Stressin$_1$-A, a Potent Corticotropin Releasing Factor Receptor 1 (CRF$_1$)-Selective Peptide Agonist†

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The potencies and selectivity of peptide CRF antagonists is increased through structural constraints, suggesting that the resulting ligands assume distinct conformations when interacting with CRF$_1$ and CRF$_2$ receptors. To develop selective CRF receptor agonists, we have scanned the sequence -Gln-Ala-His-Ser-Asn-Arg- (residues 30−35 of [DPhe^{12},Nle^{21,38}]Ac-hCRF$_{4-41}$) with an $i-(i+3)$ bridge consisting of the Glu^{i}-Xaa-Xbb-Lys^{i+3} scaffold, where residues i = 30, 31, and 32. When i = 31, stressin$_1$-A, a potent CRF$_1$ receptor-selective agonist was generated. In vitro, stressin$_1$-A was equipotent to h/rCRF to release ACTH. Astressin$_1$-A showed a low nanomolar affinity for CRF$_1$ receptor (K_i = 1.7 nM) and greater than 100-fold selectivity versus CRF$_2$ receptor (K_i = 222 nM). Stressin$_1$-A released slightly less ACTH than oCRF in adult adrenal-intact male rats, with increased duration of action. Stressin$_1$-A, injected intraperitoneally in rats, induced fecal pellet output (a CRF$_1$ receptor-mediated response) and did not influence gastric emptying and blood pressure (CRF$_2$ receptor-mediated responses).

Introduction

In previous reports describing the rationale used in the discovery of astressin (a potent nonselective antagonist at both CRF$_1$[a] and CRF$_2$ receptors) and astressin$_2$-B (a CRF$_2$ receptor-selective antagonist), we emphasized the importance of subtle structural constraints that led to the desired compounds. Whereas a Glu^{30}-Lys^{33} side chain to side chain covalent lactam constraint increased affinity of linear CRF antagonists (astressin) for CRF$_1$ and CRF$_2$ receptors,[2] we found that a Glu^{32}-Lys^{35} side chain to side chain bridge in h/rCRF fragments and the corresponding Glu^{31}-Lys^{34} bridge in sauvagine fragments yielded potent ligands (astressin$_2$-B) that are highly selective for the CRF$_2$ receptor.[3] This selectivity (>100-fold) was demonstrated using radioreceptor assays with cloned receptor cell lines and autoradiographic studies on rat brain slices. Additionally, we identified unique substitutions (CαMeLeu) that conferred on these antagonists long duration of action in vivo.[4]

Interestingly, extension of these sequences to full length CRF analogs yielded CRF$_1$/CRF$_2$ receptor (astressin-derived) agonists that were only 2- to 5-fold more potent than the parent analogues, as well as agonists that had lost some of their CRF$_2$ receptor selectivity.[1] However, the discovery of urocortin 2 (Ucn 2)[5] and urocortin 3 (Ucn 3),[6] which are potent CRF$_2$ receptor-selective agonists, provided the needed tools to study the physiological role of the CRF$_2$ receptor. Whereas a large number of studies used oCRF as a preferential CRF$_1$ receptor-selective ligand, we disclosed the structure of stressin$_1$-A[1], which was equipotent to oCRF at CRF$_1$ receptor and about 4 times less potent at CRF$_2$ receptor than oCRF, resulting in a 4-fold increase in selectivity. Independently, Tezval et al. described cortagine with about 200-fold selectivity for the CRF$_1$ receptor.[7] Although any of these molecules may be used for the study of the pharmacological or physiological CRF$_1$-mediated activities, we hypothesized that fragments of the most potent and selective analogue might yield a potent and CRF$_1$ receptor-selective antagonist. It is noteworthy that nonpeptide CRF$_1$ receptor-selective antagonists were available at the time of the initiation of this research, yet they are not necessarily the best tools for unraveling the physiological role of the CRF$_1$ receptor because they do not limit their action to the peripheral or central compartments. Although one would expect that peptide CRF$_1$ receptor-selective agonists administered in the periphery would not cross the blood brain barrier, it is known that centrally administered peptides may leak to the periphery.

We disclosed the structure of stressin$_1$-A (cyclo(31−34)-[DPhe^{12},Nle^{21,38},Glu^{31},Lys^{34}]Ac-hCRF$_{(4-41)}$), a CRF$_1$ receptor-selective agonist, at the 31st Annual Meeting of the Society of Neuroscience held in San Diego, CA.[1] When it comes to nomenclature, we proposed to call CRF antagonists, astressins[3] and use stressins for agonists with no subscript or subscript 1 or 2 (as for the antagonists) to indicate no selectivity or selectivity for either the CRF$_1$ receptor or the CRF$_2$ receptor, respectively. The essential role of the CRF$_1$ receptor in mediating ACTH release from the pituitary in response to stress

† The structure of stressin$_1$-A was first disclosed in a neuroscience meeting poster.[1]

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[a] Abbreviations: [The abbreviations for amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37)] BP, blood pressure; BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium-hexafluorophosphate; BSA, bovine serum albumin; h/rCRF, human/rat corticotropin releasing factor; oCRF, ovine CRF; CRF$_1$, CRF receptor 1; CRF$_2$, CRF receptor 2; mCRF$_2$, mouse CRF$_2$; CZE, capillary zone electrophoresis; Fmoc, 9-fluorenylmethoxycarbonyl;" HPLC, high performance liquid chromatography; ip, intraperitoneal; iv, intravenously; MS, mass spectrometry; Ofm, O-fluorenylmethyl; RPHPLC, reversed phase high performance liquid chromatography; SAR, structure−activity relationships; TBTU, *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEAP, triethylammonium phosphate; Xaa, any amino acid.

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Table 1. Physical and Biological Properties of CRF Analogues

	cmpd	HPLC[a]	CZE[b]	MS[c] calcd	MS[c] found	CRF_1[d] (nM)	CRF_2[d] (nM)	relative potency[e]
1	r/hCRF	>98	96	4755.5	4755.5	1.0 (0.2−4.6)	6.2 (2.0−19)	1.0
2	oCRF	99	98	4668.50	4668.5	1.2 (0.9−1.6)	52 (21−128)	1.0
3	cortagine	96	>98	4439.35	4440.3	3.4 (2.5−4.8	102 (46−225)	0.63 (0.37−1.1)
4	cyclo(30−33)[DPhe^{12},Nle^{21,38},Glu^{30},Lys^{33}]-Ac-hCRF$_{(4-41)}$	97	95	4440.52	4440.4	0.7 (0.2−2.0)	1.3 (0.5−3.6)	4.3 (2.5−7.8)
5	linear[DPhe^{12},Nle^{21,38},Glu^{30},Lys^{33}]-Ac-hCRF$_{(4-41)}$	96	95	4458.54	4458.6	0.5 (0.2−1.3)	1.6 (1.5−1.8)	4.5 (2.7−7.6)
6	linear[DPhe^{12},Nle^{21,38},Glu^{31},Lys^{34}]-Ac-hCRF$_{(4-41)}$	98	>98	4488.54	4488.5	2.7 (2.2−3.3)	252 (114−560)	1.0 (0.47−2.3)
7	cyclo(31−34)[DPhe^{12},Nle^{21,38},Glu^{31},Lys^{34}]-Ac-hCRF$_{(4-41)}$ (stressin$_1$-A)	>98	98	4470.53	4470.3	1.7 (1.1−2.6)	222 (137−361)	1.1 (0.53−2.2)
8	linear[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{31},Lys^{34}]-Ac-hCRF$_{(4-41)}$	>98	98	4516.57	4516.5	2.7 (0.7−10)	261 (105−654)	1.1 (0.3−5.5)
9	cyclo(31−34)-[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{31},Lys^{34}]-Ac-hCRF$_{(4-41)}$	97	94	4498.56	4498.3	0.8 (0.5−1.1)	47 (21−102)	2.2 (0.8−6.2) NP
10	cyclo(31−34)[DPhe^{12},Nle^{21},Glu^{31},Lys^{34}]-oCRF	98	94	4704.57	4704.55	66 (28.5−154)	>1000	
11	cyclo(31−34)[DPhe^{12},Nle^{21},MeLeu^{27},Glu^{31},Lys^{34}]-oCRF	95	94	4718.6	4718.29	50 (18−139)	>1000	
12	cyclo(31−34)[DPhe^{12},Nle^{21},Glu^{31},Lys^{34}]-Ac-oCRF$_{(9-41)}$	95	91	3895.15	3895.13	>500	>500	
13	cyclo(32−35)[DPhe^{12},Nle^{21,38},Glu^{32},Lys^{35}]-Ac-hCRF$_{(4-41)}$	96	95	4362.45	4362.5	1.8 (0.6−5.0)	1.8 (1.2−2.7)	2.7 (1.4−5.3)
14	cyclo(32−35)-[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{32},Lys^{35}]-Ac-hCRF$_{(4-41)}$	95	90*	4390.48	4390.6	1.3 (0.6−2.9)	1.5 (1.0−2.2)	2.8 (1.4−5.8)
15	cyclo(32−35)-[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{32},Lys^{35}]-Ac-hCRF$_{(6-41)}$	>98	95*	4196.37	4196.4	3.9 (2.2−7.0)	3.0 (1.0−9.2)	6.1 (3.8−9.7)
16	cyclo(32−35)-[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{32},Lys^{35}]-Ac-hCRF$_{(7-41)}$	>98	90*	4083.29	4083.3	11 (8.0−15.0)	1.6 (1.1−2.4)	0.32 (0.21−0.48)
17	cyclo(32−35)-[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{32},Lys^{35}]-Ac-hCRF$_{(8-41)}$	>98	95*	3996.26	3996.2	15.6 (9.0−26.0)	2.0 (1.6−2.4)	0.025 (0.013−0.043)

[a] Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH_3CN/40% A with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm. [b] Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE System 2050 or 5500. Field strength of 15 kV at 30 °C, mobile phase: 100 mM sodium phosphate (85:15, H_2O/CH_3CN), pH 2.50, on a Supelco P175 capillary (75 μm ID × 50 cm length). *CZE basic; field strength of 13 kV at 30 °C; mobile phase: 100 mM sodium borate (85:15, H_2O/CH_3CN), pH 8.50, on an Agilent μSIL-FS capillary (75 μm ID × 50 cm length). Detection at 214 nm for both systems. [c] The observed *m/z* of the monoisotope compared with the calculated $[M + H]^+$ monoisotopic mass. [d] The numbers given in Table 1 reflect the inhibitory binding constants, K_i, for the analogues binding to the cloned $hCRF_1$ and $mCRF_{2\beta}$ receptors derived from competitive radioligand displacement assays using the nonselective ^{125}I-labeled agonist [Tyr^0,Glu,^{1}Nle^{17}]sauvagine as the radioligand. K_i values were calculated by PRISM software. Values in parentheses are 95% confidence limits. [e] Potencies are relative to oCRF in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses.

or exogenous administration of CRF or Ucn 1 is well established using CRF_1 receptor knockout mouse model or selective nonpeptide CRF_1 receptor subtype antagonists.[8,9] In addition, convergent in vivo and in vitro studies have emphasized the role for the CRF_1 receptor in stress and peripheral administration of CRF-induced stimulation of colonic motor function (motility, transit, defecation, and diarrhea).[10,11] In contrast, in the upper gastrointestinal tract, CRF, Ucn 1, or Ucn 2 injected peripherally exerts an inhibitory effect on gastric motility and transit that is mediated by CRF_2 receptors in rats and mice.[3,10,12−14]

Peripheral CRF_2 receptors also play an important role in the modulation of cardiovascular function.[15] Peripheral administration of Ucn 2-induced hypotension that was blocked by the selective CRF_2 receptor antagonists, astressin$_2$-B, or antisauvagine-30 in rats.[16] Conversely, CRF_2 receptor knockout mice displayed elevated blood pressure (BP) and did not respond to peripheral injection of Ucn 1 by change in BP.[17] The expression of CRF_2, but not CRF_1 receptors in the endothelial cells of arteries, further supported a direct action of urocortins within the vascular system.[15,18]

While selective CRF_2 receptor endogenous ligands, namely Ucn 2 and Ucn 3, have been characterized recently,[5,6,19] there is no evidence for the existence of endogenous CRF_1 receptor-selective ligands. Ovine CRF displays features of a preferential but not selective CRF_1 receptor agonist, and Ucn 1 has equal affinity for both CRF receptors.[19] Because there is increased evidence for a modulation of CRF_1 receptor-mediated actions by CRF_2 receptors,[20,21] it is important to probe the CRF signaling pathways using selective CRF_1 receptor agonists. Here we describe some of the SAR studies that led to the discovery of stressin$_1$-A. Additionally, we report its biological characterization in in vitro and in vivo CRF_1 receptor-mediated bioassays such as the release of ACTH and the stimulation of colonic motor function, along with the absence of CRF_2 receptor-mediated biological responses on gastric emptying and BP in rats.

Results and Discussions

Synthesis and Physicochemical Characterization. All analogues shown in Table 1 were synthesized using the solid-

phase method of Merrifield on a methylbenzhydrylamine resin using the Boc-strategy, with orthogonal protection of the side chains of the lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[22−24] Main-chain assembly was mediated in most cases by diisopropylcarbodiimide. The best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and OFm protecting groups and when the lactam formation was mediated by BOP. The peptides were cleaved and deprotected in HF and purified with reversed phase high performance liquid chromatography (RPHPLC). Peptides were characterized using RPHPLC, CZE, and MS (Table 1).

Biological Characterization In Vitro. Selected peptides were tested in the rat dispersed anterior pituitary cell culture assay[25] to determine their relative potency and in cloned receptor-based assays to determine their binding affinities for two receptors ($hCRF_1$ receptor and $mCRF_{2\beta}$ receptor)[26] to determine their selectivity (Table 1). In earlier publications, we showed that deletion of the N-terminal residues 1−3[27] up to 1−7[28] yielded agonists with somewhat reduced relative potencies as the size of the peptide decreased. For example, whereas oCRF, Ac-oCRF, and $oCRF_{(4-41)}$ were equipotent in the rat dispersed anterior pituitary cell culture assay, $oCRF_{(6-41)}$ and $oCRF_{(7-41)}$ had 11% and 0.5% of oCRF's potency, respectively.[29]

SAR Studies Based On In Vitro Assays. The assay measuring the inhibition of CRF-stimulated release of ACTH from rat anterior pituitary cells in culture was instrumental in identifying astressin as a new lead for potent CRF antagonists because it was 32 times more potent than any of its predecessors [α-hel-$CRF_{(9-41)}$ or [DPhe^{12},Nle^{21,38}]$hCRF_{(12-41)}$].[30] In the same series, cyclo(26−29), cyclo(28−31), and cyclo(29−32)[DPhe^{12},-Glu^{i},Lys^{i+3},Nle^{21,38}]$hCRF_{(12-41)}$ were ten times less potent, while cyclo(24−27), cyclo(25−28), cyclo(27−30), cyclo(31−34), and cyclo(32−35)[DPhe^{12},Glu^{i},Lys^{i+3},Nle^{21,38}]$hCRF_{(12-41)}$ were less than 2% as potent. With the availability of receptor assays for the CRF_1 receptor and the CRF_2 receptor[26] and as rationalized in the introduction, the Glu^{i}, Lys^{i+3} cyclic lactams spanning the [DPhe^{12},Nle^{21,38}]Ac-$hCRF_{(4-41)}$ sequence (i = 30, 31, and 32) were tested for their affinities for the two cloned CRF receptors (Table 1). A good correlation was found between the relative in vitro potencies derived from the measure of ACTH stimulation in rat anterior pituitary cells in culture by increasing doses of the agonists and K_i at CRF_1 receptor for analogues **4**−**9** (Table 1). Binding affinities for the CRF_2 receptor, however, diverged significantly from binding affinities for CRF_1 in the case of both linear [DPhe^{12},Glu^{31},Lys^{34},Nle^{21,38}]Ac-$hCRF_{(4-41)}$ (**6**) and cyclo(31−34)[DPhe^{12},Glu^{31},Lys^{34},Nle^{21,38}]Ac-$hCRF_{(4-41)}$ (**7**) in that they had very high affinity for the CRF_1 receptor and low affinity for the CRF_2 receptor. This was the first indication that CRF_1 receptor-selectivity measured by the ratio of the CRF_2 receptor K_i/CRF_1 receptor K_i = 93 and 130, respectively, could be modulated by the introduction of a structural constraint similar to that used to achieve CRF_2 receptor selectivity in $astressin_2$-B[3] and consistent with that used for the potent antagonists astressin and astressin B.[2,31]

Although a cyclic constraint in **7** and **9** helped identify a region of the CRF molecule that could be altered to modulate selectivity toward CRF_1 receptor selectivity, the fact that the corresponding linear **6** and **8** also showed similar selectivity suggested that intramolecular (ionic) forces could also induce preferential secondary structures. Introduction of CαMe-leucine (CαMeLeu) at positions 27 and 40 was particularly beneficial (conferred long duration of action) in the cases of astressin B and $astressin_2$-B and was well tolerated in **8** and its corresponding cyclic **9** when compared to **6** and **7** with respect to CRF_1 receptor selectivity.

Because oCRF (**2**) was by itself CRF_1 receptor-selective, we hypothesized that the introduction of the Glu^{31}-Xaa-Xaa-Lys^{34} scaffold in [DPhe^{12},Nle^{21}]oCRF would result in an even more CRF_1 receptor-selective analogue (**10**) than an analogue with the same substitution in Ac-$hCRF_{4-41}$ (**4**). Unexpectedly, this modification resulted in significant loss of binding affinity at CRF_1 receptor (K_i = 66 nM) and the CRF_2 receptor (K_i > 1000 nM). The additional CαMeLeu substitution of **10** to yield **11** resulted in a slight increase in CRF_1 receptor binding affinity in a manner similar to what was observed after CαMethylation of **7** to yield **9**.

Analogue **12**, resulting from the deletion of eight residues at the N-terminus of **10**, was hypothesized to be a potential CRF_1 receptor-selective antagonist yet was essentially inactive in both CRF_1 receptor and CRF_2 receptor binding assays.

Shifting the i−(i+3) bridge consisting of the Glu^{i}-Xaa-Xbb-Lys^{i+3} scaffold in [DPhe,^{12}Nle^{21,38}]Ac-$hCRF_{4-41}$ from positions 31−34 to 32−35 restored high binding affinity at both the CRF_1 receptor and the CRF_2 receptor (**13**) and CαMethylation at positions 27 and 40 was well tolerated (**14**). Agonists **13** and **14** were equipotent (K_i = ca. 1.5 nM) at both receptors. In view of these results, we wondered whether the favorable constraints brought about by the 32−35 bridge would also favor a bioactive conformation despite further deletions at the N-terminus as we had shown for the 30−33 bridge.[22] Indeed, analogues derived from **14** (missing residues 1−3 from the original CRF sequence) and missing residues 1−5 (**15**), 1−6 (**16**) and 1−7 (**17**) retained considerable binding affinity, yet with decreasing relative potencies (Table 1).

While this work was in progress, and after our disclosure of the structure of $stressin_1$-A, Tezval et al.[7] reported the synthesis and biological characterization of cortagine, [Glu^{21},Ala^{40}][Svg1−12]x[human CRF14−30]x[Svg30−40], a chimera derived from two fragments of sauvagine (residues 1−12 and 30−40) and hCRF(residues 14−30) with two substitutions: Glu^{21} to reduce affinity to the CRF binding protein and Ala^{40} to increase CRF_1 receptor selectivity. It is interesting that, using two different yet structurally based approaches, analogs of CRF were identified that showed CRF_1 receptor selectivity. In our assays, however, we found that cortagine was equipotent (relative potency = 0.63) to oCRF (relative potency = 1.0) in the in vitro pituitary cell culture assay and somewhat less selective than had been reported (ratio of CRF_2 receptor K_i/CRF_1 receptor K_i = 200 compared to 30 shown in Table 1),[7] which is not statistically different from the ratio of CRF_2 receptor K_i/CRF_1 receptor K_i = 43 for oCRF. This relatively minor difference may be attributed to the fact that both compounds have very low affinity for the CRF_2 receptor and, thus, their K_is are not well defined.

In Vivo Biological Characterization. ACTH Release: As shown in Figure 1, plasma ACTH levels of vehicle-injected rats remained below 80 pg/mL at all times and showed the expected circadian variations. Both CRF and $stressin_1$-A induced significant (p < 0.01) and dose-related increases in ACTH levels at the 10 min time point, but the pituitary response to 5.0 μg oCRF/kg (1810 ± 239 pg ACTH/mL) was significantly larger than that measured after 5.0 μg $stressin_1$-A/kg (752 ± 210 pg ACTH/mL, p < 0.01). Animals administered 1.0 or 5.0, but not 0.2, μg oCRF/kg still had elevated (p < 0.01) ACTH values 1 h after injection, and these values had returned to control levels by 3 h. $Stressin_1$-A, and **9** at 1.0 and 5.0 μg/kg and **6** at 5.0 μg/kg also increased (p < 0.01) ACTH concentrations 1 h after

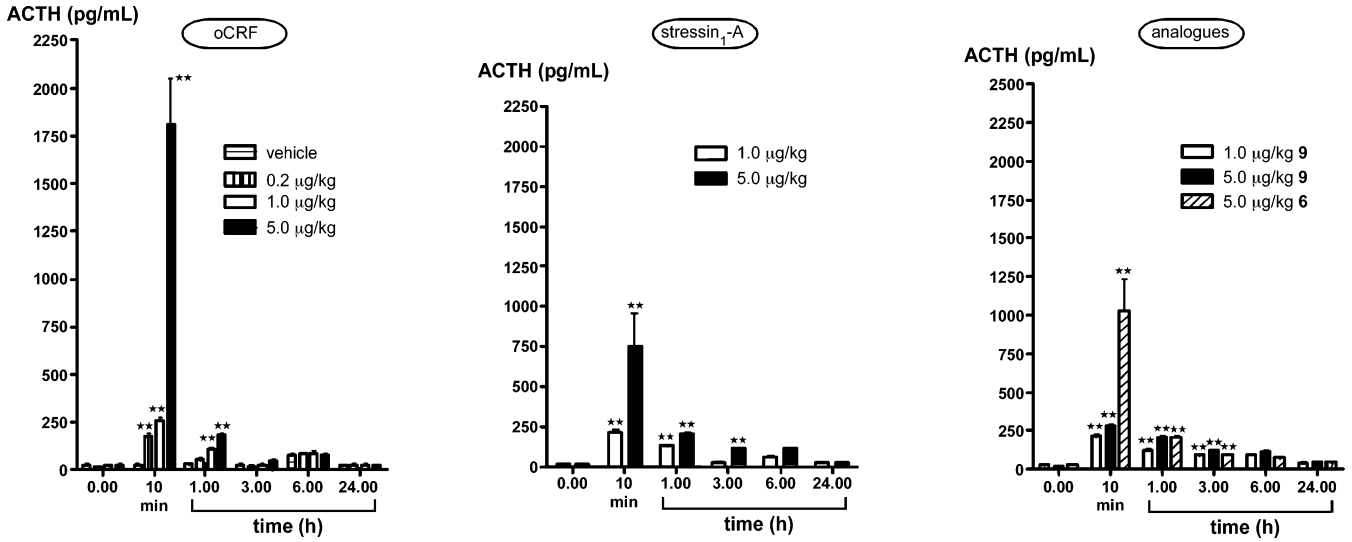

Figure 1. Plasma ACTH levels in rats injected with the vehicle, **2** (oCRF), **6**, **7** (stressin$_1$-A), and **9**. Each point represents the mean ± SEM of 6−7 animals. $^{**}p < 0.01$ vs vehicle.

treatment. The observation that the higher dose of the three peptides maintained up-regulated ($p < 0.01$) ACTH release at the 3 h time points suggests that their duration of action is slightly extended compared to that of oCRF.

Gut Motor Function and BP: Peptides were tested in three in vivo assays that are known to be mediated by either the CRF$_1$ receptor or the CRF$_2$ receptor. These are a colonic propulsive motor function test in nonfasted rat, gastric emptying of non-nutrient meal in an overnight fasted rat model and arterial BP measure in an overnight fasted and urethane anesthetized rat model. CRF$_1$ receptor activation is well established to increase colonic transit and motility,[11,14] while peripheral CRF$_2$ activation by CRF and related family members decreases gastric emptying[10,12−14] and BP[16] in rodents. Because these activities are mediated by either one or the other CRF receptor (as shown), these measurements were critical in our evaluation of the peptides' selectivity. We found that Ucn 2, but not stressin$_1$-A, injected ip, delayed gastric emptying in conscious rats. Stressin$_1$-A injected ip at 10 μg/kg had no significant effect on gastric emptying of a viscous noncaloric solution in conscious rats (51.0 ± 8.8% compared with 50.0 ± 2.5% in the vehicle group: $p = 0.92$; Figure 2A). By contrast, ip hUcn 2 given at the same dose significantly reduced gastric emptying to 26.4 ± 6.9% compared to both vehicle and stressin$_1$-A groups (Figure 2A). We also found that stressin$_1$-A injected ip stimulated colonic motor response in conscious rats. In nonfasted conscious rats, stressin$_1$-A injected ip at 10 μg/kg significantly increased the 60 min fecal pellet output when compared to saline-injected rats (8.0 ± 1.6 vs 0.5 ± 0.5 /h; Figure 2B). Time course of the cumulative effects of stressin$_1$-A on fecal pellet showed an increased response at 30 min that became significant at 45 and 60 min. In addition, stressin$_1$-A induced diarrhea in 33% of the rats, whereas none of the saline-injected rats had diarrhea as monitored 60 min postinjection (Figure 2B). Finally, stressin$_1$-A, injected iv, did not modify arterial BP. In urethane anesthetized rats, mean basal BP was 75.9 ± 3.5 mmHg and was not significantly modified after vehicle iv injection (77.4 ± 1.3 mmHg; $p = 0.14$ vs basal). Neither consecutive iv injections of stressin$_1$-A at 3, 10, or 30 μg/kg nor of cortagine at 3, 10, or 30 μg/kg produced significant changes in BP compared to vehicle (+2.3 ± 1.4%, $p > 0.05$; −0.1 ± 0.7%, $p > 0.05$; −1.6 ± 2%, $p > 0.05$ for stressin$_1$-A and 1.4 ± 0.8%, $p > 0.05$; 0.1 ± 0.3%, $p > 0.05$; −3.2 ± 0.7%, $p > 0.05$ for cortagine; data not shown).

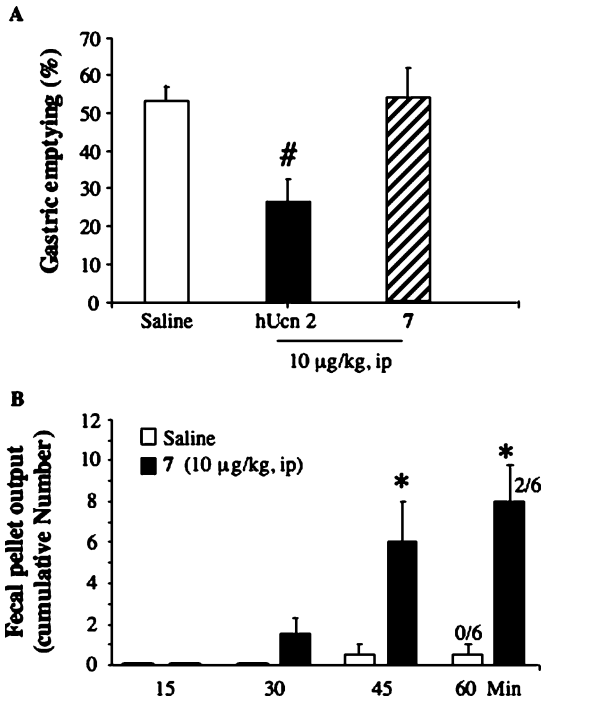

Figure 2. (A) Ucn 2 (hUcn 2) but not **7** (stressin$_1$-A) injected ip decreased gastric emptying of a viscous noncaloric meal in conscious rats. Overnight fasted rats were injected ip with saline, Ucn 2, or **7** (stressin$_1$-A) and, 10 min later, gavaged with the phenol red methyl-cellulose solution, and 20 min later, gastric emptying was monitored. Each column is the mean ± SEM of 5−6 rats/group. $\#p < 0.05$ compared with both vehicle and stressin$_1$-A groups. (B) Compound **7** injected ip stimulated colonic motor function in conscious rats. Nonfasted rats were injected ip with saline or **7** (stressin$_1$-A, 10 μg/kg) and fecal pellet output and diarrhea in conscious rats was monitored. Each column represents the mean ± SE of 6 rats/group. The numbers on the bars are the number of rats with diarrhea over the total in a group. $^{*}p < 0.05$ vs saline.

Using one in vivo bioassay (colonic fecal expulsion and diarrhea), in which the role of CRF$_1$ receptors has been well established to mediate the effects of peripheral injection of CRF and Ucn 1,[11] and two assays (decrease in gastric emptying and BP changes induced by CRF or urocortins) mediated by CRF$_2$ receptor, we showed that peripheral administration of stressin$_1$-A resulted solely in a CRF$_1$ receptor-mediated biological response.

We previously reported that the CRF_1 receptor/CRF_2 receptor agonists, Ucn 1, or CRF,[19] injected ip at 10 μg/kg, stimulated colonic transit while inhibiting gastric emptying through CRF_1 and CRF_2 receptors, respectively, in rats.[14] Therefore, the lack of change in gastric emptying together with potent stimulation of colonic motor function induced by stressin$_1$-A injected ip provided in vivo evidence for the CRF_1 receptor selectivity of stressin$_1$-A. This is further corroborated by the lack of change in BP when stressin$_1$-A was injected iv and by several literature reports that emphasize the specific role of CRF_2 receptors in the CRF or Ucn 1-induced drop in BP.[3,16,17]

To conclude, potent peptidic CRF_2 receptor agonists (urocortins 2 and 3) and antagonists (astressin$_2$-B, anti-sauvagine-30) have been described previously. Here we report the development of stressin$_1$-A and present in vitro and in vivo data providing convergent evidence that stressin$_1$-A is a novel potent and selective CRF_1 receptor agonist that will be very useful for characterizing biological actions mediated by the CRF_1 receptor.[32] Structural studies (NMR) are presently being carried out to determine the conformational differences that contribute to receptor selectivity. Still missing is a potent and long acting peptide CRF_1 receptor antagonist to complete the panoply of peptide reagents that are critical for unraveling the physiological roles of the CRF_1 receptor and the CRF_2 receptor, their possible inhibitory or potentiating interactions both centrally and peripherally and, ultimately, their pharmacological clinical potential.

Experimental Section

Animals. Adult male Sprague−Dawley rats (Harlan, San Diego, CA) weighing 250−300 g were housed in group cages under controlled illumination (12:12-h light−dark cycle starting at 6 AM), humidity, and temperature (21−23 °C) and had free access to tap water and Purina rat chow. Protocols were approved by the respective IACUCs of the University of California Los Angeles and Veteran Affairs Greater Los Angeles Healthcare System Animal Research Committees and the Salk Institute. When required by the experiments, rats were deprived of food but had free access to tap water for ∼16 h before the experiment.

Administration of Peptides. Immediately before injection, stressin$_1$-A was dissolved in vehicle (water or saline). Human Ucn 2 and cortagine were dissolved in water. Ip and iv injections were done, respectively, in 0.3 mL and in 0.1−0.5 mL.

Rat Dispersed Pituitary Cell Culture Assay. CRF analogues were tested for agonist activity in an in vitro assay measuring release of ACTH by collagenase dispersed rat anterior pituitary cells in culture.[27,29] For each in vitro experiment, approximately 50 anterior pituitary glands from male Sprague−Dawley rats were dissociated by collagenase treatment and plated (0.16×10^6 cells/well in 48-well plates) in medium containing 2% fetal bovine serum. Three days after plating, the cells were washed three times with fresh medium containing 0.1% bovine serum albumin (BSA) and incubated for 1 h. Following the 1 h preincubation, the cells were washed once more and the test peptides were applied in 1 mL final volume of media. At the end of a 3 h incubation period, the media were collected and 10 μL was tested for the level of ACTH using a radioimmunoassay (Nichols Institute Diagnostic).

Cloned Receptor-Based Binding Assays. The K_i values given in Table 1 reflect the affinities of the analogues for the cloned type 1 human and type 2β mouse CRF receptors. The values were derived from competitive radioligand displacement assays using the nonselective ^{125}I-labeled agonist [Tyr^0,Glu^1,Nle^{17}]-sauvagine as the radioligand and crude membrane fractions from CHO cells stably expressing the respective receptors.[26] Briefly, 200 000 cpm (ca. 0.5 nM) ^{125}I-[Tyr^0,Glu^1,Nle^{17}]-sauvagine were combined with increasing concentrations of peptide (0.1−1000 nM) in 0.2 mL assay buffer (50 mM Na Hepes, pH 7.5; 10 mM $MgCl_2$; 2 mM EGTA; 0.1% BSA) and incubated for 90 min at 18 °C.

Measure of Gastric Emptying of Non-Nutrient Meal. Gastric emptying of a non-nutrient viscous meal was determined by the phenol red method as described previously in conscious overnight fasted rats.[33] The noncaloric meal consisted of a viscous suspension of continuously stirred 1.5% methylcellulose (wt/vol) containing phenol red (50 mg/100 mL) given orogastrically through a stainless steel gavage tube (in 1.5 mL volume) to conscious rats. At 20 min after the administration of the solution, rats were euthanized by CO_2 inhalation. The abdominal cavity was opened, the gastroesophageal junction and the pylorus were clamped, and the stomach was isolated and rinsed in 0.9% saline. The stomach was placed into 100 mL 0.1 N NaOH and homogenized (Polytron; Brinkman Instruments, Westbury, NY). The suspension was allowed to settle for 60 min at room temperature, and then 5 mL supernatant was added to 0.5 mL of 20% trichloroacetic acid (wt/vol) and centrifuged at 3000 rpm at 4 °C for 20 min. After the supernatant was mixed with 4 mL of 0.5 N NaOH, the absorbance of the sample was read at 560 nm (Shimazu 260 spectrophotometer). The absorbance of the phenol red recovered from animals euthanized immediately after gavage of the liquid meal was taken as a standard 0% emptying. The percentage of emptying during the 20 min period was calculated with the following formula: percent emptying = (1 − absorbance of test sample/absorbance of standard) × 100.

Measure of Colonic Propulsive Motor Function. In nonfasted conscious rats, defecation was monitored as described previously[34] by counting the number of fecal pellets excreted every 15 min for 1 h. The incidence of diarrhea was assessed as percent of rats that developed watery stool from the total number of treated rats.

Measure of Arterial BP. Overnight fasted rats were anesthetized with urethane (1.5 g/kg). Each rat was then fitted with a tracheal cannula for ventilation, a jugular vein catheter for hydration through continuous perfusion of sterile saline (0.4 mL/h) and for peptide injection, and a femoral artery catheter for BP measurement. The femoral catheter was connected to a preamplifier (model 600; Millar Instruments, Houston, TX), the output of which was subsequently amplified via a single-ended connection to the transducer amplifier. BP was acquired online at a sampling rate of 300 Hz via a Micro1401 A/D interface (Cambridge Electronic Design, Cambridge, UK) connected to a Pentium II class computer running Spike 2 (Cambridge Electronic Design) version 5 data acquisition software.

Effect of iv oCRF and Stressin$_1$-A on ACTH Release In Vivo. Adrenal-intact adult (ca. 60 days old) male Sprague−Dawley rats were implanted with iv cannulae, as described earlier.[20] The test was conducted under minimum-stress conditions in fully awake and freely moving animals that were not handled for either peptide injection or blood sampling. A first blood sample was obtained, immediately followed by the iv injection of the vehicle, oCRF (0.2, 1.0 or 5.0 μg/kg) or stressin$_1$-A (1.0 or 5.0 μg/kg). Subsequently, blood samples were obtained 10 min, as well as 1, 3, 6, and 24 h later. ACTH levels were measured in plasma with an immunoradiometric assay from DiaSorin Inc. (Stillwater, MN).

Effect of Intraperitoneal hUcn 2 and Stressin$_1$-A on Gastric Emptying in Conscious Rats. Rats were deprived of food overnight, but had free access to tap water for ∼16 h before the experiment. Fasted rats were injected ip (0.3 mL) with saline, Ucn 2 (10 μg/kg), or stressin$_1$-A (10 μg/kg). At 10 min after the ip injection, the phenol red methylcellulose solution was delivered through orogastric gavage (1.5 mL), and gastric emptying was determined 20 min later. Doses of peptides were based on previous dose−response studies showing a maximal response of peripheral injection of Ucn 2 on gastric emptying in rats and mice.[12,14]

Effect of ip Stressin$_1$-A on Colonic Motor Response in Conscious Rats. Rats were injected ip with saline or stressin$_1$-A (10 μg/kg) and then immediately placed in individual cages to monitor the fecal pellet output and diarrhea response. These colonic responses were monitored every 15 min for 60 min after the ip injection. The dose of stressin$_1$-A was selected on a previous dose−response study showing maximal colonic response to h/r CRF injected ip at 10 μg/kg.[14,34]

Effect of iv Stressin$_1$-A and Cortagine on Arterial BP in Anesthetized Rats. Basal BP was monitored for 150 to 200 min using an acute catheter positioned into the femoral artery in urethane anesthetized rats ($n = 3$). Each animal received consecutive intrajugular injections of vehicle (distilled water), stressin$_1$-A at 3, 10, and 30 μg/kg, and cortagine at 3, 10, and 30 μg/kg at 15−20 min intervals. BP was recorded throughout the studies. For each treatment, mean basal BP was recorded for 5 min before and for 2 min 30 s after the injection. Normalized values were obtained by subtracting mean basal BP from the mean BP after injection to obtain the net BP response and dividing it by the mean basal BP, yielding the percent change from the basal state.

Statistical Analysis. All results are expressed as mean ± SEM. Comparison between multiple groups was performed by one-way ANOVA, followed by Student Newman−Keuls multiple comparisons.

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