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Identification and Characterization of Multiple Corticotropin-Releasing Factor Type 2 Receptor Isoforms in the Rat Esophagus

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Abstract

The rat esophagus shares some cellular features with skin squamous epithelium and striated muscle that express high levels of corticotropin-releasing factor type 2 (CRF_2) receptors or their cognate ligand urocortin (Ucn) 1, 2, and 3. We investigated the expression and cell signaling of CRF_2 receptors and ligands in the rat esophagus and lower esophageal sphincter (LES) by RT-PCR and quantitative PCR in normal and corticosterone-treated whole esophageal tissue, laser capture microdissected layers, and isolated esophageal cells. The expression of CRF_2 receptor protein and intracellular cAMP and ERK1/2 responses to CRF agonists and CRF_2 antagonist were determined in cultured esophageal cells and HEK-293 cells transfected with CRF_{2b} receptors. CRF_2 was abundantly expressed in the mucosa and longitudinal muscle layers of the esophagus and LES, whereas CRF_1 expression was scarce. CRF_{2b} wild-type transcript was predominantly expressed in the esophagus, and in addition, several new CRF_2 splice variants including six CRF_{2a} isoforms were identified. Expression of Ucn 1, Ucn 2, and to a smaller extent Ucn 3, but not CRF mRNA, was detected in the esophagus and LES. Ucn 1 and Ucn 2 stimulated dose-dependent cAMP production and ERK1/2 phosphorylation in the esophageal cells, whereas CRF and CRF_1 agonist, cortagine, had less potent effects. In addition, Ucn 2-stimulated cAMP and ERK responses were blocked by the CRF_2 antagonist, astressin$_2$-B. These data established the presence of a prominent CRF_2 signaling system in the esophagus and LES-encompassing multiple CRF_2 receptor variants and Ucn, suggesting a functional role in secretomotor activity and epithelial and muscle cell proliferation.

THE MAMMALIAN CORTICOTROPIN-releasing factor (CRF) family consists of CRF, a 41-amino acid (a.a.) peptide and three structurally related urocortins, namely urocortin

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(Ucn) 1, a 40-a.a. peptide previously called urocortin; Ucn 2, a 38-a.a. peptide and its N-terminal extended 43-a.a. peptide also known as stresscopin-related peptide; and Ucn 3, a 38-a.a. peptide synonymous to the 40-a.a. peptide stresscopin (1). The latter two members were discovered based on their sequence homology with CRF from human and mouse genome databases (2–4). Previous studies demonstrated that binding and biological actions of CRF and urocortin peptides are mediated by two G protein-coupled receptors (GPCRs), referred to as CRF_1 and CRF_2 (5). Both receptors belong to class B1GPCR subfamily and their genes share similar genomic organization and sequence homology (1, 6).

CRF displays high binding affinity to CRF_1 and binds much less potently to CRF_2, whereas Ucn 1 shows similar high binding affinity to both receptor subtypes (1, 6). In contrast, Ucn 2 and Ucn 3 exhibit high affinity and selective binding to CRF_2 but relatively low or no affinity to CRF_1 (1, 6). Functional studies also corroborate the role of CRF as the preferential agonist of CRF_1 in the central and enteric nervous systems and immunocytes (7–10). On the other hand, the activation of CRF_2 receptors by urocortins, particularly Ucn 2 and Ucn 3, exerts unique peripheral actions, and recent studies emphasized a stress-coping role of CRF_2 in modulating the recovery of the CRF_1-mediated endocrine, behavioral, and visceral stress responses (11–13).

Multiple alternative mRNA splice variants of both CRF receptor subtypes have been identified with distinct exon/intron selection and tissue-specific expression patterns in humans and rodents (5, 14–18). Whereas as many as 14–17 CRF_1 splice variants across human and rodent species have been isolated from the central or peripheral tissues such as the skin and placenta (5, 18, 19), only a few CRF_2 variants have so far been found, and their expression is highly discrete among different species (1, 16, 20). CRF_{2a} and CRF_{2b} (previously denoted as $CRF_{2\alpha}$ and $CRF_{2\beta}$, respectively) (1) are the most conserved splice variants identified from humans, rodents, and other species (15, 21). However, CRF_{2c}, a human-and primate-specific isoform, has not been found in rodents nor can its orthologs be predicted from the genomic sequences (1, 20). In contrast, three rodent CRF_{2a} splice variants encoding truncated receptors have been reported, but their human counterparts have not yet been shown (16, 17).

Convergent physiological, morphological, and genetic evidence has established the physiological role of CRF_2 receptors in direct or indirect modulation of cardiovascular and gastrointestinal (GI) functions under stressful conditions in rodents (12, 22–24). Recent studies demonstrate that CRF_2 receptors expressed in the heart, vascular tissues, and skeletal muscles are differentially regulated by glucocorticoids (25–27). Although glucocorticoid receptors have been described in the gut (28), regulation of CRF_2 isoforms by corticosteroid in GI tissues have not yet been investigated. Interestingly, the esophagus wall in rodents shares similar cellular features with skin squamous epithelium and skeletal muscle, which are known to express high levels of CRF_2 receptors and urocortin ligands (27, 29–31). However, only one report more than 2 decades ago showed dense binding of [^{125}I]CRF in the epithelial and external muscle layers of the rat esophagus (32). Heretofore, the expression and biological functions of CRF receptors and their cognate peptide ligands have not been demonstrated in the esophagus. Thus, the objectives of the present study were to: 1) determine which CRF receptor subtypes and ligands are expressed in the rat esophagus and

their mRNA regulation by corticosterone, 2) identify which CRF_2 variants and their encoded isoforms are expressed in specific esophageal cell types, and 3) examine the effects of urocortins on esophageal cell signaling with an emphasis on the activation of ERK1/2 in the MAPK family. MAPK pathways have recently been shown to be important intracellular mediators of urocortin actions in other viscera systems such as the cardiovascular and myometrial cells (33–35). Our results demonstrate that both CRF_2 receptors and ligands Ucn 1 and Ucn 2 are highly expressed in the esophagus, at which CRF_2 mediates urocortin stimulation of cAMP production and ERK phosphorylation. Moreover, we discovered multiple CRF_2 splice variants encoding novel rat CRF_{2a} isoforms.

Materials and Methods

Animals

Male Sprague Dawley rats (Harlan Laboratories, San Diego, CA) weighing 250–300 g were housed under controlled conditions (22–24 C, lights on from 0600 to 1800 h). Animals had free access to standard rodent chow (Prolab RMH 2500; PMY Nutrition International, Brentwood, MO) and tap water. Studies were conducted under the approved protocol of the Department of Veterans Affairs Animal Component of Research (99-110-06).

Peptides and chemicals

Human/rat CRF, rat Ucn 1, mouse Ucn 2, mouse/rat Ucn 3, CRF_1-selective agonist, cortagine (36), and CRF_2-selective antagonist, astressin$_2$-B (37) were kindly provided by Jean Rivier (Salk Institute, La Jolla, CA). Cell culture-grade medium and special chemicals were purchased from Invitrogen (Carlsbad, CA) or Sigma Aldrich (St. Louis, MO), unless otherwise specified.

Tissue collection for the esophagus and lower esophageal sphincter (LES)

Nonfasted rats were killed by decapitation. To avoid regional differences, in each rat, an approximately 0.5-cm length of esophagus with whole thickness tissue was dissected from the lower thoracic portion of the esophagus and LES right above the junction to the stomach. The hypothalamus, brain cerebral cortex, and heart were also harvested as control tissues. The tissues were processed immediately or frozen on dry ice and stored at −80 C before RNA extraction. For further studies, mucosal and muscle layers of the esophagus were surgically separated and stored at −80 C before RNA extraction or immediately processed for cell isolation.

Laser-capture microdissection (LCM) layers of the esophagus

The lower esophagus (~0.5 cm long) was collected approximately 0.3 cm from the LES and opened longitudinally in two naïve rats. The whole-thickness tissue samples were frozen in dry ice and sectioned longitudinally in a cryostat at 5 μm and mounted onto SuperFrost slides (Fisher Scientific, Pittsburgh, PA). The cutting direction was vertical to the opened esophagus tissues. Sections were dried in air, fixed in 70% ethanol, rehydrated in diethylpyrocarbonate-treated water and stained with hematoxylin and eosin. Different layers of the esophagus were dissected using the PixCell II laser capture microdissection system installed in an Olympus microscope (Molecular Devices, Sunnyvale, CA). The laser was set

to a laser beam of 7.5 μm in diameter and a laser power of 50 mW. Images were taken before and after the transfer of different cell layers by PixCell II Image archiving workstation. For each slide, samples were captured from at least 100 shots per layer on the CapSure LCM caps (Molecular Devices) and immersed immediately in RNA extraction solution.

Effect of corticosterone on esophageal CRF_2 expression

Corticosterone doses and timing of administration were based on previous reports showing mimicry with area under the curve of plasma corticosterone response to 1 h restraint stress in conscious rats and the modulation of CRF_2 receptor expression in the rat heart under these conditions (25). Three groups of nonfasted rats (n = 4 per group) used in the study included naïve (nontreated rats) or rats that received repeated sc injections of vehicle (200 μl, 11% ethanol containing saline) or corticosterone (125 μg/rat) at 0, 30-, 60-, 120-, and 180-min time points. Rats were killed by decapitation at 360 min and the lower esophagus (~0.3 cm above LES) and heart (left ventricle) tissues from all groups were processed immediately or frozen on dry ice and stored at −80 C before RNA extraction.

Esophageal cell isolation and primary culture

Esophageal epithelial and muscle cells from naïve rats were isolated by a combination of EDTA-calcium salt incubation and enzymatic digestion. The mucosal and muscle layers were surgically separated and sliced into smaller pieces (<2 mm). Subsequently tissue from each layer was collected in 50 ml conical centrifuge tubes and washed with PBS before shaking in buffered EDTA solution for 30 min at 37 C. The tissues were then transferred to a flask and incubated in a digestion solution containing a mixture of pronase E (0.5 mg/ml) and collagenase A (0.25 mg/ml; Roche, Indianapolis, IN) solution at 37 C with gentle stirring for 60 min. Dispersed cells were removed and digestion was repeated by adding fresh enzyme solution to the remaining tissues for an additional 60 min. Cells from each digestion were pooled after sedimentation and filtration to remove undigested tissue debris. The resulting cell preparation was then enriched by repeated suspensions and centrifugations in DMEM/F12 medium containing 1% fetal bovine serum to remove lysed cells. The final viable cells were placed onto the 6-well cell culture plates in DMEM/F12 medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin plus 50 mmol/liter streptomycin) up to 7 d before experiments.

RNA extraction

Total RNA from the esophageal tissues or isolated cells were prepared using TriZol reagent (Invitrogen) except for LCM captured samples, which were extracted using RNA-Bee (TEL-TEST, Friendswood, TX), following the manufacturer's recommended protocol. RNA pellets were resuspended in diethylpyrocarbonate-treated water and further digested with DNase I for 60 min at 37 C (Promega, Medison, WI). The quality and amount of final RNA yield were estimated based on ratio of absorbance at 260/280 nm by UV spectrophotometer (ND-1000; NanoDrop, Wilmington DE).

RT-PCR

Total RNA (1 μg) was denatured at 65 C for 5 min and used to synthesize first-strand cDNA in a total volume of 20 μl reaction by ThermoScript RT-PCR system (Invitrogen). The reverse transcriptase was terminated by incubation at 85C for 5 min. RNase H was then added to the reaction mixture to remove total RNA template. The sequences of oligonucleotide primers used in RT-PCR are listed in Table 1. Rat CRF receptor primers were designed to amplify the N-terminal-specific and full-length coding region of CRF_{1a}, CRF_{2a}, and CRF_{2b}. Rat CRF, Ucn 1, Ucn 2, and Ucn 3 primers were designed to amplify intron-spanning regions of targeted peptide transcripts. RT-PCRs were performed in a final volume of 30 μl using RedTaq System (Sigma-Aldrich). The reaction was predenatured at 95 C for 2 min and then amplified 34–40 times (94 C, 40 sec; 59 C, 40 sec; 72 C, 2 min), followed by a 5-min extension at 72 C in Thermal Cycler (PTC-200, MJ Research, San Francisco, CA). A housekeeping gene, acetic ribosomal protein (ARP), was used as an internal control to assure cDNA quality and equal loading. Negative control contained all reagents, except that 1 μl H_2O was substituted for reverse transcriptase in reverse transcriptase reaction to exclude the possibility of genomic or other DNA contamination. The PCR products corresponding to predicted CRF_{1a}, CRF_{2a}, and CRF_{2b} receptor, and Ucn 1, Ucn 2, and Ucn 3 were purified from the agarose gel (QIAquick gel extraction kit, QIAGEN Inc., Valencia, CA). The DNA fragments were inserted into pCR2.1 vector and transformed into bacterial competent cells (TA cloning kit; Invitrogen) or subjected to a second round of PCR with the same or nested PCR primers if no apparent visible bands were detected. Plasmids with positive insert were sequenced using the Big Dye terminator (version 3) in a cycle-sequencing system (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR

Real-time PCR was performed using cDNA prepared from LCM of esophageal tissue layers by the TaqMan method (Applied Biosystems). PCR primers and TaqMan probes for CRF receptors (Table 1) were selected using the Primer Express 1.0 software program (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using rodent primers and fluorescent dye-labeled VIC-probe (Applied Biosystems) were included as reference. Real-time PCR was performed for 40 cycles using the TaqMan Universal PCR master mix, 200 nM of both primers, 100 nM of TaqMan probe, and varying dilutions of normalized reverse-transcribed cDNA on the ABI PRISM 7700 sequence detection system according to the manufacturer. Data were quantified using comparative cycle threshold (C_t) method. For each sample, C_t was normalized to GAPDH and the final value ($2^{-\Delta\Delta Ct}$) was adjusted so that control had a mean relative mRNA level of 1.

DNA transfection and stable CRF_2-expressing cell lines

Rat esophageal CRF_{2b} cDNA encoding full-length CRF_{2b} protein was cloned into pcDNA3.1 expression vector (Invitrogen). Confirmed plasmid DNA was then transfected into human embryonic kidney (HEK)-293 cells (1 μ/10^6 cells) using Lipofectamine 2000 as a carrier. Stable CRF_{2b}-expressing cell lines were first selected by their resistance to G418 (Geneticin, 500 μg/ml) and then confirmed by RT-PCR and functional responses to urocortins. Representative HEK-293 cell lines from at least three positive clones were used

as controls to characterize esophageal CRF_2 in subsequent morphological and functional experiments.

Immunocytochemistry

Cultured esophageal or transfected HEK-293 cells grown on chamber slides (Nalgene; Nunc International, Naperville, IL) to discrete cell densities were fixed in 4% paraformaldehyde and stored in PBS at 4 C. The slides were preblocked in 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and then incubated overnight at 4 C with the primary antibody of goat or rabbit anti-CRF_2 (N20; Santa Cruz Biotechnology, Santa Cruz, CA, and Ab12964; Abcam, Cambridge, MA) at a 1:50 dilution. After washing in PBS, secondary antibody of donkey antigoat or donkey antirabbit IgG conjugated to Rhodamine Red-X or fluorescein (1:100; Jackson ImmunoResearch) was added for 1 h at room temperature. Slides with stained cells were sealed in antifade mounting media (Vector Laboratory Inc., Burlingame, CA) and visualized by standard fluorescence microscopy. CRF_2 transfected HEK-293 cells were used as a positive control. A primary antibody specificity test was performed following the same procedures except that the primary antibodies were preabsorbed by adding excess amounts of corresponding antigen (100 μg/ml).

Measurement of cAMP

Primary or transfected cells were grown to 1–2 × 10^5 cells/well in 12-well plates. Cells were rinsed with serum-free DMEM/F12 medium and incubated with increasing concentrations of CRF or Ucn 2 (10^{-10} to 10^{-6} M) in the presence of 3-isobutyl-1-methyl-xanthine (1 mM) and/or $astressin_2$-B (1 μM) for 30 min at 37 C. The treatment was stopped by the addition of 65% ice-cold ethanol. The cell extracts were collected and centrifuged at 2000 × g for 15 min at 4 C, and the supernatants were concentrated by evaporation under vacuum. The concentrates were dissolved in assay buffer for cAMP measurement in serial dilutions using a nonacetylation EIA procedure (cAMP Biotrak EIA system, GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunoblot analysis of ERK1/2-p44/42 MAPK

Primary esophageal cells or transfected HEK-293 cells were grown and allowed to reach 80–90% confluence on the 6-well plates. Cells were incubated in the serum-free medium for 2 h before treatment with or without $astressin_2$-B (1 μM) and increasing concentrations of CRF, Ucn 1, Ucn 2, or cortagine (10^{-10} to 10^{-6}M) for 5 min. At such a time, maximal stimulation of ERK1/2 phosphorylation was reached in CRF_{2b}-transfected HEK-293 cells and in other time course studies of ERK1/2-p44/42 activation by Ucn 1 or Ucn 2 (38, 39). At the end of the incubation period, media were removed and cells were lysed immediately in the 2× sample buffer on ice. Proteins were denatured by boiling for 5 min. SDS-PAGE analysis was performed on a standard system (Bio-Rad, Hercules, CA) using 10% gel. After electrophoresis, proteins were transferred to nitrocellulose membranes, which were then blocked using 5% nonfat dried milk in PBS (pH 7.2) and incubated overnight at 4 C with the primary antibody (phospho-p44/42 MAPK, Thr202/Tyr204; Cell Signaling Technology, Inc, Danvers, MA). The membranes were washed three times with PBS containing 0.1% Tween 20 and then incubated with secondary antibodies (horseradish peroxidase-conjugated goat antibodies to rabbit or mouse; 1:1000) for 1 h at 22 C. After washing three times with PBS-

Tween 20 solution, the immunoreactive bands were visualized using ECL detection reagents (GE Healthcare Bio-Sciences). Films were developed and scanned, and the bands were quantified using the 1-D imaging software (Kodak, Rochester, NY).

Statistical analysis

Value ($2^{-\Delta\Delta Ct}$) of real-time quantitative PCR represents mean ± SD of triplicate determinations from a serial dilution of each sample unless otherwise noted. All immunoblot image data are expressed as relative intensity (mean ± SE) to vehicle control from at least three separate experiments. Statistical analysis of the difference between groups was performed by two-tailed student's *t* test. Comparisons within multiple groups were performed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Expression of CRF receptor subtypes and novel CRF_2 variants in the rat esophagus

Both RT-PCR and real-time quantitative PCR analyses were performed to detect the presence of CRF_1 and CRF_2 transcripts in the full thickness tissues of the distal part of the esophagus and LES from the naïve rats. In addition, the rat cerebral cortex and heart, well known to express CRF_1 and CRF_2 receptors, respectively (40, 41), were taken as positive control tissues. Furthermore, CRF_{2a} and CRF_{2b} splice variants were examined using specific oligonucleotide primers designed to differentiate these two isoforms. As shown in Fig. 1A, CRF_1 transcript was barely detectable in both esophagus and LES, compared with that from the brain cerebral cortex. In rats, CRF_{2a} and CRF_{2b} have so far been regarded as the primary central and peripheral isoform, respectively (17, 40, 42). However, both CRF_{2a} and CRF_{2b} transcripts, differentiated by their N-terminal regions, were detected in the esophagus and LES (Fig. 1, B and C). A larger size band (483 bp) was also present in addition to the predicted CRF_{2a} PCR product (353 bp) in the esophagus and LES (Fig. 1B). DNA sequencing confirmed that this band represents a novel CRF_{2a} splice variant, which contains the entire intron 3 (herein referred to as $CRF_{2a\text{-}3}$, Fig. 2). Although the basal expression levels of predicted CRF_{2a} (353 bp) in the esophagus and LES were lower, compared with the hypothalamus (Fig. 1E), the expression level of CRF_{2b} (201 bp) was comparable with that in the heart ventricle (Fig. 1E). Therefore, CRF_{2b} appears to be the predominant peripheral isoform expressed in the esophagus based on the relative abundance of the CRF_{2a} and CRF_{2b} detected by RT-PCR and real-time quantitative PCR, in conjunction with the low expression of CRF_1 under the same conditions (Fig. 1E).

Rat CRF_2 gene (Crhr2, Entrez GeneID: 64680 and RGD: 70547) is currently known to consist of 14 exons. By sequencing various partial and full-length cDNA clones of CRF_{2a} and CRF_{2b} derived from the esophagus, we confirmed the identity of both $CRF_{2a\text{-}1}$ and $CRF_{2b\text{-}1}$ wild-type transcripts as well as two related splice variants with the same single codon (CAG) deletion, herein designated as $CRF_{2a\text{-}2}$ (desQ106) and $CRF_{2b\text{-}2}$ (desQ126; Fig. 2). We also detected in the esophagus a novel intron 3-including $CRF_{2a\text{-}3}$ splice variant (shown in Fig. 1B) would encode a truncated CRF_{2a} protein that lacks all seven transmembrane domains as a result of frame shift. Similarly, a C-terminal-truncated isoform, $CRF_{2\alpha\text{-}Tr}$, derived from $CRF_{2a\text{-}4}$ (with unspliced intron 8), or $CRF_{2a\text{-}5}$ (containing introns 8

and 9), was identified. Furthermore, another CRF_{2a} splice variant equivalent to the mouse brain soluble CRF_{2a} isoform ($sCRF_{2a}$) as a result of exon 6 deletion (16) was also isolated in the rat esophagus (Fig. 2). A schematic representation of presently known and novel CRF_2 splice variants identified from the rat esophagus is illustrated in Fig. 2. Taken together, at least five CRF_{2a} and one CRF_{2b} splice variants were found in addition to their wild-type counterparts, $CRF_{2a\text{-}1}$ and $CRF_{2b\text{-}1}$.

To further identify in which esophageal tissue layers CRF_2 is expressed, we analyzed enriched cDNAs prepared from epithelia, submucosa, circular muscle, and longitudinal muscle layers isolated by LCM (Fig. 3A). As shown in Fig. 3B, CRF_{2a}-related signals were detectable in all four vertical sections after a second round of nested PCR, whereas the predominant CRF_{2b} transcript was readily detected without a second nested PCR in the epithelial and longitudinal muscle layers. Consistent with RT-PCR data, distribution and abundance of CRF_2 in esophageal tissue layers were validated by quantitative PCR showing that the highest expression of CRF_{2a} and CRF_{2b} were found in the longitudinal muscle followed by the epithelia (Fig. 3C).

Expression of CRF family peptides

In view of the omnipresence of CRF_2 receptors in various esophageal cell types, we then determined the expression of endogenous CRF_2 ligands in the esophagus and LES. We used intron-spanning PCR primers for rat CRF and urocortins because all four CRF family peptide genes contain a single intron ranging from 261 bp (Ucn 1, RGD: 3929) to 667 bp (CRF, RGD: 620505), 669 bp (Ucn 2), and 5098 bp (Ucn 3, RGD: 1562213) based on the rat genomic database and by mouse and human orthologs of Ucn 2. CRF mRNA was not detectable in the esophagus or LES although abundantly expressed in the hypothalamus (Fig. 4A). On the other hand, transcripts of three urocortins were found in the esophagus and LES (Fig. 4, B–D). Ucn 1 and Ucn 2 were prominently expressed, but Ucn 3 mRNA level was relatively low in the esophagus and LES, compared with that expressed in the hypothalamus (Ucn 1; Fig. 4B) and heart (Ucn 2 and Ucn 3; Fig. 4C). Further sequence analysis identified the expression of two alternatively spliced variants containing 5′ deletion of five nucleotides (NNNAG) in the exon 2 of rat Ucn 1 and Ucn 3 in the esophagus (Table 1). Amplification of Ucn 1 also revealed a larger transcript (681 bp) that contains the entire intron sequence of rat Ucn 1. Despite extensive DNase I digestion to remove possible genomic DNA contaminants in total RNA, this unspliced form of Ucn 1 transcript persists in the esophagus and LES (Fig. 4B). However, the presence of the intron would not affect the coding sequence of either Ucn 1 or Ucn 3 because the start codon is located 13 and 11 bp downstream on the second exon.

Effects of corticosterone on CRF_2 gene expression in esophagus

To determine whether expression of esophageal CRF_2 variants is regulated by glucocorticoids, we examined acute changes of CRF_2 splice variants after 6 h after repeated sc corticosterone injections (125 μg/rat, 5 times at 0, 30, 60, 120, and 180 min). In the esophagus, corticosterone caused some but not significant changes in overall CRF_{2a} or CRF_{2b} transcript levels measured by quantitative PCR (Fig. 5A). Consistent with a previous report (25), heart ventricular CRF_{2b} level was significantly reduced in corticosterone-treated

rats, compared with that in naïve (28%, $P < 0.05$) or vehicle-treated group (40%, $P < 0.05$). To further determine whether individual CRF_2 isoforms and urocortins were differentially regulated by glucocorticoid, we performed specific RT-PCR to assess the expression patterns of each of the eight CRF_2 splice variant and three urocortin transcripts identified in the esophagus. As shown in Fig. 5B, the overall changes of $CRF_{2a\text{-}1}$ and $CRF_{2b\text{-}1}$ wild-type transcripts were not significant in the corticosterone-treated group. However, a marked decrease in both desQ isoforms, $CRF_{2a\text{-}2}$ (~64%) and $CRF_{2b\text{-}2}$ (~46%), was observed in the corticosterone-treated, compared with the naïve, group (Fig. 5B). Whereas $CRF_{2a\text{-}3}$ and $CRF_{2a\text{-}4}$ levels were not altered in either vehicle- or corticosterone-treated group, $CRF_{2a\text{-}5}$ expression was consistently elevated in corticosterone-treated group (2.4-fold over the naïve group) (Fig. 5B). Interestingly, the expression of $CRF_{2a\text{-}6}$ was significantly increased in the vehicle groups alone, but this vehicle effect was abolished in the corticosterone-treated group. On the other hand, the expression of three urocortin ligands was not significantly changed in corticosterone-treated group, although a general trend of down-regulation was observed (Fig. 5C).

Expression of CRF_2 protein in isolated esophageal cells

To determine whether CRF_2 receptor protein is expressed in the esophageal cells, we further examined CRF_2 immunoreactivity in primary cultures of isolated esophageal mucosa and muscle cells. Anti-CRF_2 antibodies specific to a mouse CRF_{2b} N-terminal epitope [RTTIGNGSGPYTYCN] (N20), or to a human N-terminal epitope common to both CRF_{2a} and CRF_{2b} variants (Ab12964) were used to detect CRF_2 isoforms by immunofluorescent staining. In agreement with LCM and PCR results, primary mucosal epithelial cells derived from the distal esophagus showed intense immunofluorescence (Fig. 6, A and C). Specificity of CRF_{2b} immunostaining could be demonstrated by preabsorption of primary antibody with the blocking peptide or in the absence of primary antibodies (Fig. 6, B and D). Furthermore, these antibodies stained specifically to rat CRF_{2b}-transfected HEK-293 cells but did not cross-react with rat CRF_1-transfected cells (data not shown).

CRF_2-mediated esophageal cell signaling: cAMP production and activation of ERK1/2-p44/42 MAPK

To further demonstrate the functionality of CRF_2 on the esophageal cells, we measured cAMP production and ERK1/2 phosphorylation stimulated by CRF and urocortins in the presence and absence of the CRF_2-selective antagonist, astressin$_2$-B. Ucn 2 at doses from 10^{-9} to 10^{-6} M, increased dose dependently intracellular cAMP levels in cultured esophageal cells with a maximal response reached at 100 nM. In contrast, CRF caused smaller but significant increases only at doses superior to 100 nM (Fig. 7A). In the control CRF_{2b}-transfected HEK-293 cells, both Ucn 2 and CRF induced dose-dependent increases in cAMP accumulation, but that of CRF was shifted to the right (Fig. 7B). In both cell types, Ucn 2-stimulated cAMP increases were inhibited by the addition of 1 μM astressin$_2$-B (Fig. 7, A and B). These data indicate that the CRF_2 receptors are functionally coupled to Gs-adenylyl cyclase effector system in both native and transfected cell types.

Phosphorylation of p44/42 MAPK at Thr202/Tyr204 by ERK kinases (MEK1/2) has been used as a reliable molecular modification indicative of activated ERK1/2 by CRF (43).

Dose-dependent increases in pERK1 and pERK2 were observed in the cultured esophageal cells stimulated with Ucn 1 and Ucn 2 after 5 min, whereas no significant effect was induced by CRF except at the highest dose (1 μM; Fig. 8A). Similar to cAMP response, maximal ERK response to Ucn 1 and Ucn 2 was reached at 100 nM, at which dose Ucn 1 stimulated 2.7- and 2-fold, whereas Ucn 2 stimulated 4.1- and 2.9-fold increases in pERK1 and pERK2, respectively, over the vehicle control levels (Fig. 8C). In CRF_{2b}-transfected HEK-293 cells, Ucn 1 and Ucn 2 caused similar dose-dependent increases in pERK1/2 (Fig. 8B). Ucn 1 caused 2.8- and 3.9-fold and Ucn 2 caused 5.5- and 7.5-fold increases pERK1 and pERK2, respectively (Fig. 8, C and D). Furthermore, dose-dependent activation of pERK1/2 by Ucn 2 in the esophageal cells was potently blocked by $astressin_2$-B pretreatment (Fig. 9). Similar to CRF, CRF_1-selective agonist, cortagine, was significantly less potent than Ucn 2 and only moderately effective in activation of ERK1/2 with 2.2- and 2-fold increases at concentrations higher than 100 nM (Fig. 9).

Discussion

In the present study, we provide novel evidence showing that multiple and functional CRF_2 isoforms are expressed in the rat esophagus and LES. Using RT-PCR and real-time PCR analyses on LCM esophageal tissue sections, we found CRF_{2b} was the predominant splice variant expressed in the epithelial and longitudinal muscle layers. On the other hand, CRF_{2a} was expressed throughout the esophageal layers, albeit at relatively lower levels than CRF_{2b}. The predominance of CRF_{2b} expression in the rat peripheral tissues (34,44) is thus expanded to the esophagus and LES. Of interest is the observation that the density of the CRF_{2b} isoforms expressed in the esophagus and LES is comparable with that in the heart, from which CRF_{2b} was originally identified (30, 40). In addition, we identified six other rat CRF_2 splice variants including two novel isoforms of CRF_{2a} (Fig. 2). The first novel variant, $CRF_{2a\text{-}2}$, has the equivalent deletion of a single codon for Glu-106 to rodent $CRF_{2b\text{-}2}$ (CRF_{2b}-$desQ_{126}$), which has previously been described in the rat aortic smooth muscle cell line and is functionally similar to the wild type (14, 45). The second novel variant, $CRF_{2a\text{-}3}$, by retaining the unspliced intron 3 (130 bp), encodes a 141-a.a. truncated receptor. This splice variant was detected in the esophagus muscle layer and LES as well as the hypothalamus and heart (data not shown). In addition, $CRF_{2a\text{-}4}$ and $CRF_{2a\text{-}5}$, two other intron-retaining variants that encode a truncated receptor isoform ($CRF_{2\alpha\text{-}Tr}$) previously found in the rat amygdala (17), was also detected in the esophagus. Truncated CRF receptors, such as $CRF_{2a\text{-}3}$ and $CRF_{2a\text{-}4}$, generated by premature termination due to frame shift, have been reported to lose either significant binding or signaling properties but may modify functions of their ligands and other wild-type receptors (5, 17). We also identified in the rat esophagus the expression of another splice variant ($CRF_{2a\text{-}6}$), which encodes a soluble CRF_{2a} isoform similar to that previously found in the mouse brain ($sCRF_{2\alpha}$) (16). This soluble $CRF_{2a\text{-}6}$ isoform displays increased binding affinity to CRF and Ucn 1, suggesting a novel role of CRF_{2a} in modulating CRF_1-dependent functions (16). Whether any similar functional role can be expected from these novel truncated or $sCRF_{2a}$ isoforms identified from the esophagus remains to be investigated.

To gain a better insight of the peptide ligand components within the esophageal CRF system, expression of four CRF family members were also investigated. There were no detectable

signals of CRF found in the normal rat esophagus or LES, whereas a strong signal was detected in the cerebral cortex as previously reported (46) (Fig. 4). This finding is not unexpected because CRF is primarily expressed in the brain and mostly absent or at low levels in the peripheral tissues (47). On the other hand, all three urocortins were peripherally expressed in the esophagus and LES. We also confirmed the presence of Ucn 1 mRNA in the hypothalamus and Ucn 2 and Ucn 3 in the heart, consistent with previous observations in the rat brain (48) and mouse cardiomyocytes (34). Sequencing data further established the identity of multiple Ucn 1 and Ucn 3 transcripts as a result of alternative splicing using a different downstream but noncoding acceptor site (NNNAG). However, the presence of an unspliced intronic sequence in the larger Ucn 1 transcript implicates a possible role in regulating the stability of mRNA or the efficiency of translation. Another possibility to account for the presence of this larger form is that this transcript may be derived from the immature or precursor form of antisense Ucn 1 mRNA. Previous studies showed that sense and antisense Ucn 1 transcripts were coexpressed not only in the rat brain but also the heart and skeletal muscle, and more recently an immature form of antisense Ucn 1 mRNA has been reported in the rat brain (49, 50). Naturally occurring antisense transcripts have been identified in rodent and human genes such as fibroblast growth factor-2 RNA and c-*myc* to regulate sense gene expression (51). Here we provide further evidence that Ucn 1 antisense RNA is also likely present in the esophagus and may play a more general role in gene silencing outside the brain.

Steroid hormone glucocorticoids regulate CRF_2 receptor and urocortin gene expression as largely investigated in the heart and skeletal muscles in rodents (25–27, 52). Activation of ligand-bound nuclear glucocorticoid receptor and *cis*-acting glucocorticoid response elements leads to an increase in Ucn 2 promoter gene activity and a decrease in CRF_{2a} and CRF_{2b} expression in the rat cardiovascular system and pituitary (25, 26, 53, 54). In the present study, rats treated with corticosterone using an acute regimen to reproduce corticosterone rise induced by acute restraint stress (25) did not result in significant changes in either esophageal CRF_{2a} or CRF_{2b} transcript levels after 6 h. However, an apparent up-regulation of CRF_{2a} was observed in animals treated with vehicle or corticosterone, suggesting sensitivity to handling and repeated sc injections (Fig. 5A). By contrast, cardiac CRF_{2b} mRNA levels were significantly reduced by such a regimen of repeated corticosterone administration consistent with previous reports (25). Because our quantitative PCR was designed to amplify the 5′ noncoding exon of CRF_{2a} or CRF_{2b}, it thus could not distinguish individual splice variants that might be differentially regulated by the glucocorticoids. Using variant-specific RT-PCR to assess the expression pattern of all eight CRF_2 isoforms, we found similar results of no significant inhibition in the wild-type $CRF_{2a\text{-}1}$ and $CRF_{2b\text{-}1}$ expression. Surprisingly, repeated injections of vehicle alone caused up-regulation of $CRF_{2a\text{-}3}$, $CRF_{2a\text{-}5}$, and $CRF_{2a\text{-}6}$ variants, which were attenuated ($CRF_{2a\text{-}3}$ and $CRF_{2a\text{-}6}$), or enhanced ($CRF_{2a\text{-}5}$) by corticosterone treatment. These data suggest that posttranscriptional processing of CRF_{2a} transcripts are more sensitive to physical stress and glucocorticoids and provide the first evidence that there is a cell- and tissue-specific regulation of CRF_2 receptor splice variants in the esophagus. Future variant-specific approach to differentiate multiple CRF_2 isoforms is therefore necessary to provide a

comprehensive expression profile of a hormonally regulated gene within a specific cell type and tissue.

To confirm a fully functional CRF_2 system that exists in the esophagus, we further used esophageal mucosal epithelial cells and stable cell lines transfected with CRF_2 receptors cloned from the esophagus to provide CRF_2 immunocytochemical evidence and characterize CRF_2-mediated intracellular signaling properties. The commercial antibodies available against CRF_2 showed specific immunoreactivity to rat GI tissues, and CRF_{2b}-transfected cells were selected for analysis (data not shown). In the present study, we demonstrated that antibody N20 and antibody Ab12964 were both immunoreactive to the cultured esophageal mucosal cells that contain wild-type CRF_{2a} and CRF_{2b} isoforms (Fig. 6). However, because both antibodies recognize the N-terminal extracellular region of the receptor, we cannot rule out the possibility that they may also react with truncated CRF_{2a} isoforms, *i.e.* $CRF_{2a\text{-}3}$ or $CRF_{2a\text{-}4}$, expressed in the native esophageal cells.

Similar to other members of the class B1 GPCR family, intracellular signals transduced by CRF receptor-ligand interaction are primarily via Gαs coupling and adenylyl cyclase activation, which lead to cAMP-dependent signaling cascades including protein kinase A and exchange proteins activated by cAMP (5, 38, 55–57). We demonstrated that Ucn 2 (10^{-9} to 10^{-6}M) induced a dose-related significant increase in cAMP production in primary cultures of isolated esophageal mucosal cells, whereas CRF resulted in a small increase only at the highest concentration. By contrast in CRF_{2b}-transfected HEK-293 cells, CRF was able to induce dose-dependent cAMP production, although with a right shift in the dose-response curve, compared with Ucn 2. The Ucn 2 effect could be abolished by the selective antagonist astressin$_2$-B, indicating the increases of intracellular cAMP were primarily mediated by CRF_2.

ERK belongs to one subfamily of MAPK composed of 42- and 44-kDa kinases, also known as p42-MAPK and p44-MAPK, respectively (58). Phosphorylation of ERK1/2 is one of the most sensitive and consistent signaling events induced by the activation of CRF receptors, although, it appears to be cell type and ligand specific (33–35, 43, 59). In rat esophageal mucosal cells, Ucn 1 and Ucn 2, and to a lesser extent CRF and cortagine, stimulated phosphorylation of ERK1/2 and Ucn 2 response was prevented by the selective CRF_2 antagonist, astressin$_2$-B. Similar action by Ucn 1 and Ucn 2, but not CRF, was observed in rat CRF_{2b}-transfected HEK-293 cells, consistent with previous data obtained from human CRF_{2b} transfected HEK-293 cells (33). Collectively, these data indicate that the increase in cAMP and ERK1/2 phosphorylation in the esophageal mucosal cells is linked with the activation of CRF_2 receptors because the weak CRF_2 ligand, CRF, and selective CRF1 agonist, cortagine (36), had less or no effect. Previous studies in transfected CHO-K1 cells have established that Ucn 2 and Ucn 3 activate ERK1/2 selectively through CRF_{2b} (38). In adult mouse ventricular cardiomyocytes, Ucn 2 and Ucn 3, and rat neonatal cardiomyocytes, Ucn 1 induces a CRF_{2b} receptor-mediated increase in ERK1/2 phosphorylation that is not dependent on the coupling with Gαs (34, 35, 60). Likewise, in cultured human pregnant myometrial cells, Ucn 1 and sauvagine, but not CRF, induce ERK1/2 phosphorylation through CRF_2 receptors coupled primarily, but not exclusively, to Gαq, whereas the Gαs pathway is not involved (33, 39, 59). In both adult rat cardiomyocytes and human pregnant

myometrial cells, Ucn 1-induced maximal ERK1/2 response occurs within 5–10 min at concentrations of 100 nM of the peptide (33, 34, 39). In rat esophageal cells, we obtained a maximal response at 10 nM, further showing the sensitivity and potency of Ucn 1 and Ucn 2 to induce ERK1/2 phosphorylation in these cells. To our knowledge, this is the first report that Ucn 1 and Ucn 2 activate the p44/42 MAPK signaling in GI cells.

The high expression of CRF_2 receptors, coupled with the presence of cognate urocortin ligands in the rat esophageal epithelium, suggest the existence of a distinct signaling network that is likely to have significant physiological significance in the regulation of esophageal functions. Activation of the ERK pathway plays an important role in processes such as cell proliferation, differentiation, and apoptosis (58). Acid is known to increase ERK activity in the esophagus that is associated with an increased rate of squamous cell proliferation during injury repair (61). Thus, CRF_2 may be involved in the stress-coping response of ERK under conditions of luminal acid exposure of the esophageal mucosa during acid reflux. Of interest was the finding revealed by our LCM and RT-PCR analysis of various esophageal tissue layers that the highest level of CRF_{2b} is localized within the longitudinal muscle layer, whereas there was little or lack of expression in the circular muscle layer. The outer longitudinal and inner circular muscle layers of the rat esophagus consist only of striated muscle and CRF_{2b} is largely expressed in striated skeletal muscles (30, 31). However, the intense signals of CRF_{2b} in the LES that is formed of smooth muscles (62–64), and the known expression CRF_{2b} in the vascular system (12, 65, 66), indicate that CRF_{2b} expression is not limited to striated muscles. The highest expression of CRF_2 in the longitudinal muscles further implies a modulation of contractile events through urocortin activation because the esophageal longitudinal muscle plays a key role in the regulation of esophageal propulsive motor function (67). In line with such a possibility, a similar Ucn 2/CRF_2 receptor system has been described in myometrial cells that regulate myometrial contractility through activation of ERK1/2 phosphorylation (39).

In conclusion, our data demonstrate that CRF_2 receptors and cognate ligands, Ucn 1, Ucn 2, and much less prominently Ucn 3, are highly expressed in the rat esophagus and LES, whereas the CRF-CRF_1 signaling system is underexpressed. The identification of multiple CRF_2 receptor isoforms, including two novel CRF_{2a} splice variants, and additional Ucn 1 and Ucn 3 splice variants further illustrate the existence of a functional CRF_2 system responsive to autocrine and/or paracrine actions of urocortins. Thus, the esophagus and LES provide a new peripheral gut target to elucidate previously unrecognized urocortin/CRF_2 signaling mechanisms that may mediate or modulate various esophageal functions, including propulsive motor activity, and epithelial cell secretion and proliferation.

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Abbreviations:

a.a.	Amino acid
ARP	acetic ribosomal protein
CRF	corticotropin-releasing factor
C_t	cycle threshold
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GI	gastrointestinal
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
LCM	laser capture microdissection
LES	lower esophageal sphincter
$sCRF_{2a}$	soluble CRF_{2a} isoform
Ucn	urocortin

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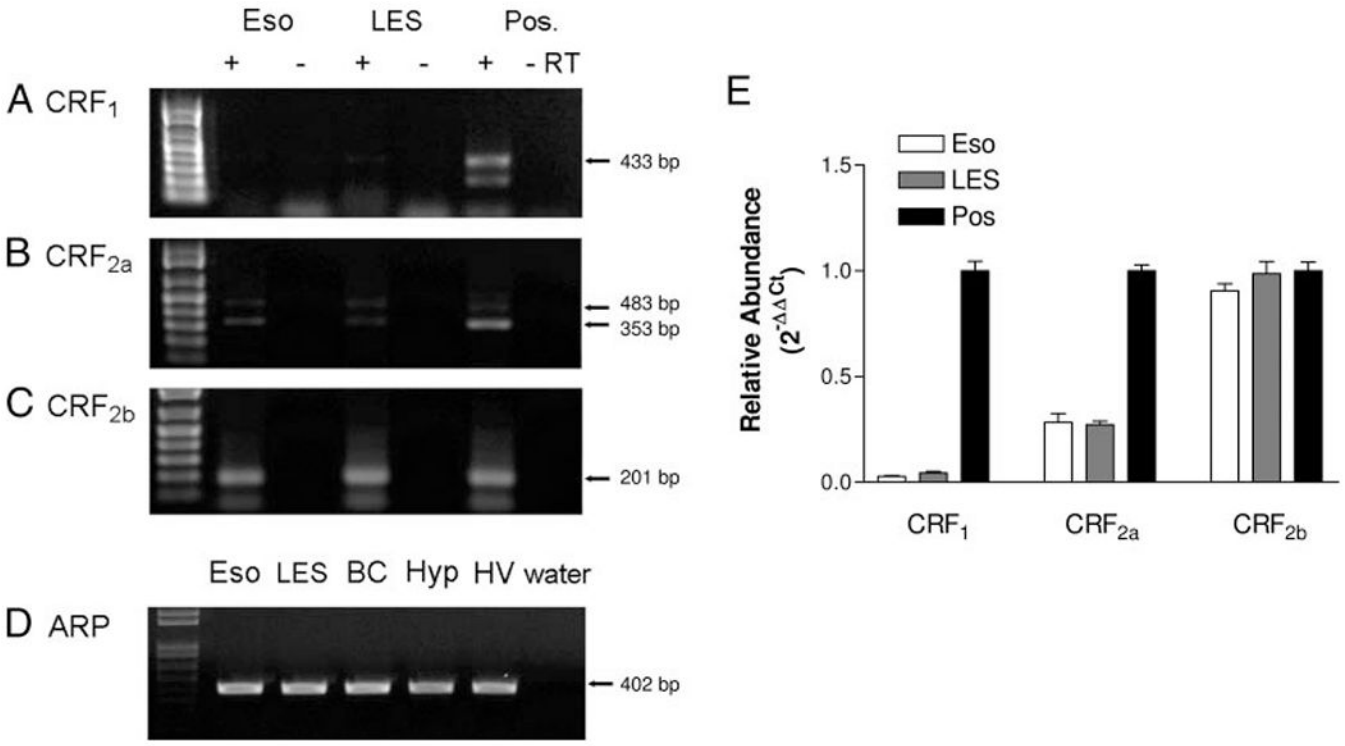

Fig. 1.

Expression of CRF receptors in the rat esophagus and LES. RT-PCR analysis was performed to detect the presence of CRF receptor transcripts in the distal esophagus (Eso) and LES cDNAs synthesized with (+) or without reverse transcriptase (–RT) of normal rats using specific oligonucleotide primers (shown in Table 1) to amplify amino-terminal coding sequences, which differentiate receptor subtypes and two major CRF_2 splice variants, 2a and 2b. Brain cerebral cortex (BC), hypothalamus (Hyp), and heart ventricle (HV) were used as positive controls (Pos.) for CRF_1, CRF_{2a}, and CRF_{2b}, respectively. Predicted CRF_1 (433 bp), CRF_{2a} (353 bp), and CRF_{2b} (201 bp) and an unpredicted CRF_{2a} variant ($CRF_{2a\text{-}3}$, 483 bp) were indicated by *arrows* next to their respective panels (A–D). Real-time PCR was performed to measure relative abundance of CRF_1 and CRF_2 using the same samples. Data were calculated by $2^{-\Delta\Delta Ct}$ method and expressed as mean ± SD of triplicate determinations from two separate experiments normalized to the internal GAPDH and positive controls, which has a mean relative mRNA level of 1.0 (E).

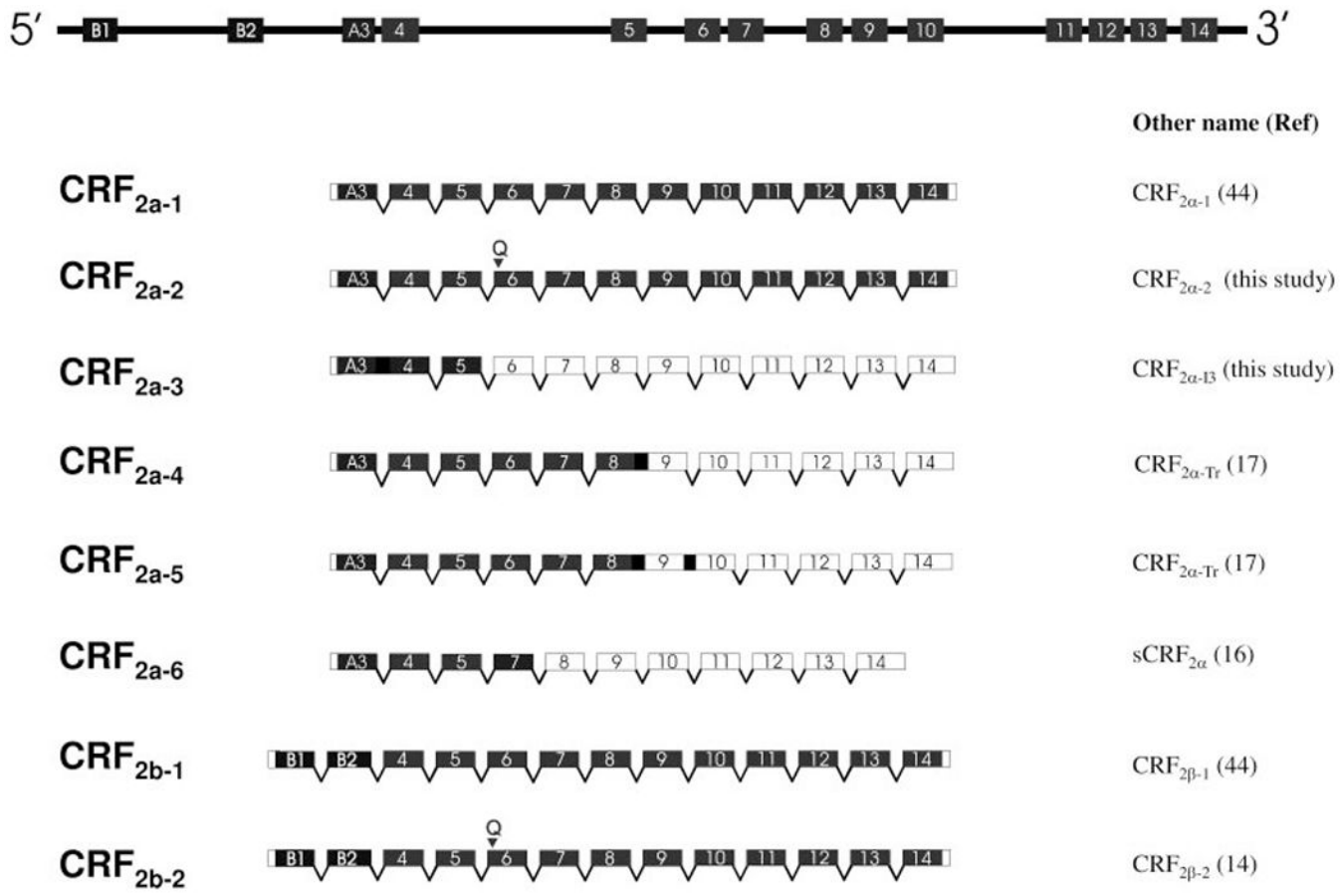

Fig. 2.

Rat CRF_2 gene organization and RNA splice variants. Schematic representation of rat CRF_2 gene organization consisting of 14 exons is diagrammed based on the latest annotation for rat Crhr2 (GenBank accession no. NM_022714, Entrez GeneID: 64680, and RGD: 70547). Wild-type and splice variants of CRF_{2a} and CRF_{2b} are indicated by inclusion or skipping of specific exons or introns in each transcript. A total of eight different CRF_2 splice variants are identified in the present and previous studies, including wild-type $CRF_{2a\text{-}1}$ and $CRF_{2b\text{-}1}$ and their respective desQ isoforms $CRF_{2a\text{-}2}$ and $CRF_{2b\text{-}2}$, a novel variant containing unspliced intron 3 and designated as $CRF_{2a\text{-}3}$ (GenBank accession no. EF078963), $CRF_{2a\text{-}4}$ and $CRF_{2a\text{-}5}$ (EF078964), two intron-including splice variants encoding $CRF_{2\alpha\text{-}Tr}$ (17), and $CRF_{2a\text{-}6}$ (EF078965) as the rat ortholog of exon-skipping variant found in mouse (16). Both $CRF_{2a\text{-}3}$ and $CRF_{2a\text{-}6}$ encode soluble CRF_{2a} isoforms lacking membrane spanning domains. *Open box* represents noncoding exon sequence in the transcript. Q, Glutamine.

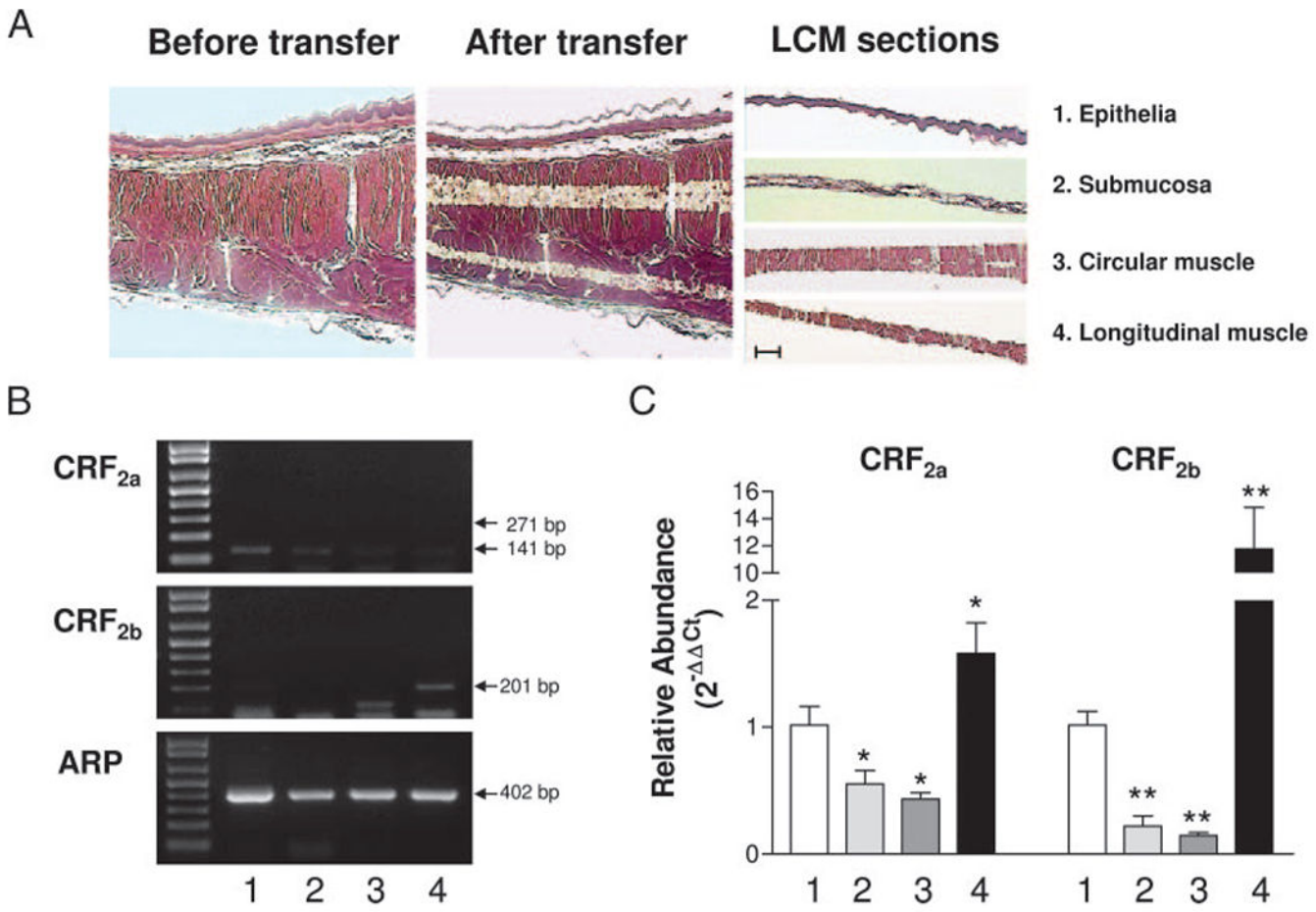

Fig. 3.

Expression of CRF_{2b} and CRF_{2a} splice variants in the esophagus from LCM layers in naive rats. A, Freshly frozen vertical sections (5μm) of esophagus were stained with hematoxylin and eosin. The surface epithelia, submucosa, and circular and longitudinal muscle sections were separately collected (*right panel*) from the whole-thickness section (*left panel*), and untargeted tissues remained attached to the glass slide (*middle panel*). Bar, 100 μm. RT-PCR and real-time quantitative PCR analyses were performed to detect the expression of CRF_{2a}- and CRF_{2b}-specific transcripts in four LCM sections captured from epithelia (1), submucosa (2), circular muscle (3), and longitudinal muscle layers (4). B, Gel image of RT-PCR analysis. PCR conditions to detect CRF_{2b} and ARP were the same as described in Fig. 1. However, detection of CRF_{2a} in LCM samples required nested PCR using NT primer pair 5′-GCGGCCCCTCATCTCCGTGAG (forward) and 5′-CTGGTCCAAGGTCGTGTTGCA (reverse) first and then replacing the forward primer with 5′-GCAATGGACGCGGCGCTGCTCCTCA (full-length forward primer) and the same reverse primer in the second PCR. Sequences of the predicted PCR product for CRF_{2a} (141 bp) and $CRF_{2a\text{-}3}$ (271 bp) were confirmed. C, Real-time quantitative PCR analysis of CRF_{2a} and CRF_{2b} for LCM sections. Data were expressed as relative abundance ($2^{-\Delta\Delta Ct}$) normalized to GAPDH and epithelia controls (section 1), which had a mean relative mRNA level of 1 and

values are mean ± SD of triplicate determinations from two rats (*, $P < 0.05$; **, $P < 0.001$ *vs.* epithelia).

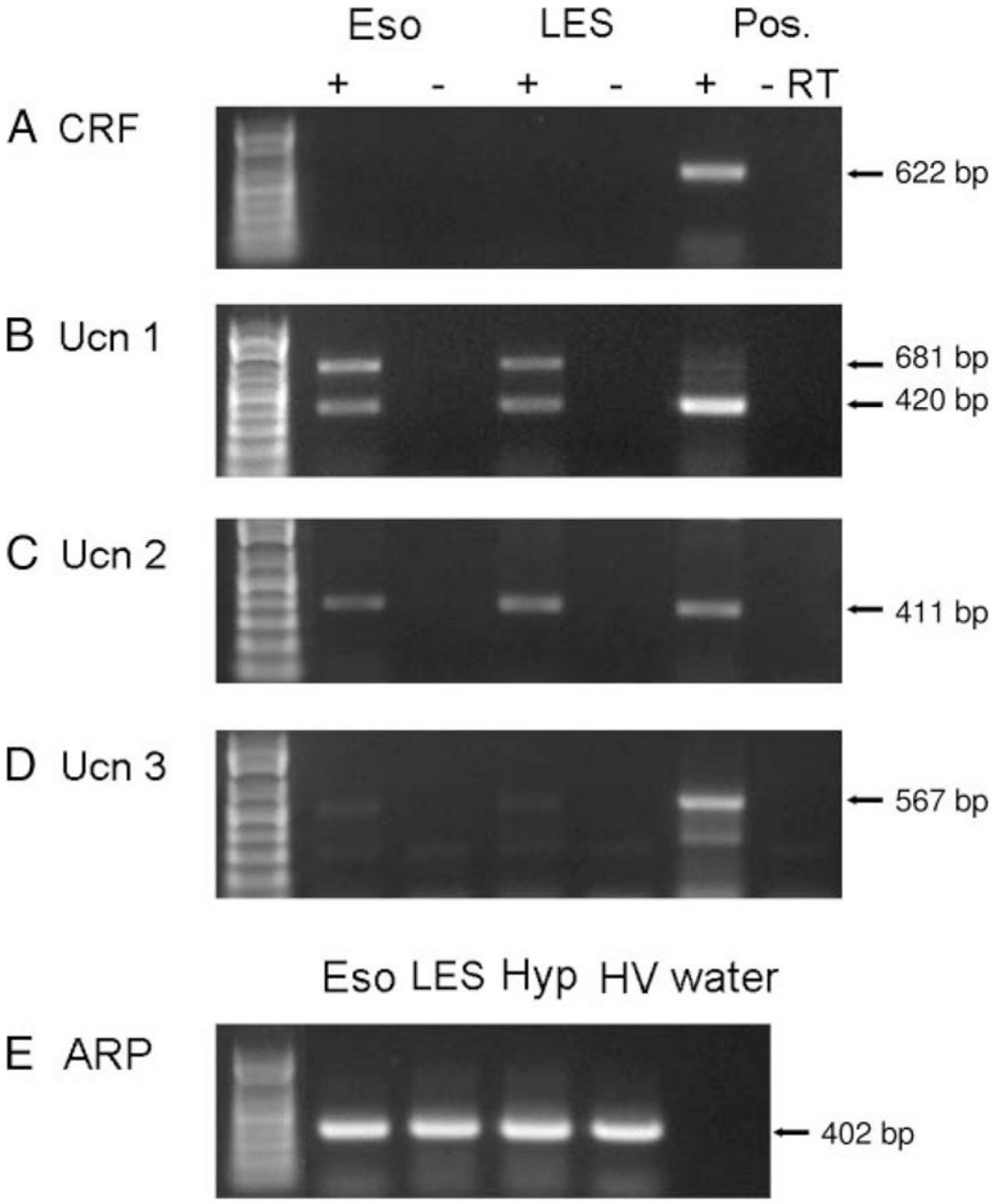

Fig. 4.

Expression of CRF and urocortins in the rat esophagus and LES. The presence and relative abundance of transcripts for CRF and urocortins including Ucn 1, Ucn 2, and Ucn 3 were determined by RT-PCR in the distal esophagus (Eso) and LES of the naive rats. Intron-spanning PCR primers were designed to amplify both noncoding (exon 1) and coding (exon 2) regions of CRF, Ucn 1, Ucn 2, and Ucn 3. Hypothalamus (Hyp) was used as positive control (Pos.) for CRF and Ucn 1 and heart ventricle (HV) for Ucn 2 and Ucn 3 to generate predicted PCR products as indicated by *arrows* next to their respective panels (A–D). CRF

(622 bp), three splice variants of Ucn 1 (415, 420, and 681 bp), Ucn 2 (411 bp), and two variants of Ucn 3 (562 and 567 bp) were identified and confirmed after DNA sequencing of the PCR products. GenBank accession numbers for rat Ucn 2 and Ucn 3 reported here are EF078966 and EF078967, respectively.

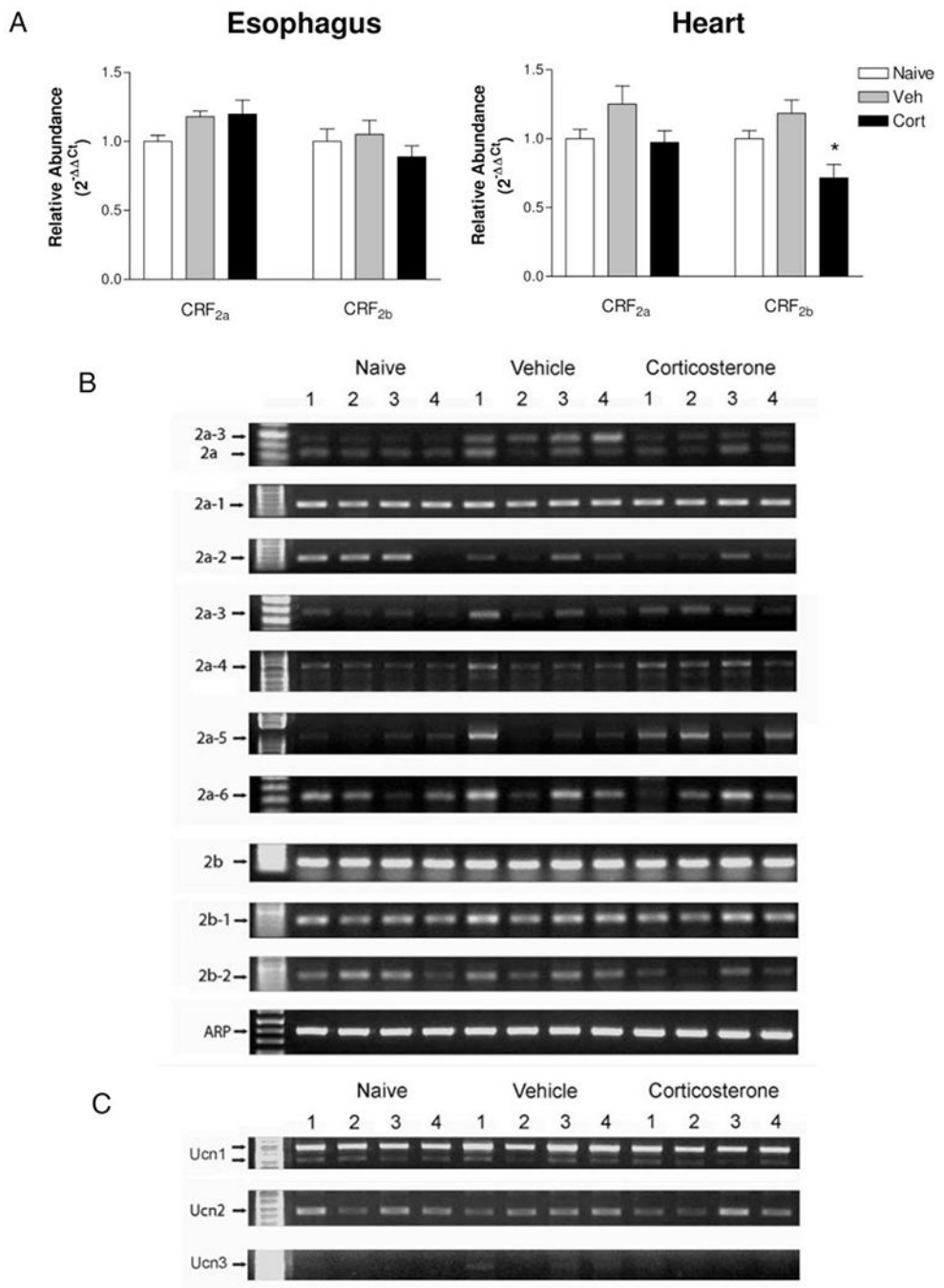

Fig. 5.

Effect of acute corticosterone injections on esophageal CRF_2 receptor and urocortin ligand transcript expression in conscious rats. Relative abundance of total esophageal and heart ventricular CRF_{2a} and CRF_{2b} transcripts (A) from the naïve and vehicle (Veh)- and corticosterone (Cort)-sc injected groups were measured by real-time quantitative PCR and expressed as mean ± SE (n = 4/group) of normalized values relative to GAPDH and naïve control (*, $P < 0.05$ *vs.* naïve and vehicle control). RT-PCR was performed to compare the expression levels of CRF_2 receptor wild-type and splice variants and CRF_2 ligand urocortins

in the esophagus between the naïve and vehicle-treated and corticosterone-treated groups. Representative gel images of CRF_2 receptor isoforms (B) and urocortins (C) are shown.

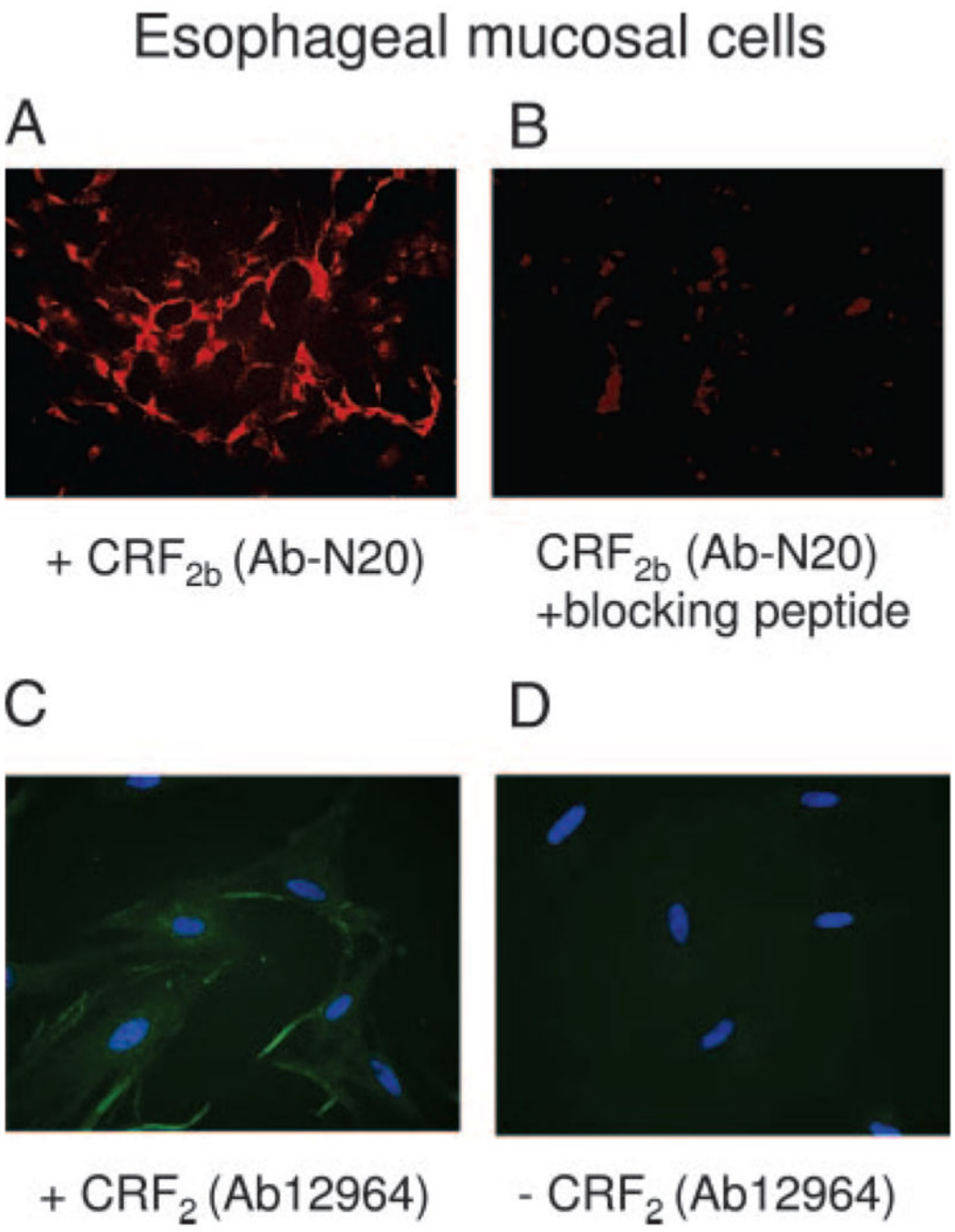

Fig. 6.

Immunofluorescence of CRF_2 receptors in rat esophageal cells. Rat esophageal cells isolated from the mucosal tissue layer and cultured for 7 d were stained with primary anti-CRF_2 antibody specific to CRF_{2b} (N20) in the absence or presence of blocking peptide (A and B) or with another antibody that recognizes both CRF_{2a} and CRF_{2b} isoforms (Ab12964, C and D). Secondary antibodies of donkey antigoat or donkey antirabbit IgG conjugated to rhodamine Red-X or fluorescin were used as distinct immunofluorescent signals.4′,6′-Diamino-2-phenylindole was used to perform nuclear counter-staining (C and D).

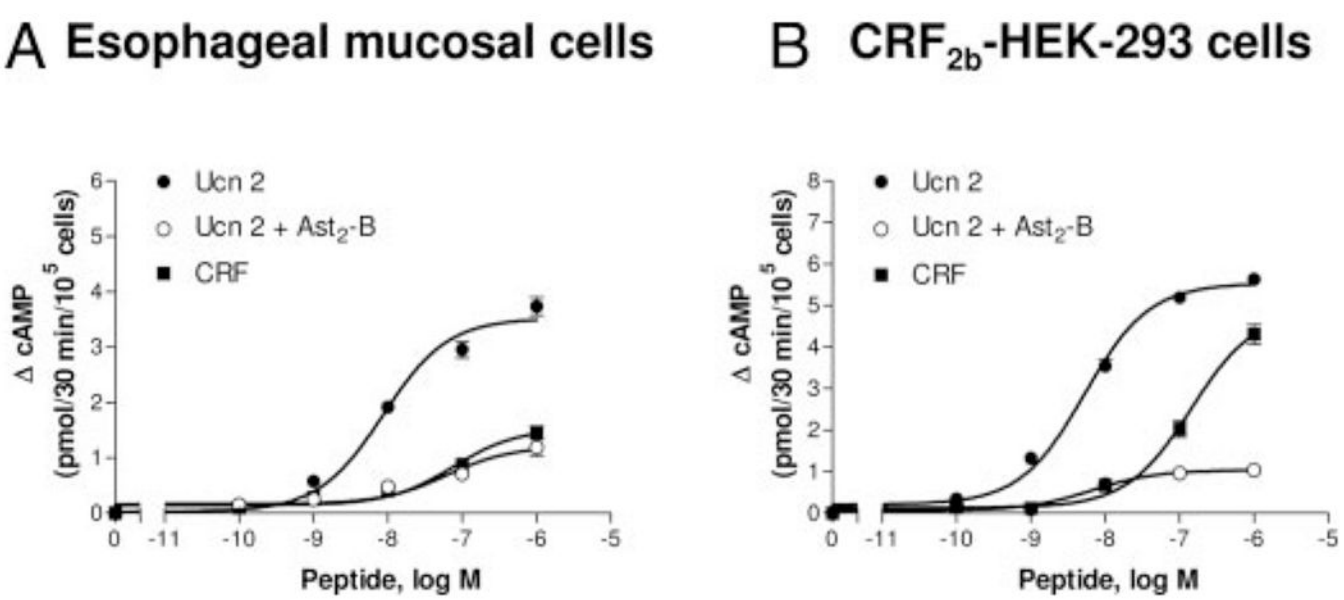

Fig. 7.

Dose-dependent intracellular cAMP production in rat primary esophageal cells and CRF_{2b}-transfected HEK-293 cells stimulated with CRF and Ucn 2. Rat primary esophageal mucosal cells or CRF_{2b}-transfected HEK-293 cells ($1–2\times10^5$ cells/well) grown on 12-well plates were treated with increasing concentrations of CRF or Ucn 2 (10^{-10} to 10^{-6} M) in the presence of 1 mM 3-isobutyl-1-methyl-xanthine for 30 min. CRF_2-selective antagonist, $astressin_2$-B, at a concentration of 1 μM was added concurrently to a separate set of Ucn-2 treated cells. Results are expressed as mean ± SE of increases over basal unstimulated levels (1.0 ± 0.2 pmol and 2.4 ± 0.3 pmol per 15 min per 10^5 cells for the esophageal cells and HEK-293 cells, respectively).

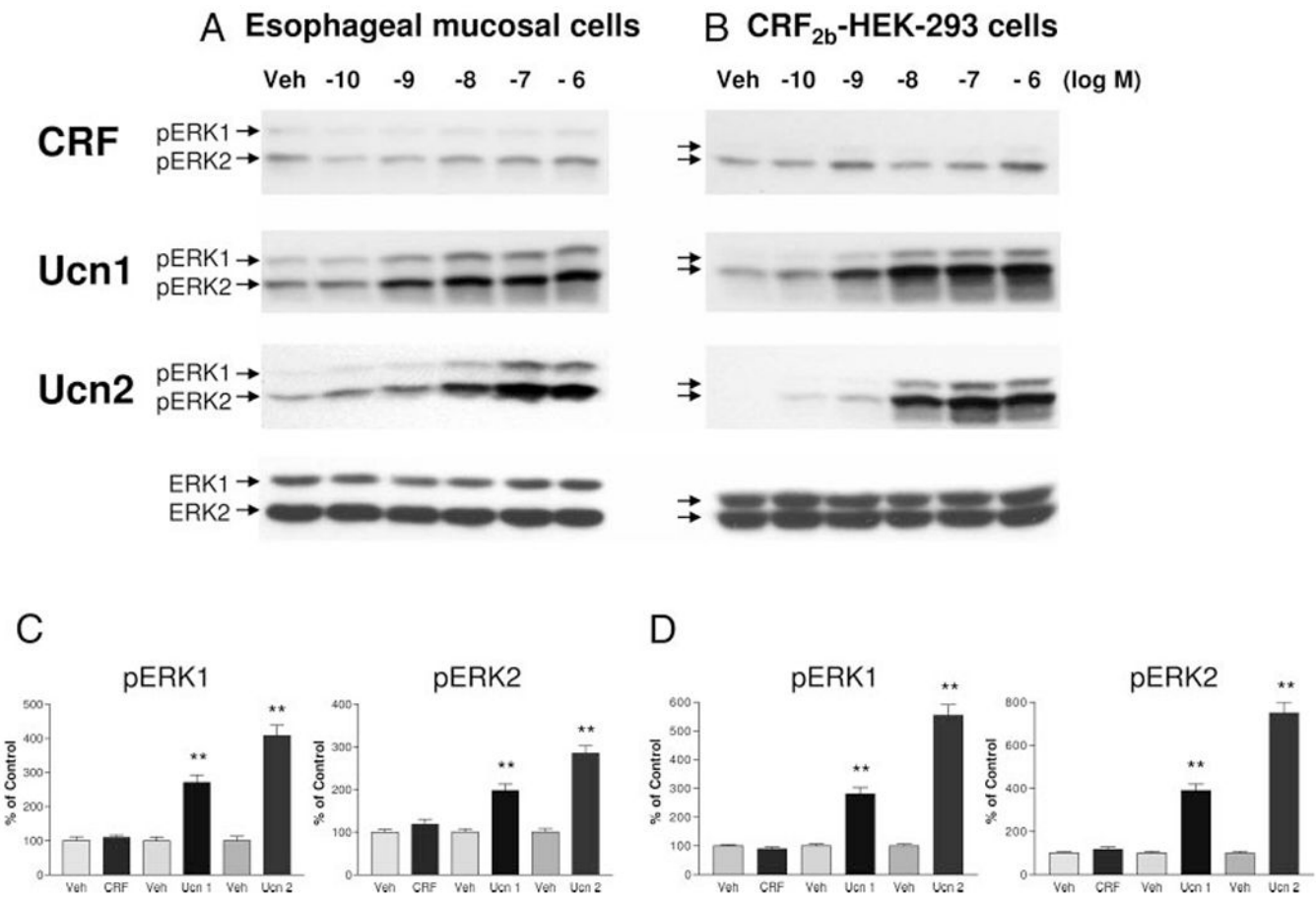

Fig. 8.

Activation of ERK1/2 in rat primary esophageal cells and CRF_{2b}-transfected HEK-293 cells stimulated with CRF receptor agonists CRF, Ucn 1, and Ucn 2. Rat primary esophageal mucosal cells (1×10^6 cells/dish) or CRF_{2b}-transfected HEK-293 cells were treated with increasing concentrations of CRF, Ucn 1, or Ucn 2 (10^{-10} to 10^{-6} M) for 5 min. Cells were then harvested and extracted cellular proteins were analyzed by Western blotting using anti-phospho-ERK1/2 or anti-ERK1/2 antibodies to detect phosphorylated or total ERK, respectively. Representative blot images are shown to demonstrate dose dependency of ERK1/2 phosphorylation in the rat esophageal mucosal cells (A), compared with CRF_{2b}-transfected human HEK-293 cells (B). Ucn 2-stimulated cell extracts were used for total ERK measurement. *Bar graphs* represent the percent of maximal change of pERK1/2 over the vehicle-control value stimulated by 100 nM of each peptide agonist in the esophageal cells (C) and CRF_{2b}-transfected HEK-293 cells (D) for 5 min. Signals were measured using Kodak 1D image analysis software, and data represent mean ± SE of at least three experiments (**, $P < 0.001$ *vs.* vehicle control).

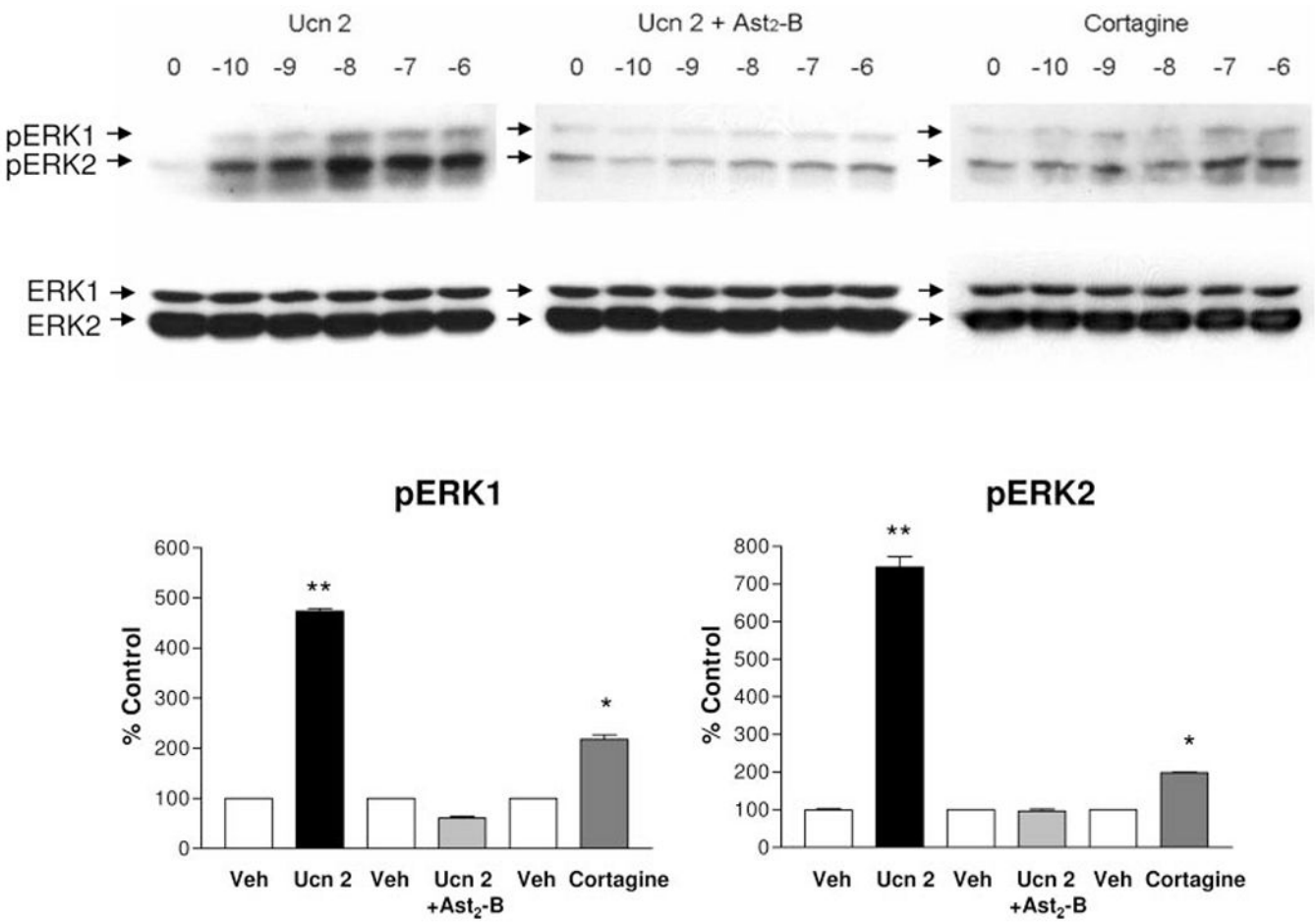

Fig. 9.

CRF_2-dependent ERK1/2 activation in rat primary esophageal cells. Primary cultures of rat esophageal mucosal cells (1×10^6 cells/well) were treated with Ucn 2 in the absence and presence of 1μM astressin$_2$-B or with CRF_1-selective agonist cortagine at the indicated concentrations (10^{-10} to 10^{-6} M) for 5 min. Cells were then harvested and extracted cellular proteins were analyzed by immunoblotting using anti-phospho-ERK1/2 and anti-ERK/1/2 antibodies to detect activated pERK1/2 and total ERK, respectively. Representative images were shown to demonstrate CRF_2-dependent ERK1/2 phosphorylation (A–C). *Bar graphs* represent the percent of maximal change of pERK1/2 stimulated by agoni6sts (100 nM) with or without astressin$_2$-B over the vehicle-control value. Signals were measured using Kodak 1D image analysis software and data represent mean ± SE of two experiments (*, $P < 0.05$; **, $P < 0.001$ *vs.* vehicle control). Veh, Vehicle.

TABLE 1.

Sequence of oligonucleotide primers used to detect the rat CRF system by RT-PCR and real-time quantitative PCR

Genes	Forward (5′–3′)	Reverse (5′–3′)	Size (bp)
CRF_1			
N-term	CTCTGGGATGTCGGAGCGATCCA	CCCAGGTAGTTGATGATGAC	433
Full-length 1a	AGGATGGGACGGCGCCCGCAGCTCC	CTCACACTGCTGTGGACTGCT	1252
TaqMan primer	CACCTCCCTTCAGGATCAGC	TTGCACTGCAGGCCAGAA	69
Probe	CTGTGAGAACCTGTCCCTGACCAGCAA		
CRF_{2a}			
N-term 2a	GCGGCCCCTCATCTCCGTGAG	CTGGTCCAAGGTCGTGTTGCA	353, 483[a]
2a-1	CGGACCCCGAAGGTCCCTA	CAGGTCATACTTCCTCTGCT	242
2a-2	CGGACCCCGAAGGTCCCTA	CAGGTCATACCTCCTCTTGT	239[b]
2a-3	CGGACCCCGAAGGTAGGCA	CAGTGGCCCAGGTAGTTGATG	413
2a-4	GCAATGGACGCGGCGCTGCTCCTCAG	GCCTCAAGATGAAGGGTGGGGA	780[c]
2a-5	GCAATGGACGCGGCGCTGCTCCTCAG	GGCCAGCCTCTCCCACTTACT	1266
2a-6	GCAATGGACGCGGCGCTGCTCCTCAG	CAGGCAGCGGATACTCCTTG	334
Full-length 2a	GCAATGGACGCGGCGCTGCTCCTCA	ATCACACGGCAGCTGTCTGCT	1240
TagMan primer	GGCCCCTCATCTCCGTG	TCTCATCGGATCACTCCTAGGA	66
Probe	CCCCGAGGCTTCTCTTGGCC		
CRF_{2b}			
N-Term 2b	CCCATGGGGACCCCAGGCTCT	CTGGTCCAAGGTCGTGTTGCA	201
2b-1	CCCATGGGGACCCCAGGCTCT	CAGGTCATACTTCCTCTGCT	411
2b-2	CCCATGGGGACCCCAGGCTCT	CAGGTCATACTTCCTCTTGT	408
Full-length 2b	CCCATGGGGACCCCAGGCTCTCT	ATCACACGGCAGCTGTCTGCT	1300
TaqMan primer	TCCTTCCGAAGCAGCCG	CCATGGGACACCAGATCCTG	70
Probe	TGGTCCCTATCCCTGAGCAAGCGAG		
CRF/Ucn			
CRF	CAGAACAACAGTGCGGGCTCACCT	ATTTCATTTCCCGATAATCTCCATC	622
Ucn 1	CTGGAGCTCCATCTTGCACTGGA	CTTGCCCACCGAATCGAATAT	415, 420,[d] 681[e]
Ucn 2	GTCTCAGCCTACCCCTGAGGA	AGGTCACCCCATCTTTATGAC	411

Genes	Forward (5′–3′)	Reverse (5′–3′)	Size (bp)
Ucn 3	CCTCGACCTGAGCACTTCCACC	CCTGCCTGGTCTTTGCTTTATTTC	562, 567[d]
ARP	GTTGAACATCTCCCCCTTCTC	ATGTCCTCATCGGATTCCTCC	402

[a] Variant CRF2a-3 (including intron 3).

[b] Variant CRF2a-2 (desQ).

[c] Variant CRF2a-4 (including intron 8).

[d] 5′ nnnag extension in the second exon.

[e] Intron containing.