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ScienceDirect
General and Comparative Endocrinology 150 (2007) 437–444
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Involvement of the corticotropin-releasing factor (CRF) type 2 receptor in CRF-induced thyrotropin release by the amphibian pituitary gland

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Received 20 September 2006; revised 9 November 2006; accepted 9 November 2006
Available online 26 December 2006

Abstract

Corticotropin-releasing factor (CRF) is considered to be a main adrenocorticotropin-releasing factor in vertebrates. In non-mammalian species, CRF and related peptides cause the release of thyroid-stimulating hormone (TSH) from the anterior pituitary. The actions of CRF peptides are mediated by two G protein coupled receptors (CRF_1 and CRF_2) that have different ligand specificities. Using ligands that bind preferentially or selectively to the CRF_2 we tested the hypothesis that TSH release by the amphibian pituitary gland is mediated by the CRF_2. Injection of frog CRF, urocortin 1 or the CRF_2-specific ligand urocortin 3 all produced significant, acute increases (by 2 h) in plasma thyroxine concentration in prometamorphic tadpoles. Chronic injections of CRF peptides accelerated tadpole metamorphosis, and the peptides with the highest affinity for the CRF_2 (urocortin 1 and sauvagine) had the greatest potency. Ligands selective for the CRF_2 (frog urocortin 3, mouse urocortins 2 and 3) all accelerated tadpole metamorphosis. We then tested frog urocortins 1 and 3, mouse urocortin 2 and sauvagine for their TSH-releasing activity using dispersed frog anterior pituitary cells in culture. All of the peptides tested markedly enhanced the release of TSH. Secretagogue-induced TSH release was completely blocked by the general CRF receptor antagonist astressin or the CRF_2-specific antagonist antisauvagine-30. Conversely, the type 1 CRF receptor-specific antagonist antalarmin had no effect on TSH secretion. Our results support the hypothesis that CRF-induced TSH release by the amphibian pituitary gland is mediated by the CRF_2.

Keywords: TSH; CRF; CRF receptor; Amphibian

1. Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid peptide originally isolated from ovine hypothalamus (Vale et al., 1981), is a potent stimulator of pituitary adrenocorticotropic hormone (ACTH), β-endorphin (Rivier et al., 1982), and α-melanocyte-stimulating hormone (Vale et al., 1981). Subsequent to the discovery of CRF, two CRF-related peptides, urotensin-I and sauvagine (SVG), were discovered in the caudal neurosecretory organ of the common sucker (Lederis et al., 1982) and in the skin of the frog *Phyllomedusa sauvagii* (Montecucchi and Henshen, 1981), respectively. Both of these peptides exhibited equivalent potencies to release ACTH from the mammalian pituitary (Rivier et al., 1983). Other CRF-related peptides, urocortin 1 (Vaughan et al., 1995), urocortin 2 (Reyes et al., 2001), and urocortin 3 (Lewis et al., 2001), have since been identified in the mammalian brain. Of these, urocortin 1 was also shown to have ACTH-releasing activity in the rat pituitary (Asaba et al., 1998). Recently urocortin 1 and 3 were

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doi:10.1016/j.ygcen.2006.11.002

isolated from the South African clawed frog *Xenopus laevis* (Boorse et al., 2005).

Complementary DNAs for two types of CRF receptors, type 1 and 2 (CRF_1 and CRF_2) have been isolated in mammals (Dautzenberg and Hauger, 2002) and in several non-mammalian vertebrates: salmon (Pohl et al., 2001), *X. laevis* (Dautzenberg et al., 1997), the North American bullfrog *Rana catesbeiana* (Ito et al., 2006) and the chicken (Yu et al., 1996; De Groef et al., 2003); three different cDNAs representing CRF receptor types (CRF_1 and two forms of CRF_2) have been isolated in catfish (Arai et al., 2001). Both CRF_1 and CRF_2 are expressed in the CNS and pituitary, and in diverse peripheral tissues in mammals and in frogs (Boorse and Denver, 2006). In mammals, CRF_1 is expressed in pituitary corticotropes and mediates the stimulatory actions of CRF on ACTH secretion (Aguilera et al., 2004). The CRF_2 is expressed as two alternatively spliced isoforms $CRF_{2\alpha}$ and $CRF_{2\beta}$ in the rat pituitary (Lovenberg et al., 1995), and mRNA expression patterns are distinct from CRF_1 (Chalmers et al., 1996).

The CRF_1 and CRF_2 have been structurally and functionally characterized in mammals (Lovenberg et al., 1995; Perrin et al., 1993) and in the frog *X. laevis* (Boorse et al., 2005). Both urocortin 1 and CRF bind to mammal or frog CRF_1 with high affinity (Vaughan et al., 1995; Boorse et al., 2005); in the frog CRF has approximately twice the potency of urocortin 1 in activating the CRF_1. Both mammal and frog CRF_2 exhibit higher affinity for urocortin 1 than for CRF (Vaughan et al., 1995), and urocortin 2 and urocortin 3 are selective agonists for the CRF_2 (Lewis et al., 2001; Boorse et al., 2005). Interestingly, SVG has the highest affinity of all CRF-related peptides tested for the frog CRF_2 ($\sim$40 times higher affinity than urocortin 1); by contrast, SVG binds to the frog CRF_1 with $\sim$40 times lower affinity than CRF (Boorse et al., 2005).

In non-mammalian vertebrates, CRF is considered to be both a potent stimulator of pituitary ACTH (teleost, Tran et al., 1990; Van Enckevort et al., 2000; amphibian, Tonon et al., 1986; bird, Carsia et al., 1986) and a potent thyrotropin (TSH)-releasing factor (teleost fishes: Larsen et al., 1998; amphibians: Denver, 1988; Okada et al., 2004; Okada et al., 2005; reptiles: Denver and Licht, 1989; birds: Geris et al., 1996). In the bullfrog, we have shown that CRF has greater potency than either thyrotropin-releasing hormone or gonadotropin-releasing hormone in stimulating the release of TSH from the pituitary *in vitro* (Okada et al., 2004). Moreover, we found that endogenous CRF accounts for approximately 40% and 50% of the total TSH-releasing activity in the adult and larval hypothalamus, respectively (Ito et al., 2004). De Groef and colleagues (2003) demonstrated that, in the chicken, CRF-induced TSH release is mediated by CRF_2 in that urocortin 3, which is a specific agonist for CRF_2 in mammals (Lewis et al., 2001), evokes the release of TSH, while the CRF_2-specific antagonist, antisauvagine-30 (Rühmann et al., 1998), blocks CRF-induced TSH secretion. In the same experiments, these researchers found that the pituitary thyrotropes express the mRNA for CRF_2, while the corticotropes express the mRNA for CRF_1.

The CRF_2 gene is markedly upregulated at metamorphic climax in the tadpole pituitary (Manzon and Denver, 2004), which is correlated with enhanced TSH responses to CRF at this stage of tadpole development (Kaneko et al., 2005). These findings, considered in the context of findings in the chick that the thyrotropes express the CRF_2, but not the CRF_1 led us to hypothesize that CRF-induced TSH release by the amphibian pituitary gland is mediated by the CRF_2. We found that injections of CRF-related peptides that bind preferentially or selectively to the CRF_2 elevate plasma thyroxine (T_4) and accelerate tadpole metamorphosis. Furthermore, we show, using a frog pituitary cell culture system and specific radioimmunoassay (RIA) for frog TSH, that CRF-related peptides act directly on amphibian pituitary cells to stimulate TSH release via the CRF_2.

2. Materials and methods

2.1. Animals

Adult bullfrogs (*R. catesbeiana*), weighing approximately 600 g each were supplied by Oh-uchi A.A.S. (Misato, Saitama, Japan) and kept in plastic containers under a 12L:12D photoperiod and constant temperature (23 °C) for 1 week prior to the experiments. *Spea hammondii* egg clutches were collected March 2005 in Riverside County, CA under California scientific collecting permit #802003-01 issued to R.J.D. Tadpoles were raised in the laboratory in aquaria at 20 °C on a 12L:12D photoperiod and fed a mixture of rabbit chow, agar, and gelatin molded into small cubes (Rugh, 1962) Tadpole developmental stages were assigned according to Gosner (1960). All animal experiments were approved by the Steering Committee for Animal Experimentation at Waseda University or the University Committee on the Care and Use of Animals at the University of Michigan.

2.2. Peptides

The *X. laevis* urocortin 1 and 3 were synthesized according to their cDNA sequences (GenBank accession #AY 943910 and #AY596826, respectively) on an Applied Biosystems 433A peptide synthesizer (Foster City, Calif., USA) using F-moc solid phase peptide chemistry and purified by reverse phase-high performance liquid chromatography (HPLC). Mouse urocortin 2 was obtained from the Peptide Institute, Osaka, Japan or from Bachem Bioscience, Inc. (Bubendorf, Switzerland), mouse urocortin 3 was from Bachem, SVG was from Bachem or kindly provided by Drs. Jean Rivier and Wylie Vale (Salk Institute, La Jolla, CA), bullfrog CRF was synthesized as described by Ito et al. (2004), and Okada et al. (2005), *X. laevis* CRF was provided by Drs. Jean Rivier and Wylie Vale (Salk Institute), astressin was from Bachem, and antisauvagine-30 and antalarmin hydrochloride were from Sigma (St. Louis, MO).

2.3. Animal treatments and morphological measurements

Tadpoles of the Western spadefoot toad (*S. hammondii*) were given intraperitoneal (i.p.) injections of CRF-related peptides and the effects on plasma T_4, plasma corticosterone (CORT) and the rate of metamorphosis were monitored. This species was chosen for study because previous work showed that they exhibit robust endocrine and developmental responses to injections of CRF peptides (Denver, 1997).

To test whether ligands that bind preferentially or selectively to the CRF_2 can influence thyroid or interrenal activity we administered i.p. injections of saline vehicle, *X. laevis* CRF (xCRF; 0.5 μg), xUrocortin 1 (0.5 μg) or xUrocortin 3 (2 μg) to Gosner stage 37–39 tadpoles (BW ~ 2 g; 8–10 animals/treatment) and collected blood 2 h later. The dose of xUrocortin 3 was adjusted based on the known receptor pharmacologies on frog CRF receptors (i.e., the K_i for xUrocortin 3 on the CRF_2 is ~4× less than for xUrocortin 1) (Boorse et al., 2005). A follow-up experiment was conducted to determine if a lower dose of xUrocortin3 (0.5 μg/animal) could activate the thyroid or interrenal axes. At the termination of each experiment tadpoles were anesthetized by immersion in 0.02% benzocaine and blood was collected into heparinized capillary tubes via the gill veins. Plasma was frozen and stored at −80 °C until assay for T_4 and CORT by RIA (see below).

We also tested whether injections of CRF-related peptides, specifically those that either preferentially or selectively activate the CRF_2, can accelerate tadpole metamorphosis. Tadpoles of *S. hammondii* in Gosner stages 31–32 (BW ~ 1.2 g) were injected daily i.p. with either saline vehicle or 0.5 μg of xCRF, xUrocortin 1, xUrocortin 3 or SVG (8–10 animals/treatment). A separate group of animals received no injections. At days 0, 4, 7 and 10 tadpoles were lightly anesthetized by immersion in 0.005% benzocaine and measurements of hind limb length were taken using a digital caliper as an indicator of the rate of metamorphosis. At the end of the experiment (day 10) we also captured digital images of all tadpoles and conducted morphometric analyses (see below).

We conducted a follow-up experiment in which we focused only on ligands that are known to be selective for the CRF_2 (xUrocortin 3, mUrocortin 2 and mUrocortin 3). These peptides were injected every other day at 2 μg per injection beginning with tadpoles in Gosner stages 33–34 (8 animals/treatment.) We also injected xUrocortin 1 at the same dose to serve as a positive control. Measurements were the same as described for experiment 2 and were taken at days 0, 8 and 15.

2.4. Morphometric analysis

Pre- and prometamorphic tadpoles of *S. hammondii* have an oval body shape when viewed dorsally, which changes dramatically during spontaneous or thyroid hormone-induced metamorphosis due primarily to the resorption of the gills and the restructuring of the craniofacial architecture. We developed a measure of tadpole shape to quantify shape changes that occur during metamorphosis. At the termination of the experiment tadpoles were euthanized by immersion in 0.02% benzocaine and dorsal images captured using a Retiga 1300R Fast digital video camera mounted on a Leica MS 5 stereoscope. Images were analyzed using MetaMorph image analysis software (v. 6.1 Universal Imaging Corp., Downingtown, PA). The body shape of each tadpole was evaluated based on its fit to a perfect ellipse that was drawn directly over the tadpole from the snout to the vent (where tail meets body) and extending to the most distal points on each side of the body. To determine the area of the tadpole that was out-of-oval, the exterior body shape was mapped and the total area was subtracted from the area of the perfect ellipse. To account for variation in tadpole size, the area out-of oval was then expressed as a percent of the total ellipse area and this value was used as the morphometric unit for comparison.

2.5. Radioimmunoassays

Radioimmunoassay (RIA) for bullfrog TSH was conducted as described by Okada et al. (2004). The RIAs used for plasma T_4 and CORT were as described by Denver (1998).

2.6. Pituitary cell culture

Dispersed anterior pituitary cells of adult bullfrogs were prepared according to the procedure described by Oguchi et al. (1996). In brief, following decapitation, the anterior lobes were rapidly dissected under sterile conditions, and the pituitaries were cut into small pieces and transferred into a mixture of 0.2% collagenase (248 U/mg; Wako Chemicals, Osaka, Japan) and 0.1% deoxyribonuclease I (Sigma). After mechanical and enzymatic dispersion, the suspension was centrifuged at 100*g* for 5 min, and the supernatant was removed. The completely dispersed cells were then resuspended in 70% Medium 199 (M199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% bovine serum albumin (BSA, Fraction V; Sigma). A sample of the cell suspension was used to determine the cell number. The volume of the suspension was adjusted so that 1 ml contained 350,000 pituitary cells. Two hundred-microliter aliquots of the suspension, each containing 70,000 cells, were plated in wells of a 96-multiwell plate (Asahi Techno Glass, Tokyo, Japan) and the plate incubated at 23 °C in a humidified atmosphere of 95% air/5% CO_2 for 24 h. This 24-h preincubation period was adopted because bullfrog pituitary cells cultured in the absence of TSH-secretagogues are known to release TSH continuously during this period (Okada et al., 2004). Following this preincubation period, the medium in each well was replaced with 200 μl of 70% M199 containing one of the substances to be tested: xUrocortin 1, mUrocortin 2, xUrocortin 3, SVG, or bullfrog CRF with or without astressin, antisauvagine-30, or antalarmin hydrochloride. All test substances except antalarmin were dissolved directly in 70% M199. Antalarmin were first dissolved in dimethyl sulfoxide (DMSO; Kanto Chemical, Tokyo, Japan) and then diluted with 70% M199. The final concentration of DMSO in both experimental and control cultures was 0.05%. The xUrocortin 3 is a specific ligand for *X. laevis* CRF_2 (and also human CRF_2); whereas, xUrocortin 1 binds to and activates the *X. laevis* and human CRF_1 and CRF_2 (Boorse et al., 2005). The test substances were incubated for 24 h unless otherwise stated. At the end of the incubation period, the medium was collected from each well and centrifuged, and the TSH concentration in the supernatant was analyzed by homologous RIA for bullfrog TSH (Okada et al., 2004). The values, given as the mean ± SEM, were expressed as ng/10,000 cells.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. Data were Log_{10} transformed before analysis when the variances were found to be heterogeneous (using Bartlett's test) or when derived values (e.g., morphometric measures, see above) were analyzed. Significant differences among treatment means within an experiment were determined using Scheffe's or Fisher's Least Squared Differences test (Fisher's LSD). A *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. Effects of injections of CRF-related peptides on plasma T_4 and CORT in tadpoles

Intraperitoneal injections of xCRF, xUrocortin 1 or xUrocortin 3 each produced robust elevations in plasma T_4 concentration by 2 h after injection in *S. hammondii* tadpoles when compared to uninjected or saline-injected tadpoles (ANOVA $F_{(4,39)} = 20.389$, $P < 0.0001$; $n = 8$/treatment; Fig. 1). Each of the CRF-related peptides also tended to elevate plasma CORT concentration, but owing to large variation among individuals the differences were not statistically significant (data not shown). The dosages of xCRF and xUrocortin 1 chosen for this experiment (0.5 μg/animal; BW ~ 2 g) were based on prior dose response studies with xCRF activation of the thyroid and interrenal axes in *S. hammondi* tadpoles (Denver, 1997), and the dose of xUrocortin 3 was adjusted (2 μg/animal) based on its 4-fold lower affinity for the frog CRF_2 compared with xUrocortin 1 (Boorse et al., 2005).

In a follow-up experiment, we compared a lower dose of xUrocortin 3 (0.5 μg/animal) to the dose that we tested previously (2 μg/animal). Both doses of xUrocortin 3 produced statistically significant elevations in plasma T_4 (compared to saline injected controls; ANOVA $F_{(2,21)} = 7.958$,

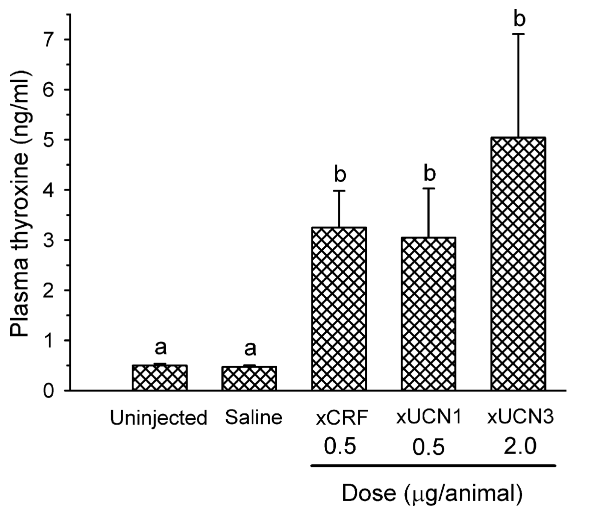

Fig. 1. Effects of i.p. injections of CRF-related peptides on plasma T_4 in tadpoles of *S. hammondii.* Gosner stage 37–39 tadpoles (BW ~ 2 g) received i.p. injections (50 μl) of either saline vehicle, *X. laevis* CRF (xCRF; 0.5 μg), xUrocortin 1 (xUCN1; 0.5 μg) or xUrocortin 3 (xUCN3; 2 μg). Two hours after injection animals were anesthetized and blood was collected for analysis of plasma T_4 by RIA. Bars represent mean ± SEM ($n = 8$–10 animals/treatment). Values with the same superscript do not differ from each other at the 5% level of significance (Fisher's LSD test).

$P = 0.003$; multiple comparisons by Fisher's LSD at $P < 0.05$; $n = 7$/treatment). The elevation of plasma T_4 with the 0.5 μg dose was 2.8-fold, while the 2 μg dose produced a 6.1-fold elevation (data not shown). As in the previous experiment, although the higher dose of xUrocortin 3 tended to elevate plasma CORT the differences were not statistically significant (data not shown).

3.2. Effects of injections of CRF-related peptides on tadpole metamorphosis

We tested if injections of CRF-related peptides that bind either preferentially or selectively to the CRF_2 could accelerate tadpole metamorphosis. Each of the CRF-related peptides tested caused significant acceleration of metamorphosis as measured by hind limb length (Fig. 2A; ANOVA: $F_{(5,51)} = 19.305$, $P < 0.0001$; $n = 8$–9/treatment) or body morphology (Fig. 2B; $F_{(5,49)} = 26.268$, $P < 0.0001$; $n = 8$–9/treatment). Testing all the peptides at the same dose (0.5 μg/animal) allowed us to evaluate potency differences. Importantly, the two ligands with greatest potency on the CRF_2, xUrocortin 1 and SVG, caused the greatest acceleration of metamorphosis. The xUrocortin3, which has significantly lower potency on the CRF_2 but is nevertheless a selective CRF_2 ligand accelerated metamorphosis compared with saline injected controls.

In a second experiment we tested only ligands that selectively bind to the CRF_2. In this experiment we chose a higher dose (2 μg/animal) to compensate for the approximately 4-fold lower affinity that these peptides have for the CRF_2 compared with xUrocortin1. We found that each of the peptides tested, xUrocortin 3, mUrocortin 2 and

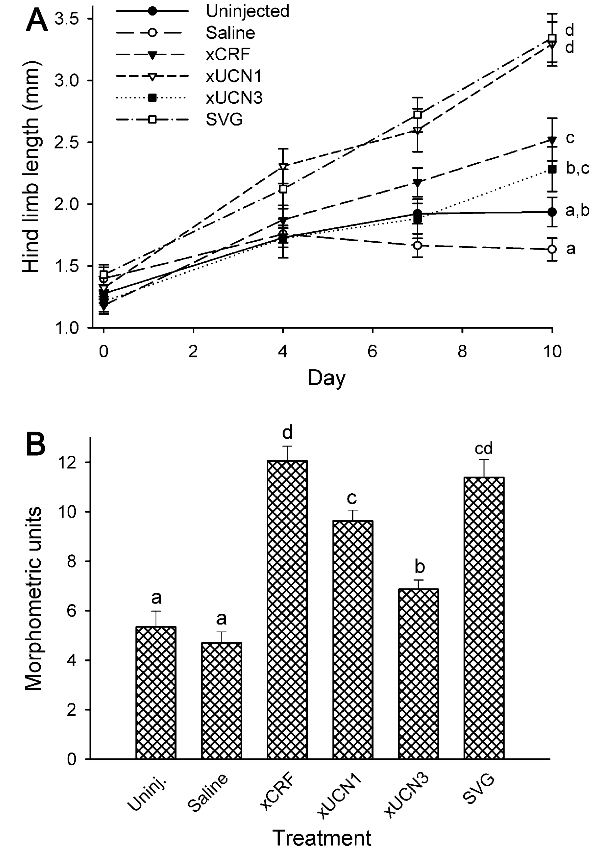

Fig. 2. Effects of i.p. injections of CRF-related peptides on metamorphosis in tadpoles of *S. hammondii.* Tadpoles in Gosner stages 31–32 (BW ~ 1.2 g) received daily i.p. injections (50 μl) of either saline vehicle or 0.5 μg of xCRF, xUrocortin 1 (xUCN1), xUrocortin 3 (xUCN3) or sauvagine (SVG). A separate group of animals received no injections. (A) Effects of injections of CRF-related peptides on tadpole hind limb length. At days 0, 5, 8 and 12 tadpoles were anesthetized and measurements of hind limb length were recorded. (B) At the termination of the experiment on day 10 digital images of tadpoles were collected and body shape was analyzed; morphometric units correspond to out-of-oval shape changes as described in Section 2. Each point or bar represents the mean ± SEM ($n = 8$–10 animals/treatment). Values with the same superscript do not differ from each other at the 5% level of significance (Fisher's LSD test).

mUrocortin3 caused significant acceleration of metamorphosis as measured by hind limb length (Fig. 3A; ANOVA: $F_{(3,27)} = 5.205$, $P = 0.006$; $n = 7$/treatment) or body morphology (Fig. 3B; $F_{(3,27)} = 9.06$, $P < 0.0001$; $n = 7$/treatment). We included a xUrocortin 1 treatment at the beginning of the experiment and these animals exhibited significant metamorphic changes within 4 days of the beginning of the injections (data not shown.) However, the 2 μg/animal dose of xUrocortin 1 was found to be toxic, likely owing to its high potency on both CRF receptors.

3.3. CRF_2 ligands stimulate TSH release by adult bullfrog pituitaries in vitro

The effects of various concentrations of the CRF-related peptides tested—xUrocortin 1, mUrocortin 2, xUrocortin 3 and SVG—on the release of TSH from dispersed pituitary cells during a 24-h culture period are shown in Fig. 4. All of

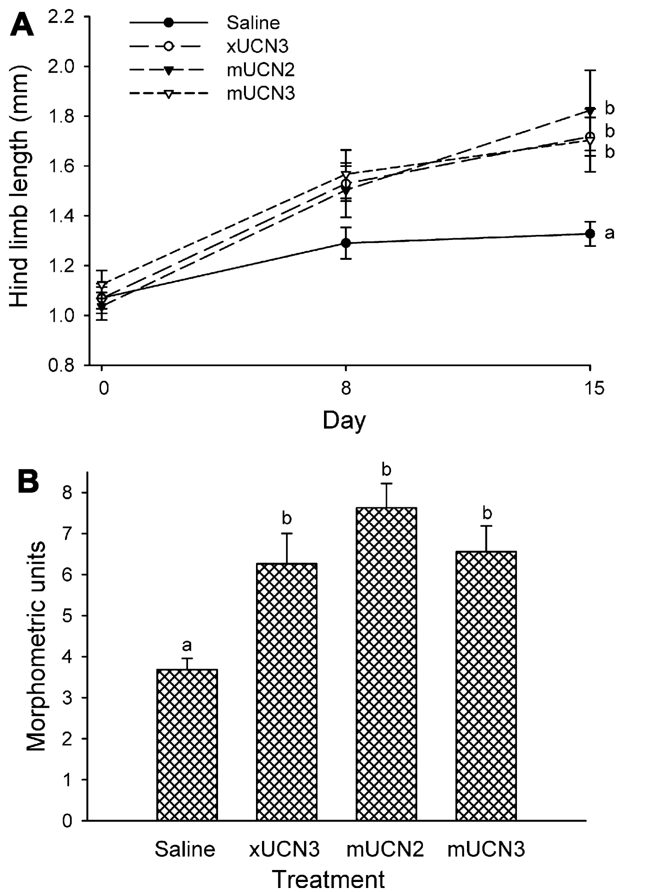

Fig. 3. Effects of i.p. injections of CRF-related peptides that bind selectively to the CRF_2 on metamorphosis in tadpoles of *S. hammondii.* Tadpoles in Gosner stages 33–34 (BW ~ 1.4 g) received i.p. injections (50 µl) every other day of either saline vehicle or 2 µg of xUrocortin 3 (xUCN3), mUrocortin 2 (mUCN2) or mUrocortin 3 (mUCN3). (A) Effects of injections of CRF-related peptides on tadpole hind limb length. At days 0, 8 and 15 tadpoles were anesthetized and measurements of hind limb length were recorded. (B) At the termination of the experiment on day 15 digital images of tadpoles were collected and body shape was analyzed; morphometric units correspond to out-of-oval shape changes as described in Section 2. Each point or bar represents the mean ± SEM ($n = 8$ animals/treatment). Values with the same superscript do not differ from each other at the 5% level of significance (Fisher's LSD test).

the peptides enhanced the release of TSH from the pituitary cells in a concentration-dependent manner. The minimum effective concentration for xUrocortin 1 and SVG were below 10^{-9} M, while those for mUrocortin 2 and xUrocortin 3 were between 10^{-9} and 10^{-8} M, respectively. Fig. 5 shows the effects of various concentrations of the CRF receptor antagonists on the frog CRF-induced TSH release from the dispersed pituitary cells during a 24-h culture period. Treatment with a general CRF receptor antagonist, astressin, or a CRF_2 selective antagonist, antisauvagine-30, counteracted the TSH-releasing activity of frog CRF in a dose-dependent manner and abolished it completely at concentrations of 10^{-7} and 10^{-6} M, respectively (Fig. 5 top, middle). Antalarmin, a CRF_1 selective antagonist, had no effect on the frog CRF-induced TSH release from the pituitary cells at any of the concentrations tested (10^{-9}–10^{-6} M) (Fig. 5 bottom).

4. Discussion

Evidence has accumulated that CRF acts as a potent TSH-releasing factor on the pituitaries of non-mammalian vertebrates (teleost fish: Larsen et al., 1998; amphibians: Denver, 1988; Okada et al., 2004; reptiles: Denver and Licht, 1989; birds: Geris et al., 1996). A recent investigation by De Groef and colleagues (De Groef et al., 2003) showed that in the chicken, TSH release is mediated by CRF_2 expressed on thyrotropes in the anterior pituitary gland. These findings led us to hypothesize that TSH release by the amphibian tadpole pituitary is mediated by the CRF_2. Here we show that CRF-related peptides that bind preferentially or selectively to the CRF_2 can elevate plasma T_4 and accelerate tadpole metamorphosis. Using dispersed frog pituitary cells in culture we show that this action of CRF_2 selective ligands is direct, and can be blocked by CRF_2 but not CRF_1 selective antagonists. On the basis of the results of our experiments we conclude that TSH release by the amphibian pituitary caused by CRF is mediated by the CRF_2.

De Groef and colleagues (2003), using chicken pituitary explant cultures, showed that human urocortin 3 which acts as a specific agonist for CRF_2 in mammals (Lewis et al., 2001) could stimulate TSH release. Furthermore, they showed that the CRF_2-specific antagonist, anti sauvagine-30 (Rühmann et al., 1998) could block CRF-induced TSH release. Using *in situ* hybridization histochemistry they also found that mRNA for CRF_2 is expressed in thyrotropes, while mRNA for CRF_1 was expressed in corticotropes in the chick pituitary gland.

We found that injections of CRF-related peptides caused rapid and robust increases in plasma T_4 concentration. The activity of xUrocortin 3 in this regard argues that the CRF_2 is involved in mediating this response, given that xUrocortin 3 exhibits negligible binding affinity for the frog CRF_1 ($K_i \sim 4$ µM; Boorse et al., 2005). A similar situation is observed in mammals, where urocortin 3 is a selective CRF_2 agonist (Lewis et al., 2001; Aguilera et al., 2004). Similarly, injections of CRF-related peptides accelerated tadpole metamorphosis, a thyroid hormone-dependent process. The high potency of xUrocortin 1 and SVG, which bind preferentially to the frog CRF_2 (Boorse et al., 2005), and the activity of the CRF_2 selective ligands in this assay (urocortins 2 and 3) also support the involvement of the CRF_2 in mediating CRF-related peptide actions on the amphibian thyroid axis. In previous work with *S. hammondii* tadpoles we found that i.p. injections of xCRF induced rapid elevations in whole body thyroid hormone and corticosterone, with half maximal doses between 0.2 and 0.5 µg/animal and maximal doses between 0.3 and 1 µg/animal (BW ~ 2 g; Denver, 1997). The doses used *in vivo* in the current study are within this range. We adjusted the dose of xUrocortin 3 to account for its ~4-fold lower affinity for the CRF_2 compared with xUrocortin 1 (Boorse et al., 2005). We found low and variable responses of plasma CORT to CRF-like peptides. In this regard, it is noteworthy that

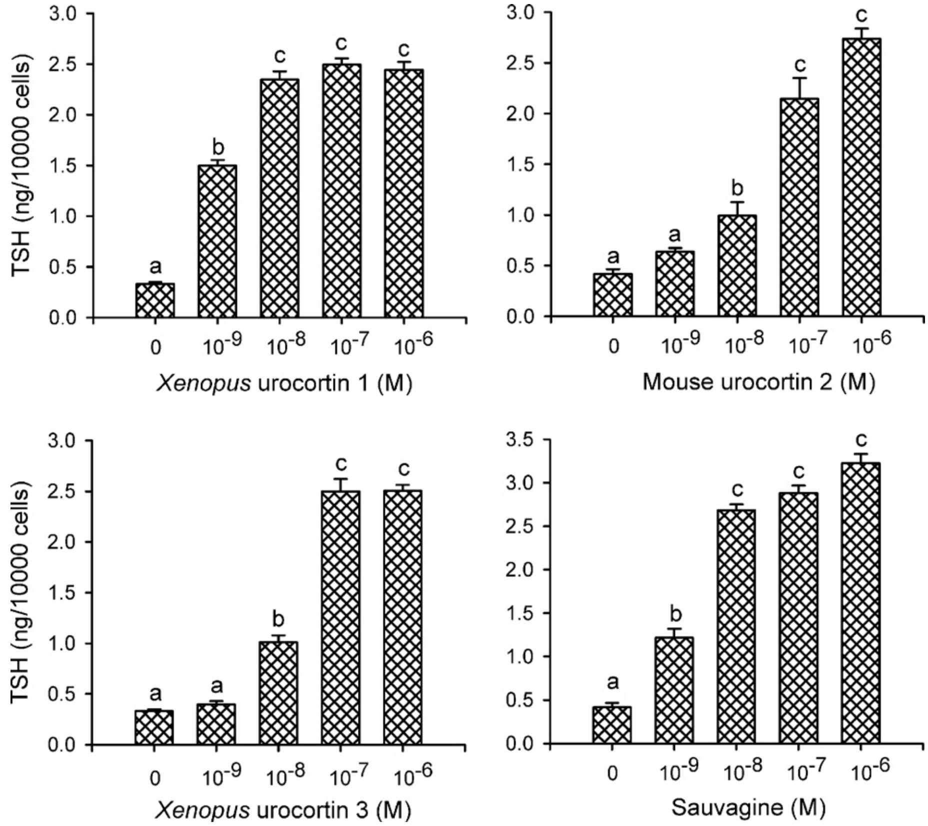

Fig. 4. Effects of CRF-related peptides on the release of TSH from dispersed bullfrog pituitary cells. Dispersed bullfrog pituitary cells (70,000 cells suspended in 200-μl aliquots of 70% Medium 199) were preincubated in wells of a 96-multiwell plate at 23 °C in a humidified atmosphere of 95% air/5% CO_2 for 24 h. The original medium in each well was then replaced with aliquots of fresh 70% Medium 199 to which one of the substances to be tested had been added. After a 24-h incubation under the same conditions, the medium was collected from each well and centrifuged, and the supernatant was subjected to the RIA for bullfrog TSH. The values, given as means ± SEM, were expressed as ng/10,000 cells ($n = 7$). Values with the same superscript do not differ from each other at the 5% level of significance (Scheffe's test).

earlier we observed smaller and variable changes in whole body CORT with administration of CRF-like peptides to tadpoles of *S. hammondii* and *X. laevis* compared with whole body T_3 or T_4 (Denver, 1997; Boorse and Denver, 2004a).

In this study we employed homologous CRFs synthesized according to the amino acid sequences predicted from cDNA sequences for bullfrog (Ito et al., 2004; Okada et al., 2005) and *X. laevis* (Stenzel-Poore et al., 1992); note that the mature CRF peptide is identical in *X. laevis* and *S. hammondii* (Boorse and Denver, 2004b). The urocortins 1 and 3 that we tested for their TSH-releasing activity were of amphibian (*X. laevis*) origin; a urocortin 2 has not yet been isolated in amphibians (see Boorse et al., 2005) so we used mouse urocortin 2. In our primary bullfrog pituitary cell culture system xUrocortins 1 and 3, mUrocortin 2 and SVG caused concentration-dependent stimulation of TSH release. The TSH-releasing potencies of xUrocortin 1 and SVG were higher than xUrocortin 3 or mUrocortin 2, which correlates with the higher affinities that the former two peptides have for the frog CRF_2 receptor compared with xUrocortin 3 (Boorse et al., 2005). We have also observed that human urocortin 1 was more potent than human urocortin 3 in stimulating the TSH release by bullfrog pituitary cells (R. Okada unpublished data). While urocortin 1 and SVG bind to both CRF_1 and CRF_2, both have greatest potency on the CRF_2 (Boorse et al., 2005). By contrast, urocortins 2 and 3 are selective CRF_2-specific agonists. It is important to note that the minimum effective dose of xUrocortin 3 tested in this study on *in vitro* TSH release (10 nM) is ~50 times lower than its EC50 for cAMP accumulation in HEK293 cells expressing the frog CRF_1, and ~200 times lower than its K_i for binding to the frog CRF_1 (Boorse et al., 2005). This strongly argues against any involvement of the CRF_1 in xUrocortin 3-mediated TSH release.

We further confirmed the CRF_2 mediation of CRF-induced TSH release by dispersed frog pituitary cells using CRF receptor antagonists. The CRF_2-specific antagonist antisauvagine-30, and the non-selective CRF receptor antagonist astressin both attenuated the TSH-releasing activity of CRF in a concentration-dependent manner; whereas, the CRF_1-selective antagonist antalarmin did not affect CRF-induced TSH release. The efficacy of antalarmin in blocking signaling by the CRF_1 in frogs was shown by Boorse and colleagues (2006) who used antalarmin to block CRF actions on the tadpole tail. The tail expresses CRF_1, but not CRF_2, and antalarmin blocked the cytoprotective

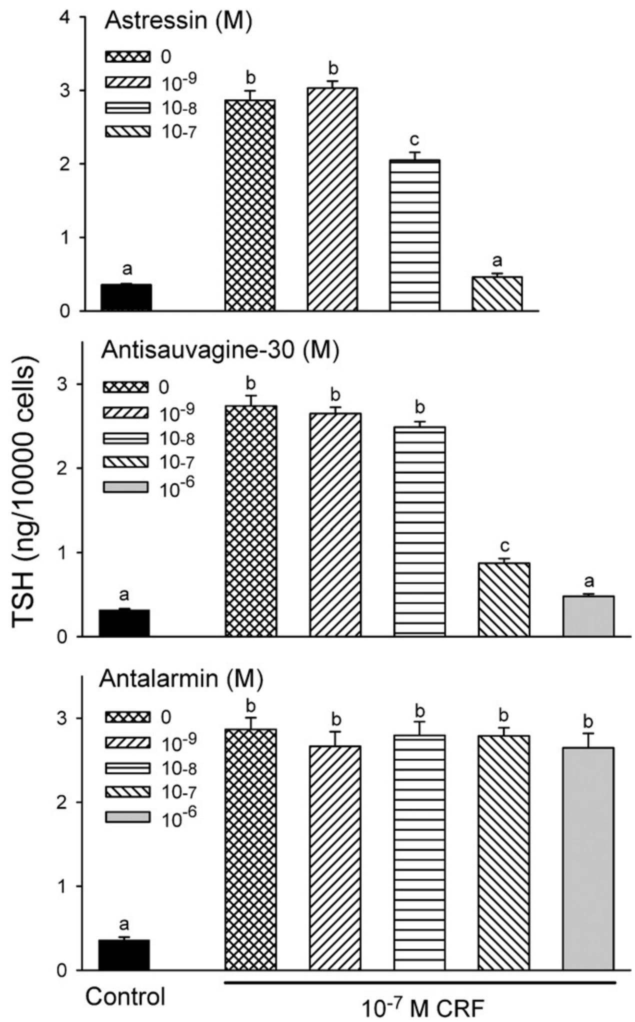

Fig. 5. Effects of CRF receptor antagonists on the frog CRF-induced TSH release from dispersed pituitary cells from adult bullfrogs. The cells (70,000 cells/well) were preincubated at 23 °C in a humidified atmosphere of 95% air-5% CO_2 for 24 h. After preincubation, the medium was replaced with fresh medium containing 10^{-7} M frog CRF and 10^{-9}–10^{-6} M astressin (top), antisauvagine-30 (middle), or antalarmin hydrochloride (bottom). Following a 24-h incubation under the same conditions, the medium was collected from each well and centrifuged, and the supernatant was subjected to the RIA for bullfrog TSH. The values, given as means ± SEM, were expressed as ng/10,000 cells ($n = 7$). Values with the same superscript do not differ from each other at the 5% level of significance (Scheffe's test).

actions of CRF on the tadpole tail *in vitro* (Boorse et al., 2006). Furthermore, antalarmin blocked the effects of CRF on cAMP accumulation in the tadpole tail muscle-derived cell line XLT-15, which also expresses the CRF_1 but not the CRF_2.

In summary, we show that CRF-related peptides that bind preferentially or selectively to the CRF_2 can elevate plasma T_4 when injected i.p. into prometamorphic tadpoles, and can also accelerate tadpole metamorphosis. We also provide strong evidence that CRF-related peptides, acting directly on amphibian pituitary cells can stimulate TSH release via the CRF_2. The CRF-induced TSH release could be completely blocked by the selective CRF_2-specific antagonist antisauvagine-30 but not by the CRF_1-specific antagonist antalarmin. Our results thus support the hypothesis that CRF-induced TSH release from the amphibian pituitary gland is mediated by CRF_2 expressed on thyrotropes, which is similar to the situation reported in the chicken pituitary (De Groef et al., 2003). The CRF_2 gene is strongly upregulated at metamorphic climax in the tadpole pituitary (Manzon and Denver, 2004). Furthermore, the tadpole pituitary exhibits enhanced TSH responses to CRF at later stages of tadpole development (Kaneko et al., 2005). We thus hypothesize that the regulation of expression of the CRF_2 in the tadpole pituitary gland plays an important role in establishing competence to respond to neurohormonal signals originating in the hypothalamus that determine the timing and rate of metamorphosis.

Acknowledgments

This study was supported by a Grant-in-Aid from JSPS to S.K. (18570063) and R.O. (18001847), Waseda University Grant for Special Research Projects (2004B-839, 2003C-009), and Grant from Inamori Foundation to R.O. This research was supported by grant IBN 0235401 from the National Science Foundation of the USA to R.J.D. This work utilized the Protein Structure Core of the Michigan Diabetes Research Training Center funded by NIH5P60 DK20572 from the National Institute of Diabetes and Digestive Kidney Disease. We are very grateful to Jeff Arendt for supplying *S. hammondii* egg masses.

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