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Cloning and functional pharmacology of two corticotropin-releasing factor receptors from a teleost fish

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Abstract

Although it is well established that fish possess corticotropin-releasing factor (CRF) and a CRF-like peptide, urotensin I, comparatively little is known about the pharmacology of their cognate receptors. Here we report the isolation and functional expression of two complementary DNAs (cDNAs), from the chum salmon *Oncorhynchus keta*, which encode orthologues of the mammalian and amphibian CRF type 1 (CRF_1) and type 2 (CRF_2) receptors. Radioligand competition binding experiments have revealed that the salmon CRF_1 and CRF_2 receptors bind urotensin I with ~8-fold higher affinity than rat/human CRF. These two peptides together with two related CRF-like peptides, namely, sauvagine and urocortin, were also tested in cAMP assays; for cells expressing the salmon CRF_1 receptor, EC_{50} values for the stimulation of cAMP production were between 4.5 ± 1.8 and 15.3 ± 3.1 nM. For the salmon CRF_2 receptor, the corresponding values were: rat/human CRF, 9.4 ± 0.4 nM; urotensin I, 21.2 ± 2.1 nM; sauvagine, 0.7 ± 0.1 nM; and urocortin, 2.2 ± 0.7 nM. We have also functionally coupled the *O. keta* CRF_1 receptor, in *Xenopus laevis* oocytes, to the endogenous Ca^{2+}-activated chloride conductance by co-expression with the G-protein α subunit, $G_{\alpha 16}$. The EC_{50} value for channel activation by rat/human CRF (11.2 ± 2.6 nM) agrees well with that obtained in cAMP assays (15.3 ± 3.1 nM). We conclude that although sauvagine is 13- and 30-fold more potent than rat/human CRF and urotensin I, respectively, in activating the salmon CRF_2 receptor, neither receptor appears able to discriminate between the native ligands CRF and urotensin I.

Keywords: Adenylate cyclase; Corticotropin-releasing factor receptor; $G_{\alpha 16}$; Urocortin; Urotensin I; *Xenopus* oocyte expression

1. Introduction

Corticotropin-releasing factor (CRF) is a 41-residue neuropeptide that was originally isolated from ovine hypothalamus by virtue of its ability to stimulate the secretion of adrenocorticotropic hormone (ACTH) and β-endorphin from anterior pituitary cells (Spiess et al., 1981; Vale et al., 1981). This molecule, which coordinates the body's response to stressors (see Ito and Miyata, 1999; Koob and Heinrichs, 1999), binds to two main receptor subtypes, CRF_1 and CRF_2, that are members of the G-protein-coupled receptor superfamily (Chalmers et al., 1996; Radulovic et al., 1999). The latter receptor exists, in several species, in two forms ($CRF_{2\alpha}$ and $CRF_{2\beta}$) that arise by alternative splicing and which have different amino-terminal sequences (Chalmers et al., 1996). In addition, a third CRF_2 receptor isoform ($CRF_{2\gamma}$) has been described in man (Kostich et al., 1998), and the amino terminus of this is quite distinct from those of the $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors. Binding of CRF to either the CRF_1 or CRF_2 receptor increases intracellular cAMP levels by the stimulation of adenylate cyclase activity (Chalmers et al., 1996; Dieterich and DeSouza, 1996).

Several years ago, a mammalian peptide that exhibits 44% identity to CRF was identified and named urocortin (Vaughan et al., 1995). Although this was demonstrated to bind with nanomolar affinity to both the CRF_1 and the CRF_2 receptor, immunocytochemical data suggested that urocortin, rather than CRF, might be an endogenous ligand for the latter receptor. However, a subsequent study shed

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doubt on this notion by showing that most of the major sites of expression of the CRF_2 receptor gene are poorly innervated by urocortin-containing projections (Bittencourt et al., 1999). Very recently, two mammalian urocortin-like peptides have been identified and characterised. One of these has been named urocortin II by Reyes et al. (2001) and stresscopin-related peptide by Hsu and Hsueh (2001); the other has been called urocortin III (Lewis et al., 2001) and stresscopin (Hsu and Hsueh, 2001). Both of these neuropeptides appear to be highly selective for the CRF_2 receptor and they, as well as urocortin, are able to induce an increase in intracellular cAMP levels.

Two other peptides also bind to the mammalian CRF_1 and CRF_2 receptors, namely, sauvagine and urotensin I. The former was first characterised from the skin of *Phyllomedusa sauvagei*, a frog native to Central and South America (Montecucchi and Henschen, 1981), while the latter was originally sequenced from two teleost fish, the white sucker *Catostomus commersoni* (Lederis et al., 1982) and the carp *Cyprinus carpio* (Ichikawa et al., 1982). CRF is also found in non-mammalian species such as fish and frogs (see Lovejoy and Balment, 1999), and two urocortin-like peptides have very recently been identified, by database searches, in the pufferfish *Fugu rubripes* and *Tetraodon nigroviridis* (Lewis et al., 2001).

The presence of multiple ligands for two CRF receptor subtypes in a given species raises the question as to how the corresponding genes co-evolved (see Darlison and Richter, 1999). Although there exists a significant body of information on the sequences of CRF and CRF-like peptides in different chordate species (see Lovejoy and Balment, 1999; Lewis et al., 2001), much less is known about the sequences and pharmacologies of CRF receptors in lower vertebrates. One of the best studied non-mammalian species is *Xenopus laevis*, for which two CRF receptors have been identified by complementary DNA (cDNA) cloning (Dautzenberg et al., 1997). Interestingly, while the two amphibian receptors have a similar affinity for *Xenopus* CRF ($K_D = 7.8 \pm 1.6$ and 9.4 ± 2.1 nM for the CRF_1 and CRF_2 receptors, respectively), the *Xenopus* CRF_2 receptor has an almost 60-fold higher affinity for sauvagine than the *Xenopus* CRF_1 receptor ($K_D = 0.9 \pm 0.1$ and 51.4 ± 6.6 nM, respectively). During the preparation of this manuscript, the cloning of cDNAs for three distinct CRF receptors from the brown bullhead catfish, *Ameiurus nebulosus*, was reported (Arai et al., 2001). While two of the catfish receptors appear to be orthologous to the mammalian and amphibian CRF_1 and CRF_2 receptors, the evolutionary origin of the catfish CRF3 receptor is unclear. Furthermore, in functional assays, neither CRF nor urotensin I appeared to be selective for any of the three receptors. To gain further insight into the pharmacology and phylogeny of CRF receptors, we have cloned cDNAs from the chum salmon, *Oncorhynchus keta*, and expressed these in human embryonic kidney 293 (HEK 293) cells and *X. laevis* oocytes.

2. Materials and methods

2.1. Cloning of O. keta full-length CRF receptor cDNAs

Total RNA was isolated from *O. keta* brain and heart using RNAClean™ (AGS, Heidelberg, Germany), digested with RNase-free DNase (Promega, Mannheim, Germany), and used as template for first-strand cDNA synthesis with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) and random nonamer primers (Stratagene, Amsterdam Zuidoost, The Netherlands). This cDNA was amplified, in the polymerase chain reaction (PCR), using *Taq* DNA polymerase (Promega) and two degenerate primers: 5′-TGCTCTAGAAA(C/T)AT(A/C/T)AT(A/C/T)CA(C/T)TGGAA-3′ and 5′-CGGGTCGAC(A/G)AA(A/G)AAXA(A/G)CAT(A/G)TAXGT-3′, where X = A, C, G and T; these oligonucleotides recognise the nucleotide sequences that encode amino acids 149 to 157 (CLRNIIHWN; single-letter code) and 326 to 334 (TYMLFFVNP), respectively, of the rat CRF_1 receptor (Chang et al., 1993; Perrin et al., 1993). A product of ~560 bp was subsequently cloned, as an *Xba*I–*Sal*I fragment, into pBluescript SK(+) (Stratagene), taking advantage of restriction endonuclease recognition sites (underlined) that were incorporated into the 5′ ends of the PCR primers. DNA sequencing revealed the presence of two distinct cDNAs which derive from the chum salmon CRF_1 and CRF_2 receptor mRNAs (see "Results").

To obtain the 5′ and 3′ sequences that were missing from the salmon CRF_1 and CRF_2 receptor cDNAs, we initially screened 5.6×10^6 bacteriophage of an *O. keta* whole-brain cDNA library, constructed in λZAPII (Heierhorst et al., 1990), with the partial sequences. Positively hybridising clones were only detected for the CRF_1 receptor; three of these were purified and found to contain the same insert, which encoded from amino acid 294 to the carboxy-terminus (Fig. 1) and included all of the 3′-untranslated sequence. To isolate the 5′ ends of the salmon CRF_1 and CRF_2 receptor cDNAs and the 3′ end of the CRF_2 receptor cDNA, we applied the rapid amplification of cDNA ends (RACE) technique to *O. keta* brain and heart first-strand cDNA essentially as described (Harvey et al., 1991; Stühmer et al., 1996). The missing 3′ sequence of the CRF_2 receptor cDNA was obtained in one PCR step, while the missing 5′ ends of the CRF_1 and CRF_2 receptor cDNAs were each generated in two steps. Finally, two full-length cDNAs were amplified, for expression purposes, using *Pfu* DNA polymerase (Stratagene) and oligonucleotide primers, the sequences of which either flank or overlap the initiating methionine and stop codons of the *O. keta* CRF_1 and CRF_2 receptor cDNAs (5′-AGTG(C/G)AGTCTAGAGA(A/T)ATCAGCAAATAAAC-3′ and 5′-AGAGGTTTCTAGACGGGGGCTTTCAGCCAA-3′ for salmon CRF_1, and 5′-TTAACGGATCCATTAACCATGGATGGATGCTACC-3′ and 5′-CCAAAGGTGAATTCTGGATTTGGTCAAAC-3′ for

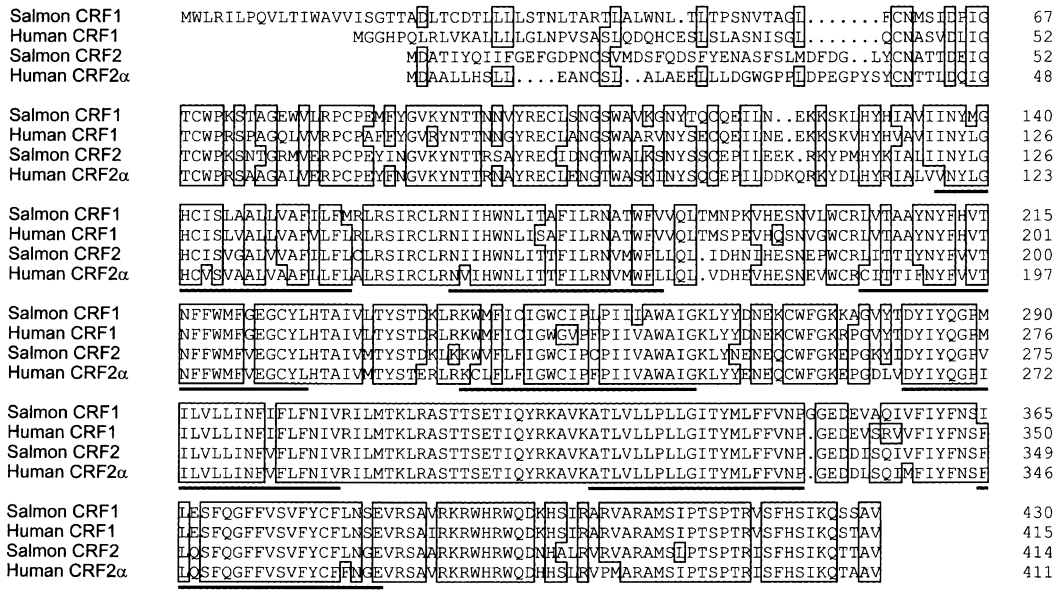

Fig. 1. Alignment of the *Oncorhynchus keta* (salmon) CRF_1 and CRF_2 receptor sequences with the human CRF_1 and $CRF_{2\alpha}$ receptor sequences. The four sequences (shown in single-letter code) were aligned using the computer programme PILEUP (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI, USA); dots denote gaps that have been introduced to maximise the alignment. Positions at which either three or four of the sequences are identical are boxed, and the seven putative membrane-spanning segments are underlined. The sequences of the cDNAs, from which the *O. keta* CRF_1 and CRF_2 receptor sequences have been deduced, have been given the EMBL accession numbers AJ277157 and AJ277158, respectively; the human CRF_1 (Chen et al., 1993) and $CRF_{2\alpha}$ (Liaw et al., 1996) receptor sequences have been taken from Swiss-Prot accession numbers P34998 and Q13324, respectively.

salmon CRF_2). The CRF_1 receptor cDNA was then cloned as an *Xba*I fragment into the *Xba*I site of pGEMHE (Liman et al., 1992), while the CRF_2 receptor cDNA was cloned as a "blunt-ended" (5′ end)-*Eco*RI (3′ end) fragment into the *Sma*I and *Eco*RI sites of the same vector. This was facilitated using restriction endonuclease recognition sites that were incorporated into the PCR primers (underlined). Both pGEMHE constructs were fully sequenced using an automated Applied Biosystems Model 377 DNA sequencer.

For expression in mammalian cells, the salmon CRF_1 receptor cDNA was excised from pGEMHE using *Xba*I and subcloned into the *Xba*I site of pcDNA3 (Invitrogen, Groningen, The Netherlands). The salmon CRF_2 receptor cDNA was excised using *Bam*HI (5′ end; this site is present in the 5′ primer, see above) and *Eco*RI (3′ end), and subcloned into the corresponding sites of pcDNA3. For use as a control, the rat CRF_1 receptor cDNA, in pBluescript SK(+) (a kind gift of Drs. T. Liepold and J. Spiess, Göttingen, Germany), was excised using *Bam*HI (5′ end) and *Sac*I (3′ end) and subcloned as a "blunt-ended" fragment into the *Eco*RV site of pcDNA3.

2.2. Transfection and competition binding

Human embryonic kidney 293 (HEK 293) cells were transfected with either the salmon CRF_1 receptor cDNA, the salmon CRF_2 receptor cDNA, or the rat CRF_1 receptor cDNA, in pcDNA3, using the calcium phosphate method (Kingston, 1987). Stable transfectants were selected in the presence of 500 μg/ml geneticin® (G418; Life Technologies, Karlsruhe, Germany) and cloned. Membranes were prepared from individual clones and used (80–100 μg protein per reaction) in a centrifugal binding assay. Reactions were incubated, at room temperature, for 2 h in 50 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM EGTA, 100 U kallikrein inhibitor (Trasylol®, Bayer, Leverkusen, Germany), 0.1 mM bacitracin (Sigma-Aldrich Chemie, Deisenhofen, Germany), adjusted to pH 7.2, and 200 pM [^{125}I-Tyr^0]-rat/human CRF (2200 Ci/mmol; NEN™ Life Science Products, Köln, Germany). 0.1 nM to 1 μM of either rat/human CRF or urotensin I (both from Bachem Biochemica, Heidelberg, Germany) was used as competitor. Reactions were terminated by centrifugation in a microcentrifuge (12,000 × *g*), at 4 °C for 5 min, and pellets were washed with 1 ml ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich) containing 0.01% (v/v) Triton X-100. The radioactivity remaining in the pellets was then measured in a γ counter (Berthold LB 211, Bad Wildbad, Germany).

2.3. cAMP assay

Individual stably transfected clones were cultured in 24-well plates that had been pretreated with poly-D-lysine. Confluent cell monolayers were incubated with 500 μM

3-isobutyl-1-methylxanthine (Sigma-Aldrich) in serum-free Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) at 37 °C for 1 h. The medium was then replaced with DMEM containing different concentrations of five peptides, namely, rat/human CRF, urotensin I, sauvagine, urocortin and [Arg^8]vasopressin (all from Bachem Biochemica). After incubation at 37 °C for 30 min, the medium was removed and the cells were washed with PBS and extracted with 70% (v/v) ethanol at −20 °C overnight. The cell extracts were then collected and lyophilised, and the cAMP content determined using a scintillation proximity assay (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

2.4. Functional expression in X. laevis oocytes

pGEMHE plasmids containing either the salmon CRF_1 or CRF_2 receptor cDNA were linearised by digestion with *Nhe*I, a pBluescript SK(+) plasmid harbouring a rat CRF_1 receptor cDNA (a kind gift of Drs. T. Liepold and J. Spiess, Göttingen, Germany) was linearised with *Sac*I, a pcDNA3 plasmid containing a rat GIRK1 cDNA (a kind gift of Prof. Dr. Volker Höllt, Magdeburg, Germany) was digested with *Xho*I, and a pAMP1 plasmid harbouring a human $G_{\alpha 16}$ cDNA was linearised with *Not*I. In each case, RNA was subsequently synthesised using T7 RNA polymerase (Promega). CRF receptor RNAs were mixed in a 1:1 ratio with either the GIRK1 or the $G_{\alpha 16}$ RNA and injected (each RNA at a concentration of 40 ng/μl) into stage V–VI *Xenopus* oocytes. Whole-cell voltage-clamp recordings were made 2–4 days later essentially as described (Mahlmann et al., 1994; Darlison et al., 1997). Oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$ and 5 mM HEPES, pH 7.5) and clamped at either −60 mV (for measurements with $G_{\alpha 16}$) or −80 mV (for measurements with GIRK1).

2.5. Reverse transcription-PCR (RT-PCR)

To assess the tissue distribution of the salmon CRF_1 and CRF_2 receptor transcripts, we performed RT-PCR. For this, total RNA was isolated from nine different tissues or organs using RNAClean™, and converted to first-strand cDNA using M-MLV reverse transcriptase and random nonamer primers. Each cDNA was then amplified with appropriate primers (see below) for either 35 cycles (CRF receptors) or 25 cycles (β-actin) as follows: 94 °C for 1 min (denaturation), either 65 °C (CRF receptors) or 60 °C (β-actin) for 1 min (annealing), then 72 °C for 1 min (extension). Products were separated in a 1.5% (w/v) agarose gel and photographed under ultra-violet light. The primer sequences used were: 5′-CACGTCGACAACTTCTTCTGGATGTTTGGG-3′ and 5′-GTAGAATTCATCTGTGTACACTCCTGCCTT-3′ for the *O. keta* CRF_1 receptor cDNA (these amplify the nucleotide sequence that encodes amino acids 213 to 286); 5′-GAGGGATCCTGTCGCCTTATAACAACGATA-3′ and 5′-ATAAAGCTTGTCAATATACTTTCCAGGTTC-3′ for the *O. keta* CRF_2 receptor cDNA (these amplify the nucleotide sequence that encodes amino acids 184 to 271); and 5′-GACAACGGTATGTGCAAAGCCGGA-3′ and 5′-TTGGGGTTGAGGGGGGCCTCGGTGAGCAGG-3′ for the *O. keta* β-actin cDNA (S. Pohl and M.G. Darlison, unpublished).

3. Results

Using degenerate oligonucleotide primers in the PCR, followed by a combination of conventional library screening and the RACE technique, we have isolated two *O. keta* full-length cDNAs that encode putative G-protein-coupled receptors. The deduced amino-acid sequences each contain seven putative membrane-spanning domains and exhibit strong similarity to those of previously identified vertebrate CRF receptors (Fig. 1). Thus, for example, the salmon CRF_1 receptor (430 amino acids; Mr = 49,595 Da) displays 82%, 83%, 81% and 87% identity, respectively, to the human, rat, *Xenopus* and catfish CRF_1 receptor sequences, while the salmon CRF_2 receptor (414 amino acids; Mr = 48,329 Da) shows 78–82%, 78–80%, 84% and 88% identity to the human ($CRF_{2\alpha}$, $CRF_{2\beta}$ and $CRF_{2\gamma}$), rat ($CRF_{2\alpha}$ and $CRF_{2\beta}$), *Xenopus* and catfish CRF_2 receptor sequences. The salmon CRF_1 and CRF_2 receptor sequences display 77% and 71% identity, respectively, to that of the catfish CRF3 receptor (Arai et al., 2001). Finally, when compared with one another, the two *O. keta* polypeptides exhibit 71% identity. The relationships between the various CRF receptor sequences are depicted, in Fig. 2, in the form of a dendrogram. Note that despite employing the 5′ RACE technique to determine the amino-terminal sequence of the salmon CRF_2 receptor, only one type of cDNA was obtained. Furthermore, although we used degenerate primers in the PCR with *O. keta* brain first-strand cDNA and screened an *O. keta* brain cDNA library with CRF receptor sequences under condi-

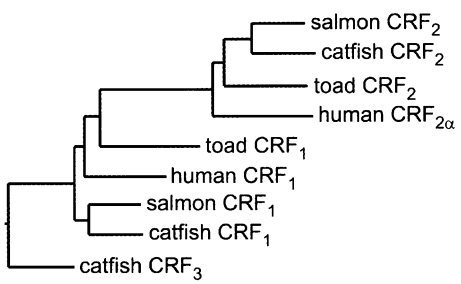

Fig. 2. Phylogenetic tree showing the sequence relationships between the salmon CRF_1 and CRF_2 receptors and CRF receptors from man, toad (*Xenopus laevis*) and catfish (*Ameiurus nebulosus*). This was generated using the Clustal algorithm of the DNAStar software package.

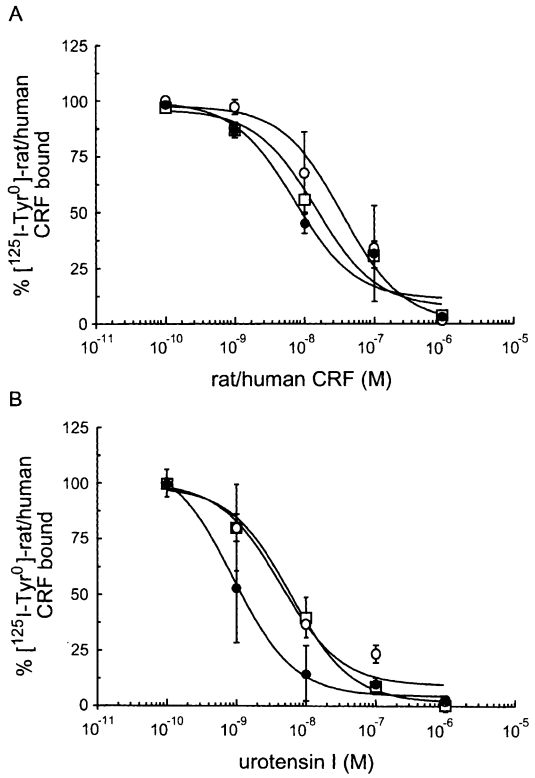

Fig. 3. Displacement of [^{125}I-Tyr^0]-rat/human CRF binding to the salmon CRF_1 and CRF_2 receptors by rat/human CRF and urotensin I. The binding of 200 pM [^{125}I-Tyr^0]-rat/human CRF to membranes prepared from HEK 293 cells expressing either the *O. keta* CRF_1 receptor (filled circles), the *O. keta* CRF_2 receptor (open circles) or the rat CRF_1 receptor (open squares) was determined in the presence of 0.1 nM to 1 μM of either rat/human CRF (A) or urotensin I (B). The data (mean values ± S.E.M.) derive from two or more experiments performed in triplicate.

tions of low stringency, we did not find any evidence for a third CRF receptor subtype in salmon.

To establish whether the *O. keta* CRF_1 and CRF_2 receptors were capable of binding CRF receptor ligands, we performed competition binding experiments (Fig. 3) in which we incubated membranes from transfected cells with 200 pM [^{125}I-Tyr^0]-rat/human CRF and increasing concentrations of either rat/human CRF or the fish peptide, urotensin I. For this, we selected stably transfected clones that produced high levels of either the salmon CRF_1 or CRF_2 receptor mRNA (data not shown). In the case of the salmon CRF_1 receptor, the binding of [^{125}I-Tyr^0]-rat/human CRF could be completely displaced by both rat/human CRF ($IC_{50} = 7.2 \pm 0.3$ nM) and urotensin I ($IC_{50} = 0.9 \pm 0.1$ nM). Similarly, the binding of the radioligand to the salmon CRF_2 receptor could be completely competed by rat/human CRF ($IC_{50} = 34.9 \pm 0.2$ nM) and urotensin I ($IC_{50} = 4.3 \pm 0.3$ nM). In control experiments, rat/human CRF and urotensin I displaced [^{125}I-Tyr^0]-rat/human CRF binding, from membranes isolated from cells transfected with the rat CRF_1 receptor cDNA, with IC_{50} values of 14.7 ± 0.3 and 6.0 ± 0.1 nM, respectively.

To study the result of agonist binding to the two fish receptors, and their pharmacologies in greater detail, we determined the effect of various peptides on cAMP levels in stably transfected HEK 293 cells. Incubation of salmon CRF_1 receptor-expressing cells with 1 μM of either rat/human CRF, urotensin I, sauvagine or urocortin produced a dramatic increase in the accumulation of intracellular cAMP, indicating the activation of adenylate cyclase (Fig. 4A). Incubation of HEK 293 cells, expressing the salmon CRF_2 receptor, with the same peptides similarly yielded significant increases in cAMP levels (Fig. 4B). In contrast,

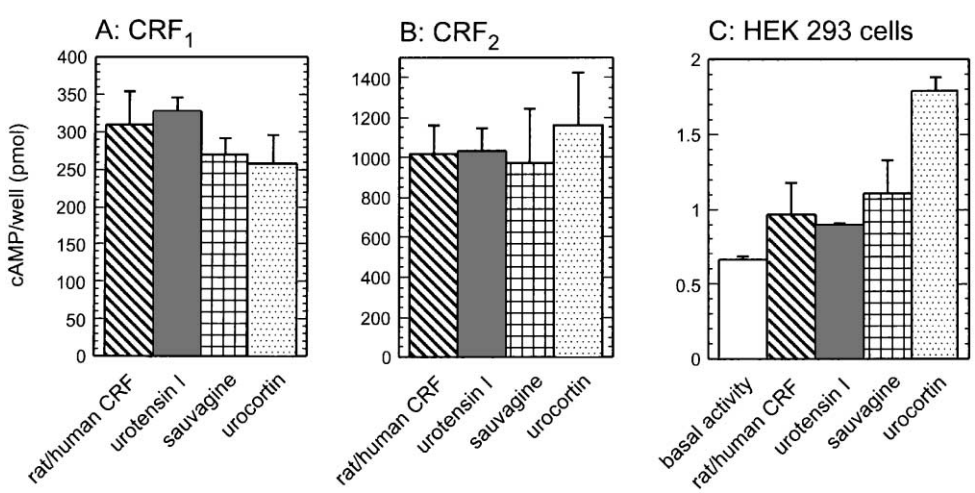

Fig. 4. Functional coupling of the salmon CRF_1 and CRF_2 receptors to the stimulation of adenylate cyclase. Four peptide agonists (rat/human CRF, urotensin I, sauvagine and urocortin; each at 1 μM) were tested for their ability to stimulate cAMP accumulation in HEK 293 cells expressing either the *O. keta* CRF_1 receptor (A) or the *O. keta* CRF_2 receptor (B). Bars representing basal activity are not shown in either (A) or (B) because they are too small to be visible. In control experiments (C), cAMP levels in non-transfected HEK 293 cells were determined in either the absence (basal activity) or presence of the same peptides (each at 1 μM) used in (A) and (B). The data (mean values ± the standard error) derive from three experiments performed in triplicate. Note the difference in magnitude of the dimensions on the ordinates in (A) and (B) compared to those in (C).

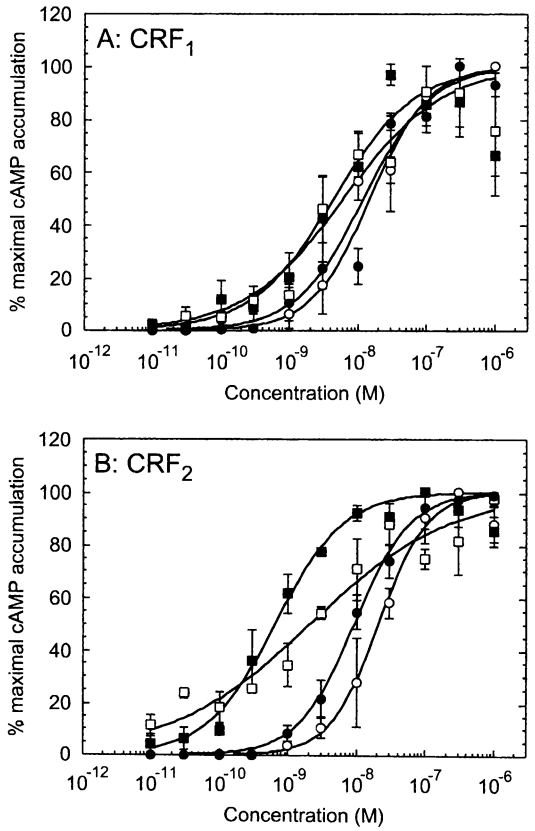

Fig. 5. Dose–response data for the stimulation of cAMP accumulation in HEK 293 cells expressing either the salmon CRF_1 or CRF_2 receptor. A range of concentrations (0.01 nM to 1 μM) of four peptide agonists, namely, rat/human CRF (filled circles), urotensin I (open circles), sauvagine (filled squares) and urocortin (open squares), were incubated with cells expressing either the *O. keta* CRF_1 receptor (A) or the *O. keta* CRF_2 receptor (B). The data derive from three experiments performed in triplicate. For each individual experiment, values were normalised to the maximal cAMP response, which was defined as 100%. Each point, therefore, corresponds to the mean ± the standard error of three determinations.

the nonapeptide vasopressin failed to augment cAMP levels in cells transfected with either receptor cDNA (data not shown). Furthermore, in control experiments on non-transfected HEK 293 cells, none of the peptides studied exhibited an effect on cAMP accumulation (Fig. 4C).

To examine the efficacy of rat/human CRF, urotensin I, sauvagine and urocortin in stimulating cAMP accumulation in HEK 293 cells expressing either the salmon CRF_1 or CRF_2 receptor, dose–response curves were generated (Fig. 5) using a range of peptide concentrations (from 0.01 nM to 1 μM). For the CRF_1 receptor, the EC_{50} values for the increase in cAMP levels were: rat/human CRF, 15.3 ± 3.1 nM; urotensin I, 12.4 ± 1.9 nM; sauvagine, 4.5 ± 1.8 nM; and urocortin, 6.3 ± 2.0 nM. For the CRF_2 receptor, the EC_{50} values were: rat/human CRF, 9.4 ± 0.4 nM; urotensin I, 21.2 ± 2.1 nM; sauvagine, 0.7 ± 0.1 nM; and urocortin, 2.2 ± 0.7 nM. Note that at very high concentrations of sauvagine and urocortin (i.e. 1 μM), the accumulation of cAMP mediated by activation of the salmon CRF_1 receptor is reduced when compared to the effect of slightly lower concentrations of these two agonists (Fig. 5A). The reason for this is currently unclear. In summary, it is evident that rat/human CRF and the three CRF-like peptides have similar efficacies at the *O. keta* CRF_1 receptor, while sauvagine is at least an order of magnitude more active than either rat/human CRF or urotensin I at the *O. keta* CRF_2 receptor.

Although CRF receptors mainly couple to the stimulation of adenylate cyclase, both native and recombinant CRF receptors have been reported to activate the phospholipase C signalling pathway (Yu et al., 1996; Grammatopoulos et al., 2001). In addition, it has been shown that a wide range of G-protein-coupled receptors can mediate inositol phosphate production when co-expressed, in mammalian cells, with the G-protein α subunit, $G_{\alpha 16}$ (Offermanns and Simon, 1995). In the *Xenopus* oocyte system, activation of phospholipase C results in the opening of endogenous Ca^{2+}-activated chloride channels. We have, therefore, investigated whether exogenously supplied

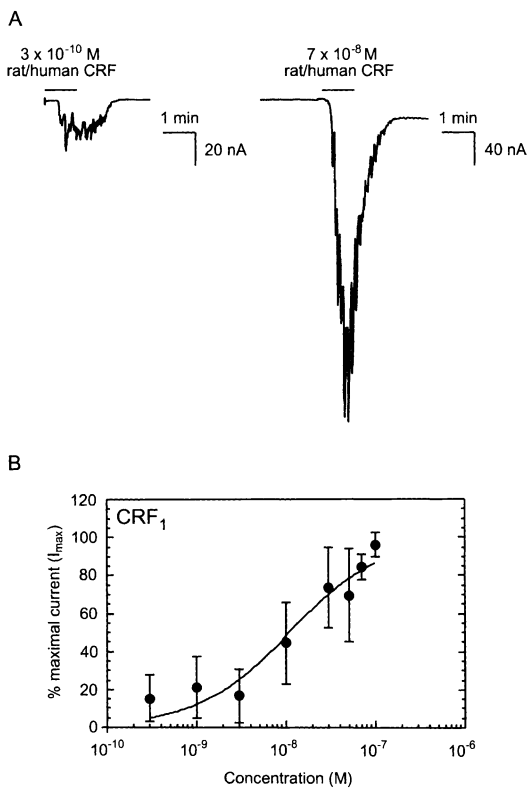

Fig. 6. Functional coupling of the salmon CRF_1 receptor to a Ca^{2+}-activated chloride conductance via $G_{\alpha 16}$ in *Xenopus* oocytes. (A) Representative current traces obtained in response to the application of 3×10^{-10} M (left) and 7×10^{-8} M (right) rat/human CRF. The horizontal bars indicate the period of peptide perfusion. (B) Dose–response curve generated using a range of concentrations of rat/human CRF (3×10^{-10} to 10^{-7} M). The data derive from seven oocytes. For each oocyte, values were normalised to the maximal current response, which was defined as 100%. Each point, therefore, corresponds to the mean ± the standard error of seven determinations.

$G_{\alpha 16}$ could transduce agonist binding, to either of the two *O. keta* CRF receptors, into a Ca^{2+}-mediated chloride conductance in *Xenopus* oocytes. We also examined, in the same system, whether the salmon CRF_1 and CRF_2 receptors could couple to the opening of the inward-rectifying potassium channel GIRK1 (via a direct interaction with free $\beta\gamma$ subunits of endogenous heterotrimeric G-proteins).

Bath application of rat/human CRF to *Xenopus* oocytes injected with in vitro-transcribed RNAs coding for the salmon CRF_1 receptor and the human $G_{\alpha 16}$ protein resulted in dose-dependent, oscillatory, inward currents at a holding potential of −60 mV (Fig. 6A and B). The EC_{50} value for channel activation was 11.2 ± 2.6 nM, which is comparable to that determined for the rat CRF_1 receptor in the same system (7.7 ± 5.4 nM; data not shown). In contrast, co-expression of the salmon CRF_2 receptor and $G_{\alpha 16}$ only yielded currents when rat/human CRF was applied at concentrations above 0.1 μM (data not shown). Lastly, no agonist-induced currents were detected upon co-expression of either *O. keta* CRF receptor with the rat GIRK1 channel (data not shown).

To examine the expression patterns of the two fish CRF receptor genes, we applied RT-PCR to total RNA samples isolated from a variety of tissues and organs. After 35 cycles of amplification, products of the correct size, corresponding to part of the salmon CRF_1 receptor cDNA, were detected (Fig. 7A) in the brain, skeletal muscle, heart, gills, ovary and the lateral line system (a sensory organ). The same number of cycles revealed expression of the salmon CRF_2 receptor gene in brain and heart (Fig. 7B). Control amplifications (Fig. 7C), using oligonucleotide primers specific for the *O. keta* β-actin cDNA (S. Pohl and M.G. Darlison, unpublished), confirmed the integrity of the RNA samples used.

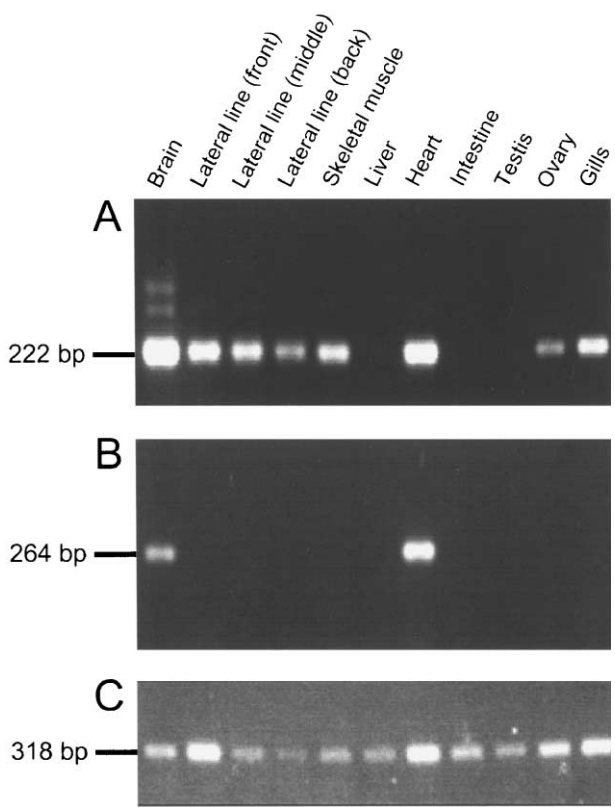

Fig. 7. Tissue expression of the salmon CRF_1 and CRF_2 receptor genes. Shown are the results of RT-PCR experiments performed using pairs of oligonucleotide primers specific for either the *O. keta* CRF_1 receptor cDNA (A), the *O. keta* CRF_2 receptor cDNA (B) or the *O. keta* β-actin cDNA (C). The sizes of the amplified fragments are shown, on the left, in basepairs. For further details, see "Materials and methods".

4. Discussion

We have described here the sequences of two members of the G-protein-coupled receptor superfamily from the teleost fish *Oncorhynchus keta*. These receptors are the orthologues of previously identified vertebrate CRF_1 and CRF_2 receptors. The salmon CRF_1 and CRF_2 receptors have been pharmacologically characterised using competition ligand-binding and cAMP assays; the latter experiments have also demonstrated that both receptors couple to the stimulation of adenylate cyclase activity, a property of other cloned (see Chalmers et al., 1996) and native (Chen et al., 1986; Dieterich and DeSouza, 1996; Hogg et al., 1996) CRF receptors. Both the salmon CRF_1 receptor and the salmon CRF_2 receptor have nanomolar affinity for mammalian CRF and the fish CRF-like peptide urotensin I (Lederis et al., 1982; Ichikawa et al., 1982), with the CRF_1 receptor having slightly higher (~5-fold) affinities than the CRF_2 receptor. However, EC_{50} values for the rat/human CRF-induced and the urotensin I-induced accumulation of cAMP were similar for the two salmon receptors. The *O. keta* CRF_1 and CRF_2 receptors can also be activated by the frog and mammalian CRF-like peptides, sauvagine (Montecucchi and Henschen, 1981) and urocortin (Vaughan et al., 1995), with EC_{50} values in the nanomolar range.

Somewhat surprisingly, the EC_{50} values for the accumulation of cAMP induced by rat/human CRF and urotensin I (9.4 ± 0.4 and 21.2 ± 2.1 nM, respectively) at the salmon CRF_2 receptor are reversed compared to their IC_{50} values for the displacement of [^{125}I-Tyr^0]-rat/human CRF (34.9 ± 0.2 and 4.3 ± 0.3 nM, respectively). Also, usually, in heterologous expression systems, EC_{50} values for receptor activation are lower than IC_{50} values derived from radioligand binding studies (see, for example, Dautzenberg et al., 1998, 1999). This was not the case here for CRF and urotensin I at the salmon CRF_1 receptor and for urotensin I at the salmon CRF_2 receptor. Although we do not know the reason(s) for these discordances, one plausible factor is that the radioligand binding experiments and the cAMP assays were performed on transfected mammalian cells that were grown at 37 °C. At this temperature,

a substantial proportion of CRF receptors, which derive from a fish that is found in cold waters, may exist in an abnormal conformation that affects either the affinity of agonist binding or the efficiency of coupling to second messenger systems. A similar discrepancy has been seen in the catfish, where rat/human CRF and urotensin I induced cAMP accumulation via the CRF_2 receptor with EC_{50} values of 2.4 ± 0.3 and 2.5 ± 0.2 nM, respectively (Arai et al., 2001), although the corresponding K_i values for the displacement of [^{125}I-Tyr^0, Gln^1, Leu^{17}]-sauvagine were more than 100-fold higher (244 ± 17 and 324 ± 49 nM, respectively). In contrast, the EC_{50} and K_i values for these two peptides at the CRF_1 and CRF_3 receptors differed much less (between 2.3- and 18.3-fold). Clearly, additional competition ligand-binding studies using sauvagine and urocortin (together with binding experiments and cAMP assays using the *O. keta* orthologues of urocortin II and urocortin III, when their sequences have been determined) are required to analyse in detail the relationships between the various IC_{50} and EC_{50} values. These experiments will form the focus of our future investigations on cloned fish CRF receptors.

Here, we have shown that the EC_{50} values, for the stimulation of adenylate cyclase, of the salmon CRF_1 receptor for rat/human CRF, urotensin I, sauvagine and urocortin are very similar, differing by no more than 3.4-fold. In contrast, the salmon CRF_2 receptor has a 13- and 30-fold lower EC_{50} value for sauvagine compared to rat/human CRF and urotensin I, respectively, and a slightly lower EC_{50} value for urocortin than for either rat/human CRF or urotensin I. It has been demonstrated (Dautzenberg et al., 1998) that five residues within the amino-terminal portion of the CRF_1 receptor influence the relative sensitivities to rat/human CRF and sauvagine. Thus, Arg^{76}, Asn^{81}, Gly^{83}, Leu^{88} and Ala^{89} in the human sequence determine similar high-nanomolar binding affinities for the two peptide ligands. In the *Xenopus* CRF_1 receptor, which has a much higher (17-fold) affinity for rat/human CRF than for sauvagine, these amino acids are replaced by Gln^{76}, Gly^{81}, Val^{83}, His^{88} and Leu^{89}. Consistent with this, the salmon CRF_1 receptor, at which sauvagine is only $\sim$3-fold more potent than rat/human CRF, possesses Lys (a conservative substitution), Asn, Val, Leu and Ser (another conservative substitution) at positions corresponding to residues 76, 81, 83, 88 and 89, respectively, in the human CRF_1 receptor sequence.

In experiments in *X. laevis* oocytes, we were able to demonstrate the coupling, via $G_{\alpha 16}$, of the salmon CRF_1 receptor, but not the salmon CRF_2 receptor, to an endogenous Ca^{2+}-activated chloride conductance. Dose–response data revealed that the EC_{50} value for channel activation by rat/human CRF was 11.2 ± 2.6 nM, a value comparable to that for stimulation of cAMP levels in transfected mammalian cells (15.3 ± 3.1 nM). These experiments represent, to our knowledge, the first demonstration of the functional expression of a CRF receptor in *Xenopus* oocytes. It is currently unclear why functional coupling between the salmon CRF_2 receptor and $G_{\alpha 16}$ was not obtained. However, an analogous situation has been observed for the two cannabinoid receptors, which normally couple to the inhibition of adenylate cyclase (Childers et al., 1993). There, the cannabinoid CB_1 receptor, but not the cannabinoid CB_2 receptor, could activate the phospholipase C signalling pathway in African green monkey kidney (COS-7) cells when co-expressed with $G_{\alpha 16}$ (Ho et al., 1999).

A further point concerning our pharmacological studies is the recent report (Dautzenberg et al., 2000) that HEK 293 cells endogenously express the CRF_1 receptor gene. During the course of the investigations described here, we did not detect either the binding of [^{125}I-Tyr^0]-rat/human CRF to non-transfected HEK 293 cells or any significant stimulation of cAMP levels, in such cells, after incubation with any CRF receptor agonist.

Although the sequence of *O. keta* CRF has not been determined, the sequences of two CRF isoforms from another teleost fish (namely, the white sucker *C. commersoni*) differ at only 2 or 3 of 41 positions from rat/human CRF (Morley et al., 1991). One may predict, therefore, that salmon CRF will activate the salmon CRF_1 and CRF_2 receptors with EC_{50} values similar to those of rat/human CRF. This being the case, neither salmon receptor appears able to differentiate between CRF and urotensin I. Similar results have recently been reported for the catfish (Arai et al., 2001), where the EC_{50} values for the stimulation of cAMP accumulation by these two peptides are comparable at the CRF_1, CRF_2 and CRF_3 receptors. Thus, neither our data, nor those of Arai et al. (2001), support the idea that urotensin I preferentially activates any of the known piscine CRF receptors. However, we cannot rule out the possibility that a specific "urotensin I receptor", that is different in sequence to CRF receptors, also exists in fish.

Lastly, the cloning of cDNAs for the salmon CRF_1 and CRF_2 receptors, and the coupling of these receptors to the stimulation of adenylate cyclase, reveal that the sequences and functional properties of CRF receptors have been conserved over a period of $\sim$400 million years, which is the evolutionary distance between teleost fish and modern mammals (Kardong, 1995). One apparent difference, however, between the *O. keta* and mammalian receptors is their tissue distribution. While expression of the salmon CRF_1 receptor gene was found to be widespread, with the corresponding mRNA being detected in brain, heart, skeletal muscle, gills, ovary and the lateral line system, transcription of the salmon CRF_2 receptor gene was only detected in brain and heart. This is in contrast to the situation in mammals (see Chang et al., 1993; Lovenberg et al., 1995; Ito and Miyata, 1999), where the CRF_1 receptor gene is predominantly expressed in brain, while the CRF_2 receptor gene is transcribed in the brain, heart, skeletal muscle, the gastrointestinal tract and epididymis. Also, although the salmon and catfish CRF_1 receptor se-

quences exhibit 87% identity, the tissue distributions of their transcripts are quite different. While, as mentioned above, the *O. keta* gene is widely expressed, the catfish gene is predominantly transcribed in the brain (Arai et al., 2001). This suggests that, in salmon, the CRF_1 receptor fulfills a much broader range of physiological functions than the corresponding receptor in mammals and catfish. In contrast, the mRNAs encoding the salmon and catfish CRF_2 receptors, which display 88% amino-acid identity, are both found in the brain and heart.

The differences in expression patterns outlined above presumably reflect the accumulation of sequence changes to the promoter regions of the genes in question during the long evolutionary history of these receptors, and point to distinct functional roles for the CRF_1 and CRF_2 receptors not only in higher vs. lower vertebrates but also in two different teleost fish (the chum salmon and the brown bullhead catfish). The recent finding of a third CRF receptor in catfish (Arai et al., 2001) is puzzling, since all other species studied to date possess only two CRF receptor genes. Since the CRF_3 receptor is more similar in sequence to the catfish CRF_1 receptor than the catfish CRF_2 receptor (Arai et al., 2001), and since it is also more similar to the salmon CRF_1 receptor than the salmon CRF_2 receptor (see Fig. 2), it is plausible that the former arose via the duplication, in catfish, of a precursor gene that ultimately gave rise to the CRF_1 and CRF_3 receptors that we recognise today. Regardless of how the CRF_3 receptor gene originated, it is evident that its pattern of expression (pituitary, urophysis and certain brain nuclei) is quite distinct from that of the catfish CRF_1 receptor (Arai et al., 2001), suggesting that these two receptors effect different physiological functions. Clearly, further studies are needed to clarify the specific biological roles and evolutionary history of these important G-protein-coupled receptors.

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