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A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle

[urotensin I/seven transmembrane domain/α-helical corticotropin-releasing factor-(9–41)]

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ABSTRACT **Corticotropin-releasing factor (CRF) mediates many critical aspects of the physiological response to stress. These effects are elicited by binding to specific high-affinity receptors, which are coupled to guanine nucleotide stimulatory factor (G_s)-response pathways. Recently, a gene encoding a receptor for CRF, expressed in pituitary and the central nervous system (PC-CRF receptor), was isolated and characterized. Here we report the identification and characterization of a second, distinct CRF receptor that is expressed primarily in heart and skeletal muscle and exhibits a specific ligand preference and antagonist sensitivity compared with the PC-CRF receptor. We refer to this second receptor as the heart/muscle (HM)-CRF receptor.**

Corticotropin-releasing factor (CRF) (1) is a member of a family of peptides from different species that act as agonists of the CRF receptor. These peptides include the frog skin peptide, sauvagine (2), and the teleost fish urophysis peptide, urotensin I (3). CRF is a 41-amino acid hypothalamic neuropeptide that plays a central role in coordinating the communications between endocrine, nervous, and immune systems to achieve homeostasis in response to environmental adversities (4, 5). The peptide was originally characterized in the hypothalamo–hypophyseal system but was later found to be widely distributed throughout the central nervous system (CNS), where it appeared to function as a neurotransmitter or neuromodulator (6). In the CNS, CRF initiates the hypothalamic–pituitary–adrenal axis by stimulating the release of adrenocorticotropin (ACTH) and β-endorphin from the anterior pituitary. ACTH stimulates adrenal cortex to secrete corticosteroids that, in turn, elicit a wide range of biological responses and exert negative feedback on the hypothalamus and pituitary (4, 5, 7). Both sauvagine and urotensin I have been shown to stimulate the hypothalamic–pituitary–adrenal axis after i.v. administration (8). Intracerebroventricular administration of CRF provokes stress-like responses including activation of the sympathetic nervous system, resulting in an elevation of plasma epinephrine, norepinephrine, and glucose, which results in increased heart rate and mean arterial blood pressure (9, 10). Outside the CNS, CRF immunoreactivity is detectable in multiple peripheral organs, including placenta, adrenal medulla, pancreas, lung, stomach, duodenum, and liver (4, 5). i.v. administration of CRF, sauvagine, and urotensin I has been shown to elicit peripheral systemic responses, including vasodilation (11).

CRF functions by binding to a membrane-bound receptor that is coupled to the guanine nucleotide stimulatory factor (G_s) signaling protein, resulting in increased intracellular cAMP levels (12, 13). The relative density of CRF receptors is highest in the anterior and intermediate lobes of the pituitary (14, 15), moderate in discrete areas of CNS (16), and lower, but detectable, in spleen (17). Recently, cDNAs encoding a CRF receptor expressed primarily in pituitary and the CNS (PC-CRF receptor) have been identified and characterized (18–20). The PC-CRF receptor belongs to a subfamily of receptors for peptides including vasoactive intestinal polypeptide [VIP (21)], secretin (22), calcitonin (23), and growth hormone-releasing factor [GRF (24–26)]. The PC-CRF receptor mRNA was found to be most highly expressed in the cerebellum and pituitary and found at lower levels in other brain areas, intestine, and testes. This receptor is undetectable in other tissues examined, including heart and skeletal muscle (18–20). However, physiological studies have indicated the presence of a CRF-responsive receptor that mediates a positive inotropic effect in isolated heart (27) and myocardium (28) and suppression of vascular leakage in skeletal muscle (29). Here we report the identification and characterization of another receptor that mediates intracellular responses induced by sauvagine, urotensin I, and CRF. This receptor is expressed specifically in heart and skeletal muscle and, as such, is termed the heart and muscle CRF receptor (HM-CRF receptor).

MATERIALS AND METHODS

Genomic DNA and cDNA Cloning. Two million plaques from a mouse 129 genomic lambda FIXII library were screened by a ^{32}P-labeled rat PC-CRF receptor cDNA (18) 1.4-kb *Hind* III/*Bam*HI fragment as a probe with 50% (wt/vol) formamide, 5× SSPE (1× SSPE is 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 3× Denhardt's solution, 0.5% SDS, and 100 μg of salmon sperm DNA per ml at 37°C. Membranes were washed in 2× SSC/0.05% SDS at 37°C. The positive clones were then subjected to restriction endonuclease mapping and DNA sequencing, identifying a PC-CRF receptor gene-related clone (HM-CRF receptor gene). A 0.5-kb *Pst* I fragment of HM-CRF receptor gene was used to screen a mouse heart, random-primed and oligo(dT)-primed cDNA library (Clontech) under the conditions described above for the genomic library screening. However, the hybridization temperature was 42°C, and membranes were washed with 0.1× SSC solution at 42°C.

Northern Blot Analysis. Poly(A)$^+$ RNAs from a selection of mouse tissues were immobilized and hybridized at 42°C with 50% formamide using a ^{32}P-labeled 0.5-kb *Pst* I fragment of HM-CRF receptor gene as a probe. This fragment spans sequence from transmembrane (TM) V to VII and includes a minimum intron sequence based on comparison to the coding sequence of PC-CRF receptor. The blot was washed twice at room temperature in 2× SSC/0.05% SDS for 20 min and twice at 50°C in 0.1× SSC/0.1% SDS for 20 min.

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Abbreviations: CRF, corticotropin-releasing factor; PC-CRF receptor, pituitary/central nervous system CRF receptor; HM-CRF receptor, heart/muscle CRF receptor; CNS, central nervous system; ACTH, adrenocorticotropin; VIP, vasoactive intestinal polypeptide; GRF, growth hormone-releasing factor; TM, transmembrane; αhel, α helical.

Expression and Functional Assay of the Cloned Receptor. The full-length cDNA (HM-CRF receptor) was subcloned into expression vector pCEP4 (Invitrogen) and was transfected into CV-1 and 293-EBNA kidney cells (Invitrogen) by calcium phosphate precipitation. Stable transfectants were selected in the presence of hygromycin B at 200 μg/ml. Cells expressing either the HM-CRF receptor or the PC-CRF receptor (18) were treated with 50 μM 3-isobutylmethyl-1-methylxanthine (Sigma) with or without the CRF receptor antagonist α-helical (αhel) CRF-(9–41) (Peninsula Laboratories) for 20 min at 37°C. Peptides were added and incubated for an additional 20 min at 37°C. The cells were extracted, and levels of intracellular cAMP were determined in triplicate by using a [^{3}H]cAMP assay system (Amersham), as described (18).

RESULTS

Cloning of HM-CRF Receptor Gene. In an attempt to identify additional members of the CRF class of receptors, we screened a mouse genomic library under low-stringency conditions by using a full-length rat PC-CRF receptor cDNA as a probe. Fourteen independent clones were obtained, of which six hybridized with an oligonucleotide derived from PC-CRF receptor sequence within TM VI–VII region. Restriction endonuclease mapping and DNA sequencing revealed, in addition to the expected PC-CRF receptor gene, a second gene containing a nucleotide sequence different from, but homologous to, the PC-CRF receptor.

To determine the expression pattern of the HM-CRF receptor gene, mRNA blot analysis was done by using a 0.5-kb *Pst* I fragment of the gene as a probe under high-stringency conditions. A single, moderately intense 3-kb mRNA band was detected in heart and muscle, and very weak hybridization was seen in brain and lung (Fig. 1), whereas no hybridization was detected in mRNA from other tissues tested. RNase protection assays confirmed the specificity of the hybridization results, revealing an identical pattern of expression (data not shown). In contrast with the HM-CRF receptor, no PC-CRF receptor gene expression was seen in heart or muscle (18, 19).

Cloning and Characterization of the HM-CRF Receptor cDNA. Several clones were obtained from a mouse heart cDNA library, with the 0.5-kb *Pst* I gene fragment as a probe. A full-length HM-CRF receptor cDNA was constructed by containing a 1293-bp open reading frame encoding a 431-amino acid protein. Amino acid sequence comparison (Fig. 2) shows that the mouse HM-CRF receptor belongs to the CRF/GRF/VIP/secretin family of G protein-coupled seven-transmembrane-domain receptors. The HM-CRF receptor shares amino acid homology to the mouse PC-CRF receptor (66%), rat secretin receptor (30%), rat VIP receptor (26%), and mouse GRF receptor (30%). Especially conserved is the sequence of the third cytoplasmic, G protein-coupling region (TM V–TM VI) of mouse HM-CRF receptor, which is completely identical to that of mouse PC-CRF receptor (20). The sequence homology throughout the TM regions is 82% between these two receptors. Four potential N-linked glycosylation sites in the N-terminus extracellular region are shown in Fig. 2. Also shown are putative phosphorylation sites for protein kinase A and casein kinase II in the third cytoplasmic region and C terminus, as well as a protein kinase C phosphorylation site in the C terminus.

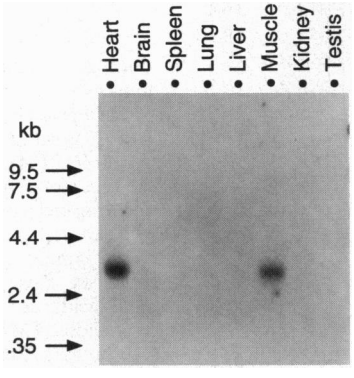

FIG. 1. Expression of the HM-CRF receptor in heart and muscle tissues. A blot immobilized with mouse tissue poly(A)$^+$ RNAs (2 μg each) was used to hybridize with an HM-CRF receptor gene-specific fragment. A single transcript (3 kb) was detected, as shown.

To evaluate whether the HM-CRF receptor functions as a CRF receptor subtype, a transcription unit encoding the entire receptor was stably transfected to CV-1 and 293-EBNA cells, which do not normally exhibit any response to CRF. The transfected cells were treated with a variety of bioactive peptides and assayed for changes in intracellular cAMP. Because both CRF and sauvagine are agonists for the PC-CRF receptor (18), it became of particular interest to compare the effects of these peptides on the HM-CRF receptor. As shown in Fig. 3*A*, cells expressing the HM-CRF receptor responded to frog sauvagine, teleost fish urotensin I, and rat CRF in a dose-dependent manner with half-maximal concentrations at ≈0.1 nM, 0.3 nM, and 3 nM, respectively. The maximal effects of each agent are virtually identical. Cells expressing PC-CRF receptor responded to CRF and CRF-like ligands with the same half-maximal concentration (0.3 nM), and the maximal effect of CRF was similar to or slightly higher than CRF-like ligands (Fig. 3*B*). No response was observed by either receptor at 1 μM of secretin, VIP, glucagon, parathyroid hormone, calcitonin gene-related peptide, amylin, adrenomedulin, or Met-enkephalin (data not shown).

A CRF receptor antagonist, αhel CRF-(9–41), preferentially blocks autonomic nervous and peripheral vasodilatory functions rather than CRF activity in the hypothalamic–pituitary–adrenal axis (30, 31). To further demonstrate the functional difference between the HM-CRF and PC-CRF receptors, we examined the inhibitory effect of αhel CRF-(9–41) on CRF-induced intracellular cAMP accumulation. CRF concentrations (3 nM, 0.3 nM) required for half-maximal stimulation of cAMP accumulation were used to evaluate the efficacy of the antagonist on HM-CRF receptor- and on PC-CRF receptor-transfected cells, respectively. As shown in Fig. 4, αhel CRF-(9–41) exerted a more potent dose-dependent inhibitory effect on the HM-CRF receptor (IC_{50} = 10 nM or less) than on the PC-CRF receptor (IC_{50} = 100 nM). These values were consistently obtained, when either sauvagine or urotensin I was used as the agonist (data not shown). This higher antagonist sensitivity of the HM-CRF receptor parallels the classical pharmacological data, demonstrating that this antagonist preferentially inhibits the peripheral effects, rather than the pituitary effects, of CRF.

DISCUSSION

Recently, *in situ* hybridization studies (32) indicated that PC-CRF receptor is only expressed in a subset of tissues found to contain CRF-binding sites by radioligand-binding studies. This difference can be partially explained by CRF-binding protein in some tissues (33). Nevertheless, PC-CRF receptor mRNA is low or undetectable in several regions implicated as important sites of CRF action. Furthermore, because CRF plays a pivotal role in integrating the functions of multiple systems, it is likely that there are multiple tissue-specific CRF receptor subtypes. Molecular cloning and characterization of the HM-CRF receptor will allow a more detailed investigation of CRF function in nonendocrine tissues.

Little information regarding CRF and CRF-binding sites in heart and skeletal muscle currently exists. Nevertheless, CRF has been shown to elicit a hypotensive effect on the cardio-

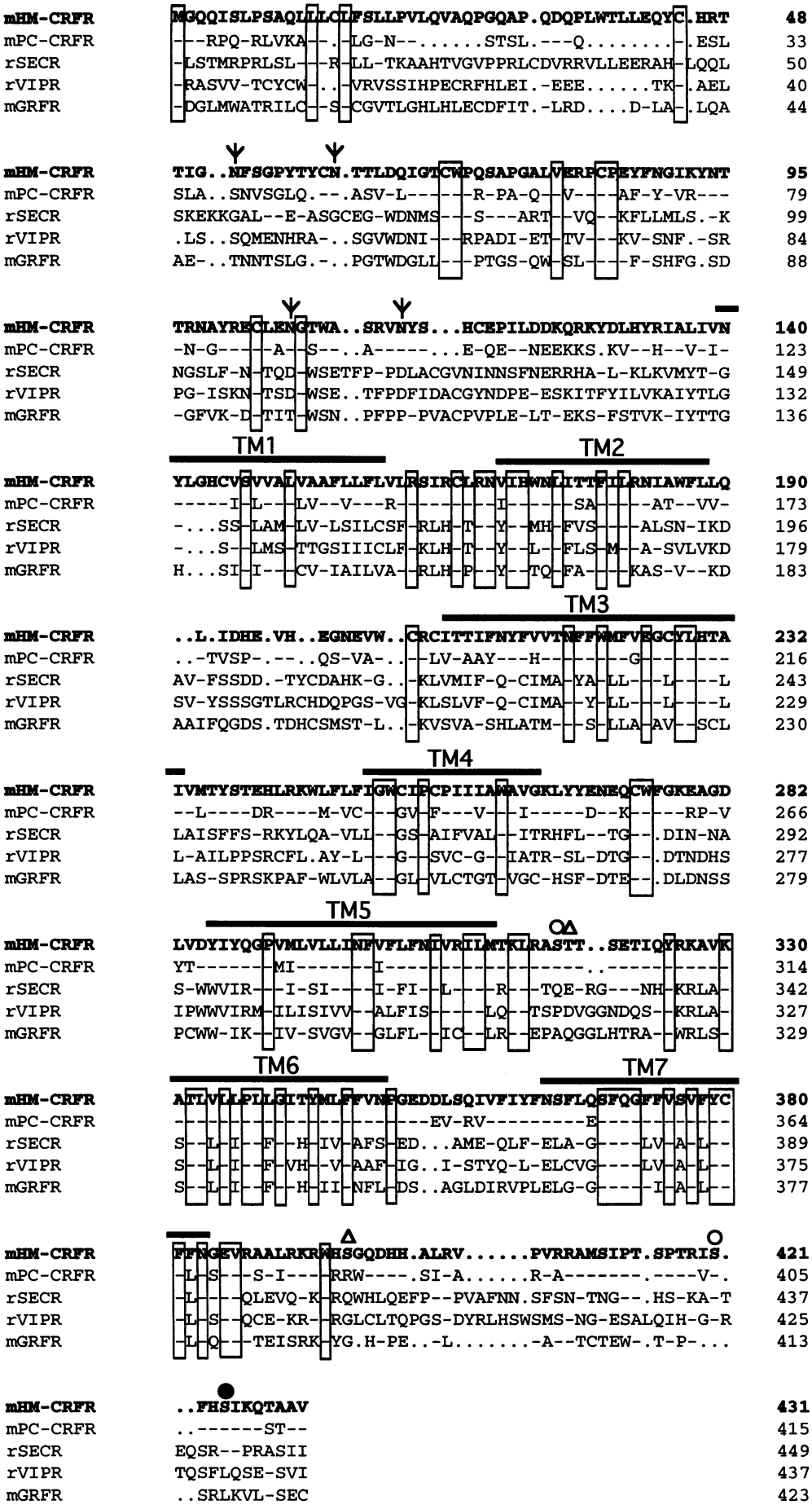

FIG. 2. Amino acid sequence comparison of the mouse (m) HM-CRF receptor (R) with mouse PC-CRF (23), rat (r) secretin (SEC) (25), rat VIP (24), and mouse GRF (27) receptors. Amino acids that are conserved with mHM-CRF receptor are represented as hyphens; deleted amino acids are represented as dots. The conserved amino acids are boxed. Putative TM regions TM1–TM7 are indicated by bars. Potential glycosylation sites (Ψ) and phosphorylation sites for protein kinase A (○), protein kinase C (●), and casein kinase II (△) are indicated in the HM-CRF receptor sequence.

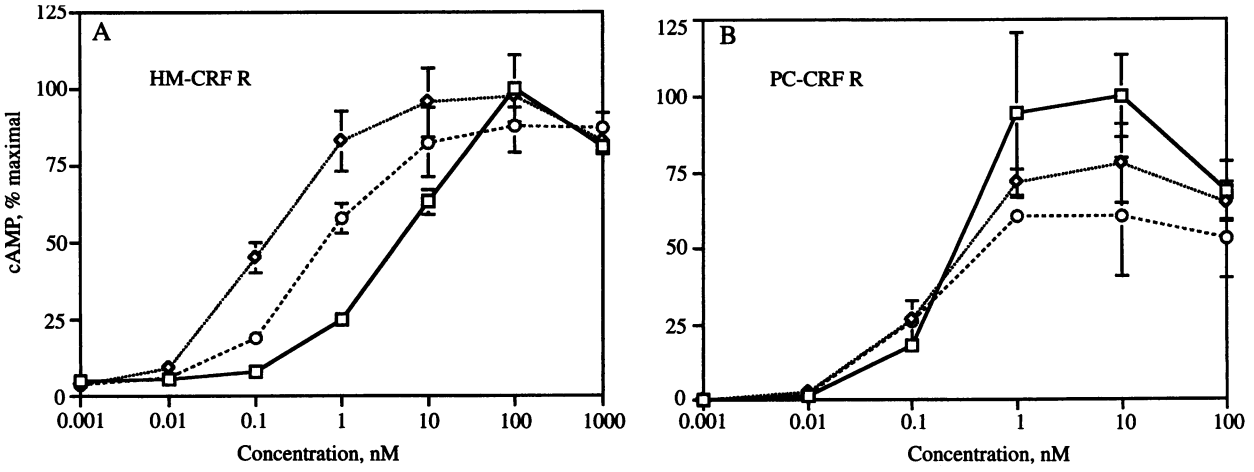

FIG. 3. Functional effects of ligands on HM-CRF receptor. Permanently transfected cells were prepared, expressing either HM-CRF receptor (R) or PC-CRF receptor, as described. Intracellular cAMP accumulations in cells transfected with HM-CRF receptor (*A*) or PC-CRF receptor (*B*) in response to CRF (□), sauvagine (◇), and urotensin I (○) are shown as percentages of maximal response.

vascular system in contrast to its hypertensive effect, which it mediates through the hypothalamic–pituitary–adrenal axis and sympathetic autonomic nervous system (4, 9–11). Additionally, a direct positive inotropic effect of CRF has been shown on isolated guinea pig ventricular myocardium (28) and on isolated working rat heart (27, 34). CRF also induces, in isolated rat heart, a short-term increase in the release of atrial natriuretic factor, perhaps supplying enough to modulate blood pressure through its diuretic, natriuretic, and vasodilatory actions (34). The identification of the HM-CRF receptor as another member of the CRF/GRF/VIP/secretin receptor family thus provides a molecular basis for understanding the mechanism by which CRF regulates the described areas of cardiovascular function.

Similar hypotensive actions and corticotropin-releasing activity in pituitary have also been observed for the structurally homologous peptides sauvagine and urotensin I (8, 11). Although CRF, sauvagine, and urotensin I are equipotent in releasing ACTH, it is important to note that sauvagine and urotensin I are 10-fold more potent than CRF as vasodilator agents (35). Our results are consistent with this, as sauvagine and urotensin I are at least 10-fold more potent than CRF as inducers of intracellular cAMP levels in cells expressing HM-CRF receptor. Taken together, these findings suggest that CRF may not be the principle endogenous ligand for the HM-CRF receptor and that a ligand, structurally closer to sauvagine or urotensin I, may be of physiological importance.

In vivo, the CRF antagonist, αhel CRF-(9–41), is a more potent inhibitor of CRF-induced hypotension and tachycardia than of CRF-induced elevations of plasma ACTH and β-endorphin (30, 31). In accordance with those studies, our results show that the antagonist αhel CRF-(9–41) is more potent in inhibiting HM-CRF receptor- rather than PC-CRF receptor-mediated cAMP response.

Although the two CRF receptors exhibit a distinct ligand preference, the 100% amino acid homology within the G protein-coupling region suggests that both receptors activate similar signal transduction components and are subject to similar mechanisms of down-regulation (36).

It was also suggested that members of this receptor subfamily might be involved in critical regulatory events in organogenesis. Analysis of the GRF receptor in dwarf mouse strains, snell (*dw*), and little (*lit*) indicated that in addition to regulating growth hormone secretion, GRF plays a crucial role in the ontogeny and proliferation of somatotropes (24, 37). CRF has also been implicated in regulating cell growth, both in corti-

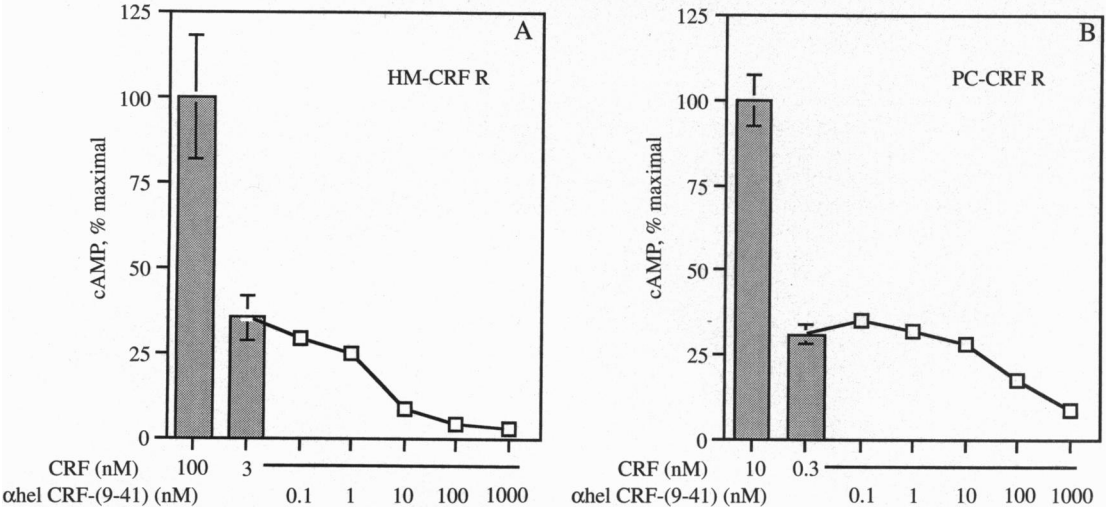

FIG. 4. Comparison of inhibitory effect of αhel CRF-(9–41) on CRF-stimulated intracellular cAMP accumulation in cells transfected with HM-CRF receptor (R) (*A*) or PC-CRF receptor (*B*). The CRF concentration required for half-maximal effect on cAMP stimulation, as determined for HM-CRF receptor- and PC-CRF receptor-transfected cells in Fig. 3, was used to test the potency of the antagonist αhel CRF-(9–41) on the two receptors.

cotrope hyperplasia (38) and in human small cell lung cancer cells, where CRF stimulates clonal cell growth that is reversed by αhel CRF-(9–41) (39). It is tempting to speculate that CRF or a mammalian sauvagine homolog is involved in the autocrine or paracrine regulation of myocyte growth and hypertrophy.

In summary, we have cloned a heart- and skeletal muscle-specific CRF receptor (HM-CRF receptor) that exhibits a >10-fold more potent stimulation by sauvagine than by CRF. With its specific expression pattern, the HM-CRF receptor may play a crucial role in the development, function, and pathogenesis of the myocardium.

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