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[D-Pro^5]Corticotropin-releasing factor analogs as selective agonists at corticotropin-releasing factor receptors

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Abstract

Corticotropin-releasing factor (CRF) acts on at least two types of CRF receptors. To search for selective CRF receptor agonists, 37 ovine CRF analogs, systematically substituted with D-amino acids, were tested for inhibitory activity on edema induced in the pentobarbital-anesthetized rat paw by heat (immersion in 58°C water for 1 min). The activity of each analog, administered 21 nmol/kg i.v. 10 min before heat, was compared to published data on the analog's potency in stimulating adrenocorticotropin (ACTH) release from cultured rat pituitary cells. In general, a positive rank correlation was found between the anti-edema and neuroendocrine activities of these analogs, however, one outlier, [D-Pro^5]ovine CRF, exhibited greater selectivity for anti-edema activity. The human/rat analog of [D-Pro^5]CRF was synthesized and found to be equipotent to human/rat CRF for suppression of heat-edema. In cells transfected with two types of cloned CRF receptors, the intracellular cAMP response to [D-Pro^5]human/rat CRF was equipotent to human/rat CRF in the heart-muscle CRF ($CRF_{2\beta}$) receptor assay but was five times less potent than human/rat CRF in the pituitary-central nervous system CRF (CRF_1) receptor assay. We conclude that changing residue Pro^5 in the CRF molecule from a L- to a D-configuration confers selectivity by decreasing second messenger activation at the CRF_1 receptor whilst retaining full potency at the $CRF_{2\beta}$ receptor.

Keywords: CRF (corticotropin-releasing factor); CRF receptor; Anti-edema; ACTH (adrenocorticotropin); Structure-activity relationship

1. Introduction

Corticotropin-releasing factor (CRF), a 41-residue neuropeptide, that regulates adrenocorticotropin (ACTH) secretion from the anterior pituitary has several direct actions on central and peripheral tissues (Owens and Nemeroff, 1991). For example, in hippocampal slices, CRF stimulates firing of pyramidal neurons, and behavioral actions are initiated after central administration of this peptide (Aldenhoff et al., 1983; Diamant and de Wied, 1993). In arterial smooth muscle preparations, CRF produces relaxation of epinephrine-contracted strips (Lei et al., 1993). CRF also affects inflammatory processes (Chrousos, 1995), one unusual action being suppression of edema formation after local injury to peripheral tissues, an effect independent of ACTH release (Wei and Thomas, 1993). In an attempt to determine if the anti-edema activity of CRF could be disassociated from ACTH-releasing activity, we have recently screened 33 ovine CRF analogs in which residues 5–41 were singly substituted with alanine (Kornreich et al., 1992; Wei and Thomas, 1994). In general, a positive rank correlation was found between the anti-edema activities and neuroendocrine potencies of these analogs, as reported by Kornreich et al. (1992). In this study, we tested an additional set of 37 ovine CRF analogs, in which residues 5–41 were singly substituted with D-amino acids (Rivier et al., 1993). The results showed that replacement of Pro^5 with D-Pro^5 conferred selectivity for the anti-edema action, and indicated that this effect was mediated by a distinct form of the CRF receptor.

2. Materials and methods

2.1. Peptides

Ovine CRF and single point D-substituted ovine CRF analogs were kindly provided by Dr. Jean Rivier (Salk Institute, La Jolla, CA). Doses of peptides were based on

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the weight of the supplied materials and were not corrected for purity which ranged from 91 to 99% (Rivier et al., 1993). Human/rat CRF was a gift from Dr. Nicoholas Ling (Whittier Institute, La Jolla, CA). [D-Pro^5]human/rat CRF was custom synthesized (Lot BC0124) by Dr. Janos Varga (California Peptide Research, Napa, CA). The purity of this peptide as determined by high-performance liquid chromatography on a Vydac C_{18} 5-μm column in two buffer systems was $>99\%$. The mass spectrum of the synthesized peptide was consistent with the calculated mean mass and the amino acid analysis gave the expected ratio of amino acids.

2.2. Bioassays

The method for measuring heat-induced edema was described previously (Wei and Thomas, 1994). Briefly, male Sprague-Dawley rats (200–250 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and saline (1 ml/kg body weight) or test substances dissolved in saline were injected i.v. via a branch of the femoral vein. 10 min after injection, thermal stimulus was applied to the hindpaw by immersion of the foot, up to the ankle joint, in 58°C water for 1 min. 30 min after heat exposure, animals were killed by overdose with concentrated sodium pentobarbital and both hindpaws were removed at the ankle joint and weighed. The degree of heat-induced edema was estimated as the difference between the weights of the heated and unheated paws divided by the weight of the unheated paw. Four to six analogs ($n \geq 3$ animals/analog) were tested on each occasion with concurrent saline controls. For some peptides, the median effective dose (ED_{50}) or the median effective concentration (EC_{50}) was calculated according to the method of Litchfield and Wilcoxon (1949).

The methods for cloning the two types of CRF receptors, the pituitary-central nervous system CRF (CRF_1) receptor and the heart-muscle CRF ($CRF_{2\beta}$) receptor, and for transfecting cells with the full-length cDNA of these receptors and for intracellular cAMP assays (using a [^{3}H]cAMP kit from Amersham Life Sciences) are described elsewhere (Chang et al., 1993; Kishimoto et al., 1995). cDNAs for the CRF_1 receptor and the $CRF_{2\beta}$ receptor were subcloned into expression vector pCEP4 and transfected into 293-EBNA cells (Invitrogen, San Diego, CA). Stable transfectants were incubated with 50 μM 3-isobutylmethyl-1-methylxanthine for 20 min at 37°C and then peptides were added and incubated for another 20 min at 37°C. Levels of cAMP were assayed in sextuplicate.

3. Results

In the saline-treated controls, immersion of the hindpaw in 58°C water for 1 min resulted 30 min later in a 73 ± 2% (mean ± S.E.M., $n = 54$) increase in paw weight, an effect

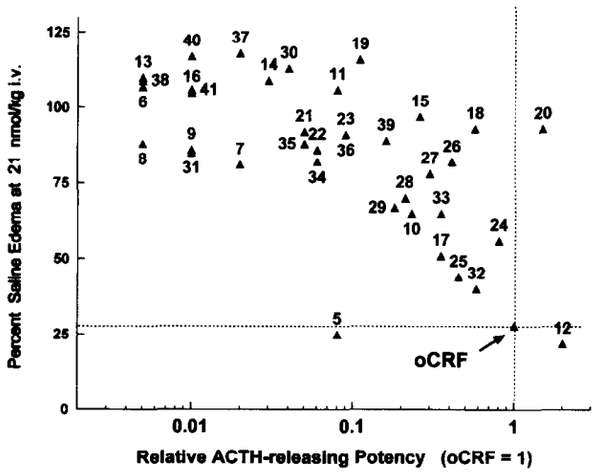

Fig. 1. Correlation of anti-edema and ACTH-releasing activities of D-substituted ovine CRF analogs. The number adjacent to each point represents the position of D-amino acid substitution. The data are heat-induced edema responses as percentage of concurrently injected saline controls ($n \geq 3$/group). Values of ACTH-releasing potencies are from Rivier et al. (1993). Spearman's rank correlation test, $r_s = 0.603$, $P \leq 0.01$.

caused by an increase in the water content of the tissues (Wei et al., 1988). About 10 out of the 37 D-substituted ovine CRF analogs, tested at 21 nmol/kg (0.1 mg/kg) i.v. injected 10 min before heat, produced significant suppression of edema (Fig. 1). The activities of the 37 analogs were positively correlated to the ACTH-releasing potencies reported by Rivier et al. (1993), as measured by the Spearman's rank correlation test, $r_s = 0.603$, $P < 0.01$ (Fig. 1) (Siegel, 1956). Two outliers, [D-Pro^5]ovine CRF and [D-Glu^{20}]ovine CRF, were detected with activities suggestive of separation between ACTH-releasing activity and anti-edema activity. [D-Pro^5]Ovine CRF was at least as potent as ovine CRF in suppression of heat-induced edema (Table 1), but its potency in releasing ACTH from dispersed rat anterior pituitary cells was reported to be 8% that of ovine CRF. [D-Glu^{20}]Ovine CRF did not produce significant suppression of edema at 21 nmol/kg i.v. but its potency in the ACTH-release assay was reported to be 150% that of ovine CRF.

Due to the limited quantities of ovine CRF analogs available for further assays, we decided to synthesize and to evaluate [D-Pro^5]human/rat CRF. This analog was equipotent to human/rat CRF in suppression of heat-induced edema (Table 1). In a more direct test of its receptor

Table 1
Potency of CRF and [D-Pro^5]CRF peptides in suppression of heat-induced edema in the anesthetized rat

Peptide	ED_{50} (95% CL) nmol/kg i.v.
Ovine CRF	8 (4–17)
[D-Pro^5]ovine CRF	4 (1–13)
Human/rat CRF	2 (0.6–6)
[D-Pro^5]human/rat CRF	2 (0.6–7)

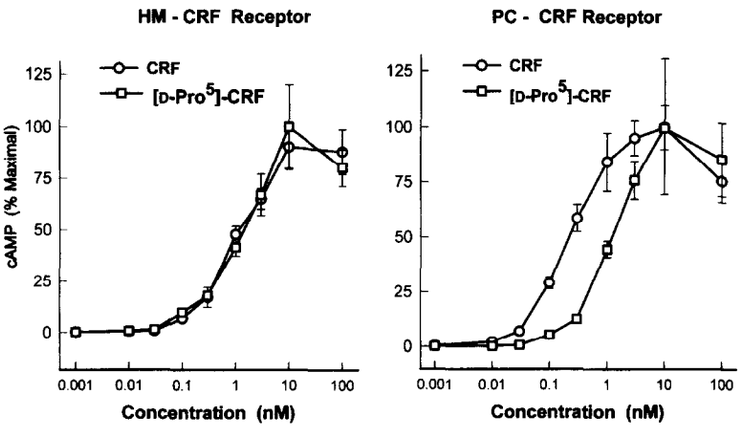

Fig. 2. Functional effects of human/rat CRF and [D-Pro^5]human/rat CRF on cells transfected with either the CRF_1 receptor or the $CRF_{2\beta}$ receptor. The baseline cAMP levels for the $CRF_{2\beta}$ receptor assay and the CRF_1 receptor assay were 4.0 ± 0.08 and 5.9 ± 1.8 pmol/well, respectively and increased to a maximum of 2000 ± 190 and 1200 ± 370 pmol/well, respectively, after stimulation with human/rat CRF. Values represent percentages of the maximal response ($n = 6$, mean $\pm$ S.D.).

activity, the interactions of [D-Pro^5]human/rat CRF with the CRF_1 receptor and the $CRF_{2\beta}$ receptor on cAMP accumulation are shown in Fig. 2. [D-Pro^5]Human/rat CRF was equipotent to human/rat CRF on the $CRF_{2\beta}$ receptor but was five times less potent than human/rat CRF on the CRF_1 receptor (EC_{50} of 0.22 nM for human/rat CRF vs. 1.1 nM for [D-Pro^5]human/rat CRF).

4. Discussion

Earlier studies by Rivier et al. (1983, 1984) reported that elimination of the first 4 residues of ovine CRF did not affect ACTH-releasing potencies in in vitro systems. Single replacement of residues 5–19 with alanine in ovine CRF resulted in a significant decrease in ACTH-releasing potency (Kornreich et al., 1992). A decline in potency was also observed when residues 5–11 and residues 13–19 were singly replaced with D-amino acids (Rivier et al., 1993). The exception was [D-Phe^{12}]ovine CRF which had twice the potency of ovine CRF on ACTH release. From this information, it was inferred that the side-chains of residues in the N-terminal region (residues 5–11 and 13–19) may be important for receptor binding and activation (Kornreich et al., 1992, and Rivier et al., 1993). With the exception of [D-Pro^5]ovine CRF and [D-Glu^{20}]ovine CRF, the anti-edema data on alanine-ovine CRF analogs and on single D-substituted ovine CRF analogs confirm the pattern of structure-activity relationships that was seen with ACTH-releasing activities. For example, ovine CRF analogs, singly substituted with alanine or D-amino acids in residues 6–9 showed a > 90% loss of activity, relative to ovine CRF, in both the anti-edema and the ACTH-release assays. The finding in this study that [D-Pro^5]ovine CRF was at least equipotent to ovine CRF on suppression of heat-induced edema whilst it was reported to retain only 8% of the ACTH-releasing potency of ovine CRF suggested that the anti-edema and ACTH-releasing actions were mediated by distinct receptor pathways.

Recently, receptors to CRF have been identified in mammalian pituitary, brain and peripheral tissues (Chen et al., 1993; Lovenberg et al., 1995; Kishimoto et al., 1995; Perrin et al., 1995). These receptors belong to a subfamily of guanine nucleotide stimulatory factor (G_s)-coupled receptors which include receptors for peptide hormones, such as secretin, calcitonin and parathyroid hormone, and for neuropeptides, such as vasoactive intestinal polypeptide. The CRF_1 receptor is linked to ACTH release (Chang et al., 1993; Chen et al., 1993) and has a smaller N-terminal domain than the $CRF_{2\beta}$ which is expressed in heart, muscle and brain (Lovenberg et al., 1995; Kishimoto et al., 1995; Perrin et al., 1995). Cells transfected with CRF receptors are sensitive to the cAMP stimulatory effects of frog sauvagine and sucker fish urotensin I, two non-mammalian peptides which show homology to CRF (Erspamer et al., 1980; Lederis et al., 1982). In two studies, the rank order of potency for cAMP stimulation in cells expressing the $CRF_{2\beta}$ receptor was sauvagine > sucker fish urotensin I > human/rat CRF (Lovenberg et al., 1995; Kishimoto et al., 1995). On the other hand, sauvagine, urotensin I and human/rat CRF were about equipotent in cells transfected with the CRF_1 receptor. For suppression of heat-induced edema in the rat, an earlier study showed that the i.v. ED_{50} values for sauvagine, sucker fish urotensin I and human/rat CRF were 0.44, 1.5 and 5.9 nmol/kg, respectively (Wei and Kiang, 1989). Thus, the potency of agonists at the $CRF_{2\beta}$ receptor paralleled anti-edema activity. The data here showed that changing residue Pro^5 in the CRF molecule from a L- to a D-configuration decreased second messenger activation at the CRF_1 receptor whilst full potency was retained at the $CRF_{2\beta}$ receptor. These results further support the functional link of anti-edema activity to the peripheral actions of the $CRF_{2\beta}$ receptor, and suggest that ligands modified at the residue Pro^5 of CRF may have selective actions on CRF receptors.

Acknowledgements

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