Biochemical Pharmacology, Vol. 52, pp. 829–833, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00300-0

Corticotropin-Releasing Hormone (CRH) Receptors in the Mesenteric Small Arteries of Rats Resemble the (2)-Subtype

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ABSTRACT. The potencies of the corticotropin-releasing hormone (CRH) agonistic peptides oCRH, h/rCRH, frog sauvagine, and carp urotensin I and of the antagonistic peptide α-helical CRH9-41 were compared in 3 different *in vitro* assays: (a) receptor binding to rat brain membranes; (b) release of ACTH/β-endorphin from rat pituitary cells; and (c) relaxation of rat mesenteric small arteries. From their potency profiles, especially from the high potency of sauvagine relative to CRH in the relaxation assay, it is concluded that the receptors mediating the hypotensive action of systemic CRH in vascular smooth muscle are different from those in the pituitary and brain, and may be identical or very similar to the recently cloned new CRH receptor type 2. BIOCHEM PHARMACOL 52;6:829–833, 1996.

KEY WORDS. CRH agonists; CRH antagonist; rat; receptor; arteries; pituitary

CRH[‖] mediates its effects through high-affinity receptors that have been characterized by radioreceptor studies and by stimulation of adenylate cyclase in membrane fractions of brain, pituitary and spleen from the rat (for review, see [1]). These receptors showed very similar kinetic and pharmacological properties, consistent with a single type of receptor [1]. However, for the peripheral actions of intravenously administered CRH, vasodilation and tachycardia, the CRH receptor agonistic peptides sauvagine and urotensin I were shown to be much more potent than CRH, itself [2], although all three peptides were nearly equipotent in their action on the release of ACTH and β-endorphin from the rat pituitary [3].

Furthermore, the antagonist α-helical CRH9-41 antagonized the sustained hypotensive effect, but not the initial decrease in mean arterial blood pressure obtained after i.v. injection of h/rCRH [4]. Using this antagonist and CRH in different *in vivo* rat bioassays, Fisher *et al.* [5] found that the activities of the antagonist in blocking the CRH-induced elevations of plasma levels of ACTH and β-endorphin were much lower than its activity in blocking CRH-induced hypotension and tachycardia.

Taken together, these results suggested the existence of multiple CRH receptor subtypes, especially of a receptor type in blood vessels differing from that in brain and pituitary. The purpose of this study was, therefore, to determine the *in vitro* potencies of four CRH agonists (oCRH, h/rCRH, sauvagine, and urotensin I) in relaxing mesenteric small arteries, to be compared with the potencies of these agonists in their binding to the CRH receptor in rat brain and their stimulation of the release of ACTH and β-endorphin from rat pituitary cells, including the effect of the antagonist α-helical CRH9-41 in these 3 systems.

Recently, a CRH receptor from rat brain was cloned and shown, when functionally expressed, to be stimulated by CRH agonists in a rank order of potency [6] quite different from that for the brain and pituitary receptors (referred to as CRH-1 receptors) described earlier [7–9]. This characteristic of the new brain receptor type (referred to as CRH-2 receptor) corresponded with the potencies of agonists in their activity on cloned CRH receptors found in heart [10, 11] and skeletal muscle [11] of mouse. In this study, we obtained results that suggest that the receptors in the mesenteric small arteries are of this second type, differing from those pharmacologically characterized in the pituitary and brain by the assays used here.

MATERIALS AND METHODS

Peptides

The agonists of the CRH receptor h/rCRH, oCRH, frog sauvagine, and carp urotensin I and the antagonist α-heli-

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‖ *Abbreviations:* CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; EGTA, ethylene glycol bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid.

Received 3 January 1996; accepted 8 April 1996.

cal CRH9-41 were synthesized as previously described [12]. [^{125}I]Tyr^{0}-oCRH (2200 Ci/mmol) was obtained from DuPont NEN (Bad Homburg, Germany).

CRH Receptor Assay

Whole brains of male Wistar rats weighing 220–250 g were homogenized with a Teflon-glass homogenizer (10 strokes at 800 rpm) in 0.32 M sucrose, 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, and 0.15 mM bacitracin at 50 mg wet weight per mL. After centrifugation at 1000 × *g* for 5 min, the supernatant was centrifuged at 26,000 × *g* for 20 min. The pellet was resuspended in 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, 0.15 mM bacitracin, and 0.0015% aprotinin (assay buffer) and, again, centrifuged. The resulting pellet was resuspended in assay buffer containing 0.32 M sucrose and stored at −20°C. All steps were carried out at 4°C. Protein concentrations were determined by the method of Bradford [13], using BSA as standard.

One hundred μg of membrane protein in 300 μL assay buffer were incubated in quadruplicates with 0.1 nM [^{125}I]Tyr^{0}-oCRH in the absence and presence of 12 different concentrations (0.2 nM up to 1 μM) of unlabelled peptides at 25°C for 2 hr. Nonspecific tracer binding was determined in the presence of 1 μM oCRH. At the end of incubation, 3 mL of ice-cold washing buffer (assay buffer without inhibitors containing 0.01% Triton X-100) was added to the assay tube and the samples were immediately filtered through GF/C filter discs (Whatman), presoaked for 2 hr in 0.1% polyethylenimine using a Brandel-Harvester. The incubation tubes and filters were then washed with 3 mL cold washing buffer. Triton X-100 in this buffer strongly reduced nonspecific tracer peptide binding. Radioactivity retained on the filter was measured by γ-counting.

Receptor affinities (Kass, Kd = 1/Kass) and capacities (B_{max}) were estimated using the nonlinear least-squares curve fitting program RADLIG (BIOSOFT, Cambridge, U.K.) and a K_d of 0.48 nM for the binding of the tracer peptide as determined from tracer saturation assays. The total amount of bound tracer was 5%, approximately 30% of which was nonspecific.

ACTH/β-Endorphin Releasing Activity of Peptides on Rat Anterior Pituitary Cells

Pituitary cells were obtained by enzymatic digestion of the anterior pituitary of male Wistar rats weighing 220–250 g following the procedure by Denef *et al.* [14]. Two hundred thousand cells in DMEM/0.25% BSA per well were seeded in cell culture plates and maintained at 37°C under 5% CO_2/95% air for 3 days. The culture medium was replaced by 0.5 mL fresh medium and, after 2 hr, by culture medium containing one of the peptides studied at different concentrations. After a stimulation period of 3 hr, the medium samples were harvested and stored at −70°C. The antagonistic activity of α-helical CRH9-41 was studied by incubating the cells with different concentrations of the antagonist in the presence of a constant concentration of oCRH.

ACTH in the samples was determined by an immunoradiometric assay (HS-ACTH-IRMA from the Nichols Institute Diagnostika GmbH, Bad Nauheim, FRG) using hACTH as standard. This assay uses two antibodies directed against the N- and C-terminal part identical in ACTH from human and rat. β-Endorphin was determined by a radioimmunoassay using camel β-endorphin as standard. EC_{50} values were calculated from the dose-response curves by a four-parameter logistic curve fitting program.

Relaxing Activity of Peptides on Rat Mesenteric Small Arteries

Ring segments of mesenteric small arteries (diameter <300 μm, length ca. 2 mm) were dissected from male MOL-Wistar rats (Møllegaard) and mounted in a dual myograph for small vessels (model 500, J. P. Trading, Aarhus, Denmark). The vessels were equilibrated in PSS (119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.026 mM EDTA, and 5.5 mM glucose, pH 7.4) at 37°C under 5% CO_2/95% O_2 for 30 min.

The preparations were normalized as described by Mulvany and Halpern [15]. The passive wall tension-internal circumference characteristics were determined and the circumference was set to a normalized internal circumference L_0 (corresponding to 90% of the internal circumference the vessel would have under a passive transmural pressure of 100 mm Hg) to obtain optimal conditions for active force development. The effective lumen diameter, l_0, was calculated as L_0/π. After normalization, the arteries were sequentially activated with 10 μM noradrenaline, 125 mM-KPSS (PSS with replacement of 125 mM KCl for NaCl), and 10 μM noradrenaline in KPSS to test the contractility of the preparations.

After the vessels were equilibrated in PSS for a further 10 min, they were contracted with 10 μM prostaglandin $F_{2\alpha}$ in a 25 mM-KPSS solution for 5 min, after which 1 nM up to 1 μM peptide was applied cumulatively for a period of 5 min for each concentration. The vessels were then washed with PSS 3 times at 10-min intervals before a new test series was begun. The antagonistic activity of α-helical CRH9-41 was determined by conducting concentration-response measurements of the peptides on the same vessel preparation in the absence and presence of 100 nM antagonist.

Relaxation responses were calculated as the percentage of the force of contraction before the first addition of peptide, and IC_{50} values were estimated from the dose-response curves by a four parameter logistic curve-fitting program.

RESULTS

A one-site binding model gave the best fit for the data of the binding of the CRH agonists h/rCRH, oCRH, sauvagine, and urotensin I, as well as of the antagonist α-helical CRH9-41 to the rat brain membrane receptor, resulting in identical capacities (47.60 fmol/mg protein ± 2.00 SE) for all peptides. The K_d values are summarized in Table 1.

The dose-response curves of ACTH and β-endorphin release from the rat pituitary cells by the agonist peptides were superimposable, leading to the same maximum release of immunoactive ACTH and β-endorphin of 247.9 ± 24.9 and 268.4 ± 18.9 (SE) ng per mg cell protein (ca. 5×10^6 cells), respectively, within 3 hr which, when compared with the basal release, corresponded to a stimulation factor for ACTH and β-endorphin of 10.1 and 7.4, respectively. Generally, there was a close relation between the release of ACTH and β-endorphin within each assay, with a molar ratio of (camel) β-endorphin/ACTH of 1.04 ± 0.03 (SE, from linear regression line); however, immunoactive β-endorphin may contain β-lipotropin [16]. EC_{50} values resulting from the curve-fitting program are given in Table 1.

The antagonist α-helical CRH9-41 dose-dependently inhibited the CRH-stimulated release of ACTH and β-endorphin. At the same time, it showed some agonistic activity, releasing at 1 μM, the highest concentration tested, 33.3% and 24.8% of the maximal CRH-stimulated amount of ACTH and β-endorphin, respectively, similar to the findings of Corder *et al.* [4]. The values for the inhibition of peptide release evoked by 1 nM oCRH, as given in Table 1 are, therefore, apparent values, because the inhibition is weakened by the increasing agonistic activity of the antagonist in concentration-inhibition curves.

All agonistic peptides exhibited identical maximal relaxing activities on rat mesenteric small arteries, diminishing the level of contraction to 20–24% of the precontracted value. The estimated IC_{50} values are given in Table 1. Earlier it was found [17] that, at 0.1 μM, the antagonist α-helical CRH9-41 inhibited the relaxation activity of h/rCRH. The Kb values obtained from the Schild equation in Table 1 reveal similar inhibitory activities of the antagonist towards all peptides. The antagonist showed no agonistic activity at concentrations of up to 3.2 μM.

To compare the activities of the CRH agonists in the different assays, normalized potencies were calculated and are shown in Fig. 1, which also includes values for cAMP production by the stimulated CRH receptor types 1 and 2 as calculated from data by Lovenberg *et al.* [6].

DISCUSSION

Comparing the orders of potency of the CRH agonists between the different assays, the ratios of these potencies as to their relaxing activity (sauvagine ≫ urotensin > r/hCRH ≫ oCRH) were quite different from those for the release of ACTH/β-endorphin from pituitary cells (Table 1, Fig. 1). In this assay, the potencies of the peptides were very similar, differing only by a maximal factor of approximately 2, similar to the results of Sutton *et al.* [18]. The ratios of the brain receptor affinities of r/hCRH, oCRH and sauvagine were comparable to those of the cell assay but, remarkably, urotensin I showed an exceptionally high affinity.

Most striking, the order of the potencies of the peptides for relaxing activities was the same as that found by Lovenberg *et al.* [6] for the activities of the peptides in cAMP accumulation in mouse Ltk− cells expressing a second member of the CRH receptor family (CRH-2 receptor). The ratios of the potencies in the pituitary cell assay, however, were close to those found for the CRH-1 receptor in Ltk− cells (Fig. 1). The latter type had already been identified in rat brain [7, 9], human pituitary [8] and mouse pituitary AtT20 cells [19], and is assumed to be the only one occurring in the pituitary [6] as well as being responsible for the activation of the corticotrophic cells by CRH. Based on these results, it is concluded that the relaxing activity of CRH on mesenteric arteries is mediated by the CRH receptor type 2 as characterized in rat brain [6]. Receptors of such a type, showing high preference for sauvagine to CRH, have also been recently cloned from mouse heart [10, 11].

CRH peripherally administered to rats evokes hypotension and tachycardia [4, 16], and the *in vitro* relaxing action of CRH on rat resistance arteries, as described earlier [17,

TABLE 1. *In vitro* activities of CRH peptides in receptor binding to rat brain membranes, release of ACTH and β-endorphin from rat anterior pituitary cells, and relaxation of rat mesenteric small arteries. Values are given as mean ± SE.

	Receptor K_d (nM)	ACTH EC_{50} (nM)	β-Endorphin EC_{50} (nM)	Arteries IC_{50} (nM)	Arteries Kb (nM)†
r/hCRH	2.85 ± 0.94	0.19 ± 0.02	0.23 ± 0.03	33.6 ± 11.7	7.1 ± 1.6
oCRH	4.31 ± 0.99	0.28 ± 0.01	0.29 ± 0.02	132.3 ± 37.0	17.3 ± 9.0
Urotensin I	1.04 ± 0.23	0.13 ± 0.01	0.23 ± 0.05	19.2 ± 6.3	20.4 ± 4.0
Sauvagine	4.54 ± 0.77	0.16 ± 0.02	0.19 ± 0.01	4.2 ± 2.3	22.2 ± 7.3
α-helical CRH9-41	20.52 ± 3.08	1800*	2000*		

* Concentration of the antagonist required for 50% inhibition of the release of ACTH and β-endorphin evoked by 1 nM oCRH.

† Dissociation constant from the Schild equation.

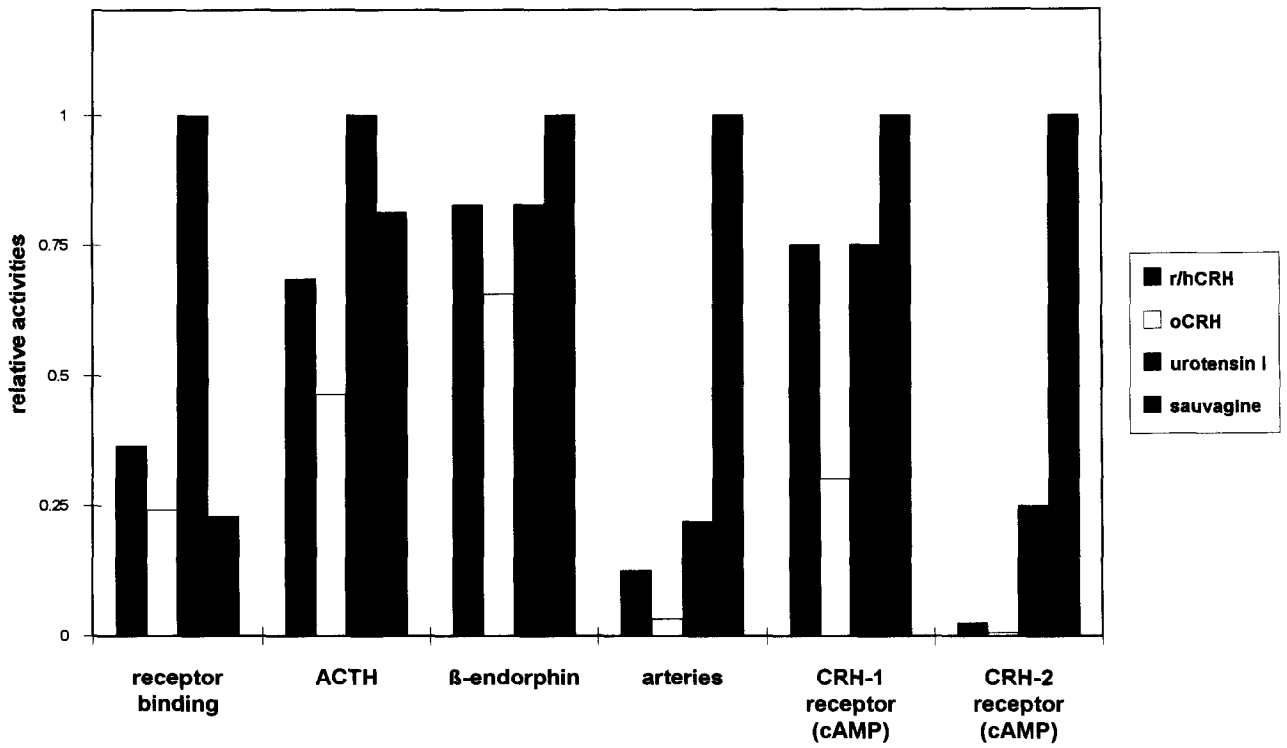

FIG. 1. Relative *in vitro* activities of CRH agonistic peptides in receptor binding to rat brain membranes, release of ACTH and β-endorphin from rat anterior pituitary cells, and relaxation of contracted rat small mesenteric arteries. Also given are their activities in cAMP accumulation by the CRH-1 and CRH-2 receptor in mouse Ltk- cells from data by Lovenberg *et al.* [6]. The potencies were normalized within each type of assay, and the potency (1 K_d, $1/EC_{50}$ or $1/IC_{50}$) of the most potent peptide set to 1.

20], suggests that the vasodilatory effect of systemic CRH is mediated through a direct action on the vascular smooth muscle [20]. From the present results, it is further concluded that the receptors mediating these effects are of the second type. In support of this conclusion is the fact that the *in vivo* vasodilatory activities of sauvagine and urotensin I were found to be much higher than with CRH [2], in accordance with the properties of the CRH-2 receptor. Furthermore, the *in vitro* concentrations of CRH and the other peptides required to relax the arteries were much higher than those required to stimulate ACTH/β-endorphin release. This is comparable to the much higher *in vivo* dose of CRH required to lower blood pressure in rats than that required to stimulate ACTH/β-endorphin secretion [16]. The high abundance of mRNA for the CRH-2 receptor in arterioles of brain and heart, recently found [21], further supports the existence of this receptor type in smooth muscle.

Because α-helical CRH9-41 showed binding to the brain CRH receptor and antagonistic activity on the release of ACTH and β-endorphin from pituitary cells, as well as on the relaxant effect on mesenteric arteries evoked by the CRH agonists (Table 1), it cannot be used to differentiate between the two CRH receptor types. This is in agreement with the finding [6] that the stimulation of adenylate cyclase activity by both receptor types, when expressed in mouse Ltk- cells, is inhibited by the antagonist at approximately equal affinities. Furthermore, the antagonist was shown to inhibit the binding of [^{125}I]Tyr^{0}-oCRH to the murine CRH receptor type 2 in transfected cells with an IC_{50} value of 40.8 nM [10], very close to that found here for the rat brain membrane receptor (Table 1). Why only the sustained hypotensive effect, but not the initial decrease in mean arterial blood pressure, after intravenous injection of CRH was blocked by the antagonist remains unclear [4].

In rats, Fisher *et al.* [5] found low antagonist activity of α-helical CRH9-41 on the elevations of plasma ACTH/β-endorphin compared with high activity on hypotension and tachycardia after i.v. CRH. This difference may be partly explained by the different partial agonistic activities of the antagonist. Whereas no agonist activity of the antagonist in relaxing the mesenteric arteries was found at concentrations up to 3.2 μM, its agonistic activity on the pituitary at high doses may explain its low antagonistic activity on the pituitary as observed *in vivo* [5], as well as here and in [4] *in vitro* (Table 1). Furthermore, for the activities on blood vessels, it should be noted that the high pharmacological affinity of the antagonist estimated from the Schild equation (Table 1) contrasts with the low activity of the agonists compared with corresponding receptor affinities in the brain (Table 1) and cell systems [6, 10].

The cloning of the two CRH receptor types from rat brain [6] could mean that both receptor proteins are also functionally expressed in rat brain membranes. The results from the receptor binding studies using membranes from

the whole rat brain did not substantiate this possibility, because the ratios in the affinities of the ligands used were not in line with the possible presence of a sufficient amount of the CRH-2 receptor to be detected (Table 1). Therefore, detailed regional brain receptor studies seem to be necessary.

In summary, from the ratios of the *in vitro* potencies of four CRH agonistic peptides on the relaxation of rat mesenteric small arteries, on ACTH/β-endorphin release from rat pituitary cells, and on rat brain receptors, it is concluded that the hypotensive action of systemic CRH is mediated by receptors in vascular smooth muscle different from those in the pituitary and from the bulk of brain receptors, and that they may be identical or very similar to the recently cloned CRH-2 receptor [6, 10, 11]. The preference of this receptor for sauvagine compared to CRH suggests the possible existence of an additional endogenous peptide that may physiologically act on the vessels. Recently, a new mammalian member of the CRH family, urocortin, was localized in the rat midbrain [22]. Urocortin was shown to be more potent than r/hCRH at binding and activating the CRH-2 receptor. Thus, urocortin could be an endogenous ligand for this receptor type, but it remains to be established if there is any specific endogenous peptide specifically activating the CRH-2 receptor in smooth muscle. Furthermore, the development of an antagonist specific for the CRH-2 receptor should greatly help to clarify the physiological significance of CRH or a CRH-like peptide in the periphery.

We thank Dr. Maria Andries (Leuven) for support in establishing the pituitary cell culture and Jenny Eichhorst, Annerose Klose, Dagmar Smettan, and Gabriela Vogelreiter (Berlin) for excellent technical assistance. This work was supported by grant BMBF 01ZZ9508.

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