; Schulz et al., 1996), and, unlike α-helical CRF (9–41), did not display partial agonism. Furthermore, CP 154,526 had no effect on the human CRF_2 receptor-mediated acidification response, confirming it is a CRF_1 receptor specific antagonist (Schulz et al., 1996). Likewise, CP 154,526 had a similar affinity (5.9 nM) at the rat CRF_1 receptor and was inactive at the rat $CRF_{2\alpha}$ receptor, indicating there were no species differences in affinity with this compound, consistent with previous in vivo findings (Mansbach et al., 1997). Similarly, the novel compounds PD 173307, PD 173602, PD 173713 and PD 174239 were shown to be CRF_1-specific competitive antagonists, with comparable affinities to that of CP 154,526, in agreement with radioligand binding studies (Wustrow et al., 1998; Suman-Chauhan et al., 1999).

In conclusion, the current data have shown that the Cytosensor microphysiometer is a valid technique for studying CRF receptor pharmacology, yielding results for both agonists and antagonists at CRF_1 and CRF_2 receptors that are consistent with the accepted pharmacology of these receptors. Moreover, the Cytosensor is a more sensitive assay with respect to agonists, and this enabled the detection of the partial agonist effects of α-helical CRF (9–41), previously only reported in vivo.

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