# Corticotropin-releasing factor (CRF) and CRF-binding protein expression in and release from the head kidney of common carp: evolutionary conservation of the adrenal CRF system

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## Abstract

Corticotropin-releasing factor (CRF) plays a central role in the regulation of the stress axis. In mammals, CRF as well as its receptors and its CRF-binding protein (CRF-BP) are expressed in a variety of organs and tissues outside the central nervous system. One of these extrahypothalamic sites is the adrenal gland, where the paracrine actions of adrenal CRF influence cortical steroidogenesis and adrenal blood flow. Although the central role of CRF signaling in the initiation and regulation of the stress response has now been established throughout vertebrates, information about the possible peripheral presence of CRF in earlier vertebrate lineages is scant. We established the expression of CRF, CRF-BP, and the CRF receptor 1 in a panel of peripheral organs of common carp (*Cyprinus carpio*). Out of all the peripheral organs tested, CRF and CRF-BP are most abundantly expressed in the carp head kidney, the fish equivalent of the mammalian adrenal gland. This expression localizes to chromaffin cells. Furthermore, detectable quantities of CRF are released from the intact head kidney following *in vitro* stimulation with 8-bromo-cAMP in a superfusion setup. The presence of CRF and CRF-BP within the chromaffin compartment of the head kidney suggests that a pathway homologous to the mammalian intra-adrenal CRF system is present in the head kidney of fish. It follows that such a system to locally fine-tune the outcome of the centrally initiated stress response has been an integral part of the vertebrate endocrine system since the common ancestor of teleostean fishes and mammals.

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## Introduction

Corticotropin-releasing factor (CRF) was initially identified and is still best known as the principle hypothalamic initiator of the stress response. CRF induces glucocorticoid secretion from the adrenal cortex indirectly, via the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. This axis is appropriately named the hypothalamus–pituitary–adrenal (HPA)-axis. Nevertheless, the expression of CRF has, since its discovery by Vale *et al.* (1981), been reported in many cells and tissues other than the hypothalamus (Karalis *et al.* 1997, Slominski *et al.* 2001, Coste *et al.* 2002). One of the sites where extrahypothalamic CRF has clear ties to the regulation of the stress response is the mammalian adrenal gland, which expresses and releases CRF (Nussdorfer 1996, Ehrhart-Bornstein *et al.* 1998). The adrenal cortex is the site of synthesis and release of the glucocorticoid hormones, which are directly responsible for many of the downstream effects of stress-axis activation, whereas the medulla of the adrenal gland is the principle site of catecholamine secretion (epinephrine and norepinephrine). The adrenal medulla in particular produces and contains many neurotransmitters and peptide hormones other than CRF, such as neuropeptide Y, serotonin (5-HT), and vasoactive intestinal peptide; the presence has been established, but the functions are incompletely understood (Nussdorfer 1996, Ehrhart-Bornstein *et al.* 1998). CRF is found exclusively in a subpopulation of medullary chromaffin cells (Hashimoto *et al.* 1984, Suda *et al.* 1984, Bruhn *et al.* 1987*a*,*b*, Minamino *et al.* 1988). Moreover, direct effects of CRF, independent of HPA-axis activation, have been demonstrated on adrenocortical steroid release (Bornstein *et al.* 1990, Jones & Edwards 1992, van Oers *et al.* 1992, Nussdorfer 1996). These direct effects of CRF on the adrenal gland require the local presence of CRF receptors. Indeed, CRF receptors, predominantly CRF receptor 1 (CRF-R1), are found within the adrenal gland (Dave *et al.* 1985, Udelsman *et al.* 1986, Aguilera *et al.* 1987, Willenberg *et al.* 2000, Muller *et al.* 2001). In addition, CRF-binding protein (CRF-BP), an important modulator of the concentration of


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free bioavailable CRF, has also been demonstrated in chromaffin cells of rat adrenal gland (Chatzaki *et al.* 2002). The absence of CRF receptors in adrenal cortex in all species investigated to date (Dave *et al.* 1985, Udelsman *et al.* 1986, Aguilera *et al.* 1987) with the exception of mouse (Muller *et al.* 2001) seems to preclude a direct effect of CRF on cortical cells. Indeed, the effects of CRF on the adrenal cortex require the presence of medullary tissue, as CRF has no effect on the steroid release from isolated adrenocortical cells *in vitro* (van Oers *et al.* 1992) or from autotransplants of cortical cells deprived of chromaffin tissue (Andreis *et al.* 1992). This implies that the actions of CRF on cortical steroidogenesis are indirect, as they apparently require an intermediate adreno-medullary component (Andreis *et al.* 1991, 1992). Collectively, this indicates the presence of a local paracrine CRF system within the adrenal gland that is capable of fine-tuning adrenal output via the modulation of either cortical steroidogenesis or adrenal blood flow.

The presence of an adrenal CRF system that modulates the output of the activated HPA-axis has now been firmly established in mammals. We know virtually nothing about the evolutionary origins of this modulatory CRF system. The central initiation of the stress response in fish, as in mammals, is controlled by CRF, CRF-R1, and CRF-BP (Huising *et al.* 2004), although the stress axis of teleostean fish differs anatomically from that of mammals. One of these anatomical differences is the location of the catecholamine-producing cells and the glucocorticoid-producing 'interrenal' cells that release cortisol as the main glucocorticoid. These cells are located within the paired head kidney, the fish homolog of the mammalian adrenal gland. The fish head kidney, however, lacks the clear cortex–medulla architecture that is characteristic of the mammalian adrenal gland. Instead, the interrenal and chromaffin cells are intermingled and lie around the cardinal veins of the head kidney, while the bulk of the head kidney tissue consists of cells of the hematopoietic lineage. This provides the opportunity for paracrine modulation of the outcome of HPA-axis activation by signals from the immune system, and vice versa. Here, we report the presence of a local CRF system resembling the one present in the mammalian adrenal gland and consisting of ligand, receptor, and binding protein. This implies that an adrenal CRF system was already present in the common ancestor to the teleost and tetrapod lineages and thus dates back at least 450 million years.

## Materials and Methods

### Animals

Common carp (*Cyprinus carpio* L.) of the R3×R8 strain were obtained from the 'De Haar Vissen' facility of Wageningen University (The Netherlands). R3×R8 are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain; Irnazarow 1995). Carp were maintained at 23 °C in recirculating u.v.-treated tap water at our fish facilities and were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0·7% of their estimated body weight. Fish were killed by anesthesia with 0·1% 2-phenoxyethanol before the collection of plasma and tissue samples. All animal experiments were carried out in accordance with national legislation.

### RNA isolation and gene expression analysis

RNA from carp tissues was isolated according to Chomczynski & Sacchi (1987). Briefly, organs were homogenized in lysis buffer (4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7·0), 0·5% sarcosyl, and 0·1 M 2β-mercaptoethanol), followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed, and dissolved in water. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1% (w/v) agarose gel. Gene expression was assessed by RT-PCR with the Superscript One-Step RT-PCR System (Gibco-BRL, Breda, The Netherlands). Briefly, 1 μg total RNA and forward and reverse primers (400 nm each; Table 1) were added to 12·5 μl of 2× reaction mix, 0·2 μl RNase inhibitor, and 1 μl Platinum Superscript II RT/Taq mix, and filled up with diethyl pyrocarbonate-treated water to a total volume of 25 μl. All primer sets span one or more introns. Reverse transcription was performed at 50 °C for 30 min. The reaction was subsequently denatured at 94 °C for 4 min and subjected to 30–40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, followed by a final extension step of

**Table 1** Primer sequences and corresponding accession numbers and amplicon lengths

| Gene | Accession number | Amplicon length (bp) | Primer | Sequence 5′→3′ |
|---|---|---|---|---|
| *CRF* | AJ317955 | 549 | CRF.fw2 | GATATATCAATTACGCACAGATT |
| | AJ576243 | | CRF.rv1 | TGATGGGTTTGCTCGTGGTTA |
| *CRF-BP* | AJ490880 | 507 | CRF-BP.fw2 | GGGTGGGTGATGAAGGGCGAGAA |
| | AJ490881 | | CRF-BP.rv1 | CACCATTCTGATCACAGTGTTATC |
| *CRF-R1* | AJ576244 | 409 | CRF-R1.fw3 | GACGTGTTGGGCCCAGGAGCAG |
| | | | CRF-R1.rv4 | CACCAGATCACATTGCTCTCAT |
| *β-actin* | CCACTBA | 708 | β-actin.fw1 | AGACATCAGGGTGTCATGGTTGGT |
| | | | β-actin.rv1 | GATACCGCAAGACTCCATACCCA |



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10 min at 72 °C. RT-PCRs were analyzed on a 1% (w/v) agarose gel. Amplicon identity was confirmed by sequencing.

### *Immunohistochemistry*

Tissue was fixed in Bouin's solution (15 ml picric acid, 5 ml formol, and 1 ml glacial acetic acid), dehydrated, embedded in paraffin, and sectioned in 5 µm sections. CRF was detected with a rabbit anti-sheep CRF antiserum (Biotrend, Cologne, Germany) at a dilution of 1:50. CRF-BP was detected with a rabbit anti-human CRF-BP antiserum (a generous gift of Dr Wylie Vale) at a dilution of 1:1000. We previously demonstrated that this antibody detects a single species of 37 kDa in western blots of lysates prepared from carp tissue and that the antibody is suitable for immunohistochemistry (Huising *et al.* 2004). Primary antibodies were incubated overnight. Goat anti-rabbit IgG –biotin (1:200, 1 h; Vector Laboratories, Burlingame, CA, USA) was used as the second antibody, followed by amplification with the Vectastain ABC amplification kit (Vector Laboratories) according to the manufacturer's protocol. The signal was visualized with 3-amino-6-ethylcarbazole (AEC; Sigma) as the substrate. Controls for the cross-reactivity of the secondary reagents and for endogenous enzyme activity were included in all experiments and were negative. Nuclei were counterstained with hematoxylin before embedding in Kaiser's gelatin.

### *Confocal laser scanning microscopy*

In a two-color immunofluorescence approach, interrenal cells were visualized either via their higher autofluorescence (in double staining with CRF) or (in double staining with CRF-BP) by staining for cortisol with an anti-cortisol antibody (1:150; Campro Scientific, Veenendaal, The Netherlands). Goat anti-rabbit IgG–HRP (Bio-Rad) was used as the second antibody at 1:200 and the signal was visualized with tyramide-fluorescein–isothiocyanate (FITC) (1:50 for 30 min; NEN Life Science Products, Boston, MA, USA). For the detection of CRF and CRF-BP, the same primary and secondary antibodies as before were used at the same dilutions. Signal was detected by incubating with avidin–Texas Red (Vector Laboratories) for 10 min. Sections were embedded in Vectashield (Vector Laboratories) and examined with a Zeiss LSM-510 laser scanning microscope. Fluorescein signal was excited with a 488 nm argon laser and detected using a band-pass filter (505–530 nm) and Texas Red was excited with a 543 nm helium–neon laser and detected with a long-pass filter (>585 nm).

### *RIAs*

Cortisol was measured by RIA, using a commercial antiserum (Campro Scientific) as previously described (Huising *et al.* 2004). As carp CRF is 93% identical to human/rat CRF, we developed an RIA for the detection of carp CRF based on a rabbit antiserum directed at human/rat $CRF_{24–41}$ (C5348; Sigma). According to the manufacturer, the antibody exhibits <0·01% cross-reactivity with rat urocortin-1, sauvagine (*Phyllomedusa sauvagei*), and human ACTH. The antibody also did not cross-react with carp urotensin-I (UI; kindly donated by Dr Jean Rivier, The Salk Institute for Biological Studies, La Jolla, CA, USA). The optimal antibody dilution was experimentally established at 1:10 000. Human/rat Tyr-CRF (H-24 551; Bachem, Bubendorf, Switzerland) was used as standard. The standard was also used as tracer following labeling with $^{125}$I (ICN, Costa Mesa, CA, USA) by the iodogen method (Salacinski *et al.* 1981) and purified through solid-phase extraction (octadecyl Bakerbond column). All constituents were in phosphate-EDTA RIA buffer of pH 7·4 (63 mM $Na_2HPO_4$, 13 mM $Na_2$EDTA, 0·02% (w/v) $NaN_3$, 0·1% (v/v) Triton X-100, 0·25% (w/v) BSA (Sigma), and 2·5% (v/v) aprotinin (Trasylol; Bayer). Samples and standards of 25 µl were preincubated in duplicate or triplicate respectively, with 100 µl primary antibody (1:10 000) for 96 h at 4 °C. Then, tracer was added at a volume of 100 µl (~4000 c.p.m.) and incubated for 24 h at 4 °C. A volume of 100 µl secondary antibody solution (goat anti-rabbit IgG; Biogenesis, Ede, The Netherlands; diluted 1:16 (v/v) in RIA buffer containing 0·007% (w/v) rabbit IgG; Sigma) was added and incubated for 30 min at room temperature. Immune complexes were precipitated by adding 1 ml ice-cold polyethylene glycol (PEG) 6000 and centrifuged at 2000 ***g*** for 10 min at 4 °C. Supernatants were aspirated and the pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The RIA has a sensitivity of 2·5–5·0 pg/tube (0·5–1·0 fmol/tube). The inter-assay variation was 5·97 ± 2·05% ($n$=6) and the intra-assay variation was 1·90 ± 1·63% ($n$=5).

### In vitro *superfusion*

To assess CRF and cortisol release *in vitro*, freshly collected head kidneys were placed on a cheesecloth filter in a superfusion chamber and superfused with 0·015 M HEPES/-Tris-buffered medium (pH 7·4) containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2·2H_2O$, 0·25% (w/v) glucose, 0·03% (w/v) BSA (Sigma), and 0·1 mM ascorbic acid. Medium was saturated with carbogen (95% $O_2$/5% $CO_2$) and pumped through the superfusion chambers at 20 µl/min with a multichannel peristaltic pump (Watson–Marlow, Falmouth, Cornwall, UK). Medium and tissues were maintained at 23 °C throughout the experiment. At the indicated times, head kidneys were stimulated by a pulse of 60 mM KCl or 8-bromoadenosine-3′-5′-cAMP (8-br-cAMP; B-7880; Sigma) dissolved in superfusion medium. Fractions were collected every 10 or 15 min, stored on ice for the immediate determination of CRF content and stored at −20 °C for the determination of cortisol content at a later time. Basal unstimulated release was calculated based on the three values preceding the (first) pulse and designated at 100%. Stimulation is expressed as a percentage of basal release.

### *Statistical analysis*

Statistical analysis was carried out with SPSS software (version 11.5.0, SPSS Inc., Chicago, IL, USA). Differences



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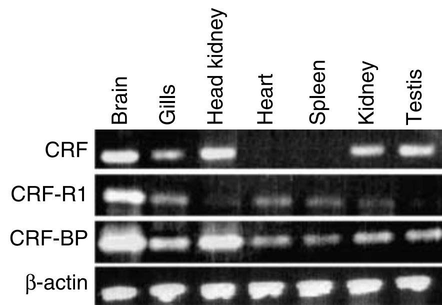


**Figure 1** Expression of CRF, CRF-R1, and CRF-BP in brain and peripheral organs of carp. Note that the most prominent gene expression of CRF and CRF-BP outside the central nervous system is observed in the head kidney. Expression of CRF-R1 is detectable in gills, head kidney, heart, spleen, and kidney, but not in testis. The β-actin gene product was included as a reference gene. PCRs for CRF, CRF-R1, CRF-BP, and β-actin were performed for 40, 30, 35, and 30 cycles respectively.

were evaluated with the non-parametric Kruskal–Wallis *H* test. When this test indicated significant differences in the dataset, the Mann–Whitney *U* test was used to determine which samples differed significantly from controls. Differences were considered significant at $P<0{\cdot}05$ (one-sided).

## Results

To investigate the presence of CRF, CRF-R1, and CRF-BP, outside the central nervous system, we compared gene expression in the brain with that in a panel of peripheral organs. In the periphery, CRF as well as CRF-BP is expressed most abundantly in the head kidney (Fig. 1). CRF expression was also present in gills, kidney, and testis, but was undetectable in heart and spleen. CRF-BP expression was detected throughout the panel of peripheral organs. CRF-R1 is expressed to some extent in all organs except testis and is weakest in the head kidney.

To investigate the cellular location of CRF and CRF-BP within the head kidney, we investigated their distribution via immunohistochemistry. This approach revealed that CRF and CRF-BP immunoreactivity is present in the endocrine compartment of the carp head kidney. The interrenal and chromaffin cells are organized in patches of endocrine tissue around the blood vessels of the head kidney, embedded in densely packed hematopoietic tissue. A small proportion of these endocrine cells is positive for CRF or CRF-BP (Fig. 2). To establish whether CRF and CRF-BP are expressed in the interrenal or the chromaffin cell compartment, we further investigated their expression via confocal laser scanning

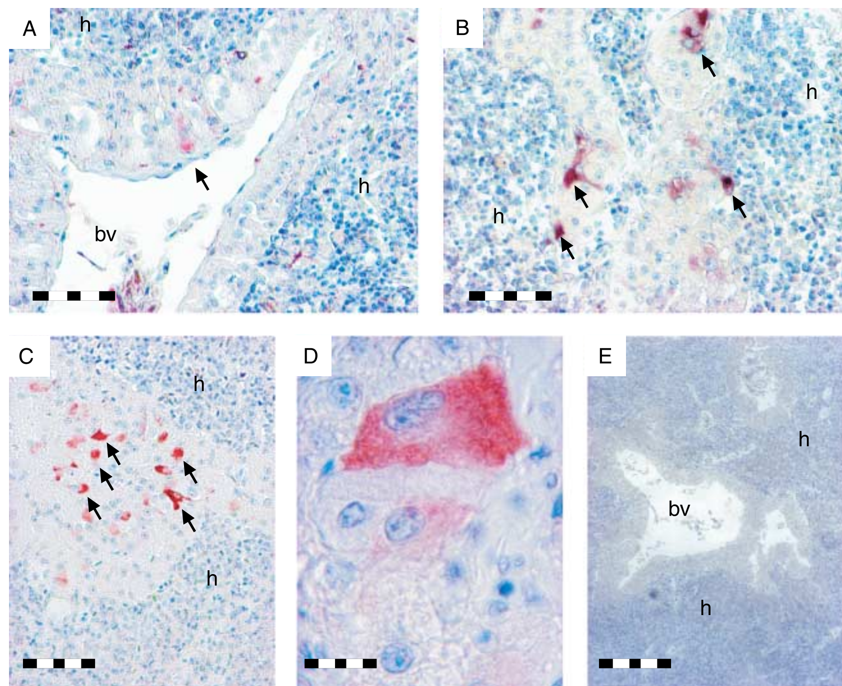


**Figure 2** CRF and CRF-BP immunoreactivity in the islands of endocrine tissue that surrounds the blood vessels of the head kidney. (A and B) Presence of CRF immunoreactivity (red) in a subset of endocrine cells. The endocrine tissue of the teleostean head kidney is organized around blood vessels (bv) and surrounded by densely packed hematopoietic tissue (h). (C and D) Presence of CRF-BP immunoreactivity. (E) Result of a negative control incubated with secondary antibody and AEC only. Nuclei were counterstained with hematoxylin. Arrows in (A–C) indicate immunoreactive cells. Scale bars are 50 μm in (A–C), 10 μm in (D), and 250 μm in (E).



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microscopy. Interrenal cells, unlike chromaffin cells, display autofluorescence following Bouin's fixation. We exploited this characteristic to establish that CRF immunoreactivity is absent from the interrenal compartment but localizes to a subset of chromaffin cells (Fig. 3A). In a similar approach, CRF-BP immunoreactivity was also demonstrated in a subpopulation of chromaffin cells and not in the interrenal cell compartment (Fig. 3B).

The release of CRF from the head kidney was studied in an *in vitro* superfusion assay. For the detection of carp CRF, we developed and validated an RIA. The carp CRF released from head kidney displaces $^{125}$I-Tyr human CRF in parallel with the standard curve (Fig. 4), which validates the RIA for the detection and relative quantitation of carp CRF. Depolarizing concentrations of $K^+$ (60 mM) did not induce the release of CRF (Fig. 5A), but did induce a modest and transient increase in cortisol secretion (Fig. 5B), demonstrating that depolarization did occur. Stimulation of protein kinase C via phorbol 12-myristate 13-acetate did not result in CRF release (not shown). Direct activation of protein kinase A (PKA) by 1 mM 8-bromo-cAMP resulted in a marginal CRF release, 10 mM resulted in a rapid and pronounced release of CRF from the head kidney (Fig. 5C). CRF release rapidly returned to baseline values upon termination of the stimulus. Simultaneously, cortisol release is induced by 1 mM 8-bromo-cAMP, although the secretion starts only towards the end of the 30 min pulse and persists until the subsequent stimulation with 10 mm 8-bromo-cAMP (Fig. 5D). To demonstrate the responsiveness of head kidney *in vitro* for the duration of the experiment, we subjected head kidneys to four subsequent 30 min pulses with 10 mM 8-bromo-cAMP. Each of the four stimulations is closely followed by a rapid, profound, and transient release of CRF (Fig. 5E). Moreover, the magnitude of the CRF responses is constant throughout the experiment, which indicates that the CRF content of the head kidney suffices to sustain repeated episodes of *in vitro* stimulation. As predicted, the repetitive stimulation with 10 mM 8-bromo-cAMP initiated a profound increase in cortisol secretion that is delayed compared with the simultaneous release of CRF (Fig. 5F). Because of the lag in the termination of the cortisol release, its enhanced secretion is maintained throughout the experiment.

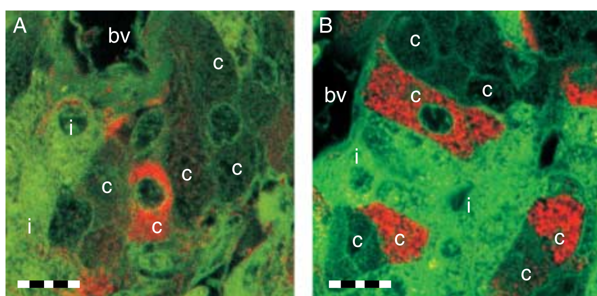


**Figure 3** CRF and CRF-BP immunoreactivity is localized to a subset of the chromaffin cells. (A) Confocal micrograph of a detail of the head kidney endocrine tissue that features a cell stained for CRF (red). The slide's autofluorescence is recorded in the green channel of the confocal microscope and highlights the interrenal cells (i), which have stronger autofluorescence that the chromaffin cells (c) that remain dark. (B) Chromaffin cells that contain CRF-BP immunoreactivity (red) amidst a group of interrenal cells that are stained with an antibody against cortisol (green). Note that most chromaffin cells do not contain CRF-BP and that 'bv' indicates the lumen of the blood vessel. Scale bars are 10 μm.

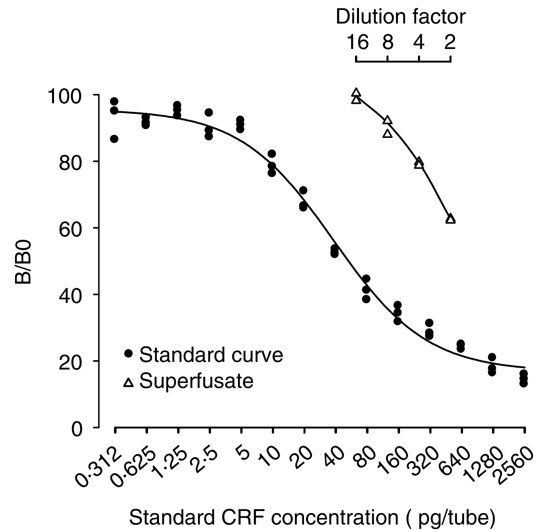


**Figure 4** The CRF RIA binding curve for standard (human) CRF and the dilution curve of carp CRF run in parallel. Carp CRF was obtained from pooled and concentrated head kidney superfusion fractions.

## Discussion

Here, we present the presence of a local CRF system in the head kidney of a teleostean fish, in analogy to the intra-adrenal CRF system of mammals. The adrenal gland is one of the several peripheral organs and tissues home to a local CRF system; CRF systems are found in the skin of mammalian species (Slominski *et al.* 2000, Slominski 2005) as well as in the skin and gills of carp (Mazon *et al.* 2006). Of the peripheral tissues examined here, CRF and CRF-BP were expressed most prominently in the head kidney, which also contained a lower level of CRF-R1 expression. Within the head kidney, CRF and CRF-BP immunoreactivity are present in a subset of chromaffin cells and were not detected in the interrenal compartment. This is similar to the situation in mammals where CRF and CRF-BP are present in the medulla, but not the cortex, of the adrenal gland (Nussdorfer 1996, Ehrhart-Bornstein *et al.* 1998, Chatzaki *et al.* 2002). The distribution of CRF in the chromaffin, but not the glucocorticoid compartment of vertebrates, is in agreement with their ontogenic roots: interrenal or adrenocortical cells are mesodermally derived, whereas the chromaffin cells originate from neural crest (Vrezas *et al.* 2003). Apparently, the expression of neuropeptides or peptide hormones in the



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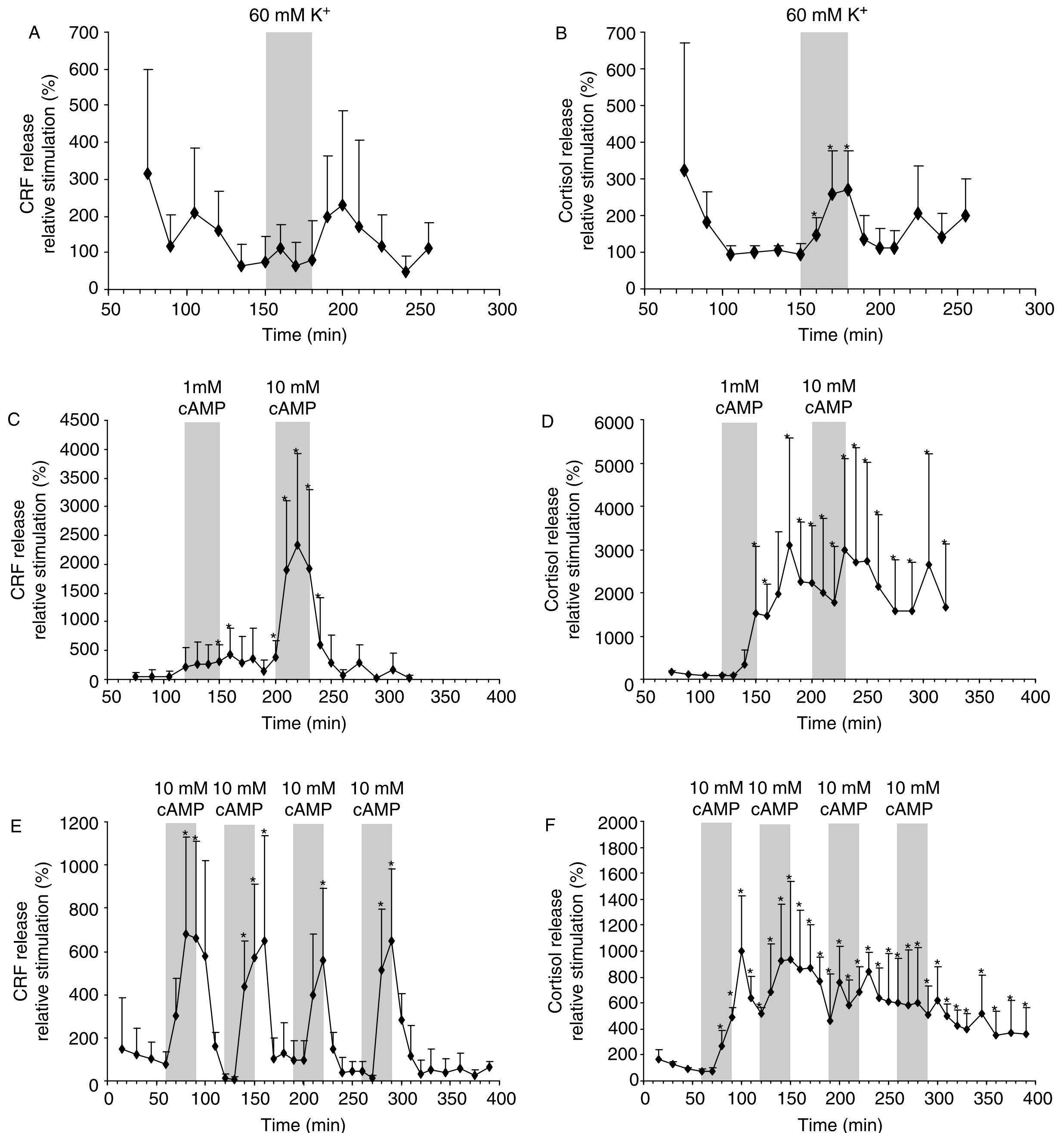


**Figure 5** The effects of $K^+$ and 8-bromo-cAMP on the release of CRF and cortisol from superfused carp head kidneys. Depolarization induced by 60 mM $K^+$ failed to induce CRF release (A), but did induce a modest release of cortisol (B), $n=5$. Direct stimulation of PKA via 8-bromo-(cAMP) induced a marginal CRF release when applied at 1 mM but resulted in the rapid and pronounced release of CRF at 10 mM (C). Stimulation via 8-bromo-cAMP also induced the release of cortisol, albeit with a considerable delay (D), $n=6$. Four consecutive 30 min pulses with 10 mM 8-bromo-cAMP result in four distinct peaks of CRF release that closely follow the application of 8-bromo-cAMP (E). The same stimulation also induced the profound release of cortisol (F), $n=3$. The cortisol response to 8-bromo-cAMP displays a delay in both its initiation and termination, which leads to the continued secretion of cortisol throughout the duration of the superfusion experiment. Note that in all experiments CRF and cortisol content is measured within the same superfusion samples. Stimulation is expressed as a percentage of basal release, which was calculated based on the three values preceding the (first) pulse. Basal CRF release corresponds to 4·1, 1·3, and 1·4 pg/min in (A, C, and E) respectively. Basal cortisol release corresponds to 199·6, 97·8, and 256·2 pg/min in (B, D, and F) respectively. Asterisks denote a significant increase from basal release (*$P<0·05$).



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fish head kidney and its homologs of later vertebrate lineage is largely restricted to the chromaffin cells of neuroectodermal origins (Nussdorfer 1996, De Falco *et al.* 2002).

The release of CRF from intact head kidneys was detectable *in vitro* and is most potently induced by 8-bromo-cAMP, which directly activates the PKA pathway. This is similar to studies on the regulation of hypothalamus and amygdala CRF in mammals that report an increase in gene expression and peptide release following stimulation with the PKA activator forskolin (Emanuel *et al.* 1990, Kasckow *et al.* 2003). The rapid response of the carp head kidney following stimulation by 8-bromo-cAMP is too fast (minutes) to involve *de novo* peptide synthesis and indicates that 8-bromo-cAMP induces the direct release of stored CRF. This is supported by the presence of CRF immunoreactivity in the cytoplasm of chromaffin cells. The kinetics of CRF and cortisol secretion following repeated stimulation with 10 mM 8-bromo-cAMP indicates that CRF is released faster than cortisol and thus independently of the latter. The differences in response time between CRF and cortisol likely stem from the different mechanisms that are responsible for their release: CRF is stored cytoplasmatically and can be released rapidly via exocytosis, whereas cortisol is the end product of an enzymatic cascade that requires more time to become maximally activated. The relatively high dose of 8-bromo-cAMP (10 mM) required to induce robust secretion of CRF is attributed to the experimental setup; although superfusion media flow over the target tissue at a constant rate, activation of the cells within the tissue depends on diffusion. Therefore, local levels of 8-bromo-cAMP within the head kidneys are likely lower than those in the surrounding media. The magnitude of 8-bromo-cAMP-induced CRF release is not diminished by a prolonged simultaneous secretion of cortisol, which indicates that direct activation of the PKA pathway overrules any potential negative feedback mechanism of glucocorticoids on the secretion of CRF.

The mammalian intra-adrenal CRF system is considered to exert local paracrine effects that modulate the overall adrenal glucocorticoid response. A similar paracrine function seems likely for teleostean head kidney CRF too as the number of CRF and CRF-BP-positive cells is relatively small compared with the bulk of head kidney endocrine cells. And although CRF was clearly detectable at the peak of its release in an *in vitro* superfusion setup, where release is measured immediately downstream of the source, it is plausible that head kidney CRF will be diluted beyond detection in the general circulation and before it can induce systemic effects. We detected only a relatively modest amount of CRF-R1 expression in the head kidney. This level of expression (which was the result of only 30 cycles of amplification) apparently suffices for the mediation of paracrine effects within the head kidney. Alternatively, direct effects of CRF may be mediated by CRF-R2 or a potential third, as yet unidentified CRF receptor in carp (Arai *et al.* 2001). Nevertheless, the direct corticotropic effect of CRF on co-cultures of human glucocorticoid and chromaffin cells is completely inhibited by the specific CRF-R1 antagonist antalarmin, suggesting that the CRF-R1 is the most important CRF receptor in the adrenal CRF system (Willenberg *et al.* 2000).

Whether the local presence of CRF-BP in the carp head kidney serves the sole purpose of modulating the paracrine response to local CRF is presently unclear. It is conceivable that the local presence of CRF-BP is intended for the modulation of the head kidney response to CRF that is derived from sources outside the head kidney such as the hypothalamus or the pituitary pars intermedia that in fish contains many CRF-positive nerve fiber bundles (Yulis & Lederis 1987, Huising *et al.* 2004). In tilapia (*Oreochromis mossambicus*), high concentrations of CRF are detected in circulation following acute stress (Pepels *et al.* 2004). It is also conceivable that CRF-BP modulates the response of the head kidney to UI, which is a member of the CRF family of peptide hormones. The major source of UI in fish is the caudal neurosecretory system that in flounder (*Platichthys flesus*) also contains CRF (Lu *et al.* 2004). Indeed, UI enhances the steroidogenic actions of ACTH on the head kidney of flounder (Kelsall & Balment 1998), although UI, in contrast to CRF, is not expressed in the flounder head kidney (Lu *et al.* 2004). Finally, it is possible that additional CRF paralogs such as urocortin-2 and urocortin-3 (Boorse *et al.* 2005) are expressed in the carp head kidney, although the genes that encode them have not yet been identified in carp. In mammals, however, urocortin-2 and urocortin-3 signal exclusively via CRF-R2 and neither peptide consistently binds to CRF-BP with high affinity in all species investigated (Hillhouse & Grammatopoulos 2006).

Based on i) the presence of CRF as well as its modulator CRF-BP in a subset of chromaffin cells and ii) the demonstration of cAMP-dependent CRF release from the head kidney *in vitro*, we conclude that a local CRF system is present in the head kidney of teleostean fish. The intra-adrenal CRF system of mammals is implicated in the modulation of glucocorticoid release by the effects on glucocorticoid release as well as adrenal blood flow. Our *in vitro* superfusion setup will allow us to further investigate the effects of CRF on the modulation of cortisol release from the carp head kidney, independently of any potential modulatory effect of CRF on blood flow. The presence of a local CRF system in the head kidney of fish indicates that the capacity to locally modulate the output of systemic stress-axis activation at the level of glucocorticoid release has apparently provided an adaptive advantage to the early vertebrate ancestor.

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