

Corticotropin-releasing factor family peptide signaling in feline bladder urothelial cells

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Abstract

Corticotropin-releasing factor (CRF) plays a central role in the orchestration of behavioral and neuroendocrine responses to stress. The family of CRF-related peptides (CRF and paralogues: urocortin (Ucn)-I, -II, and -III) and associated receptors (CRFR1 and CRFR2) are also expressed in peripheral tissues such as the skin and gastrointestinal tract. Local signaling may exert multiple effects of stress-induced exacerbation of many complex syndromes, including psoriasis and visceral hypersensitivity. Interstitial cystitis/painful bladder syndrome (IC/PBS), a chronic visceral pain syndrome characterized by urinary frequency, urgency, and pelvic pain, is reported to be exacerbated by stress. Functional changes in the epithelial lining of the bladder, a vital blood–urine barrier called the urothelium, may play a role in IC/PBS.

This study investigated the expression and functional activity of CRF-related peptides in the urothelium of normal cats and cats with feline interstitial cystitis (FIC), a chronic idiopathic cystitis exhibiting similarities to humans diagnosed with IC/PBS. Western blots analysis showed urothelial (UT) expression of CRFR1 and CRFR2. Enzyme immunoassay revealed release of endogenous ligands (CRF and Ucn) by UT cells in culture. Evidence of functional activation of CRFR1 and CRFR2 by receptor-selective agonists (CRF and UCN3 respectively) was shown by i) the measurement of ATP release using the luciferin-luciferase assay and ii) the use of membrane-impermeant fluorescent dyes (FM dyes) for fluorescence microscopy to assess membrane exocytotic responses in real time. Our findings show evidence of CRF-related peptide signaling in the urothelium. Differences in functional responses between FIC and normal UT indicate that this system is altered in IC/PBS.

Key Words

- ▶ urothelium
- ▶ CRF-signaling
- ▶ stress
- ▶ interstitial cystitis

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Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid hypothalamic neuropeptide (Vale *et al.* 1981), plays a central role in the orchestration of behavioral and neuroendocrine responses to stress (Stengel & Tache 2010). This complex process of stress adaptation is

fine-tuned by several related peptides, called urocortins (UCNs: Ucn I, II, and III), which exhibit various degrees of amino acid sequence homology to CRF. CRF and UCNs exert their actions in target cells via activation of the G protein-coupled receptors, CRFR1 and CRFR2, which are

encoded by two separate genes (Grammatopoulos 2012). CRFR1 and CRFR2 have distinct pharmacological properties and agonist selectivity; CRF exhibits high affinity for CRFR1 (with a tenfold lower affinity for CRFR2) (Jappelli *et al.* 2014) and Ucn III preferentially binds and activates CRFR2 (Lewis *et al.* 2001).

In addition to their distribution in the CNS, CRF-related peptides and their receptors are widely expressed in peripheral tissues, including the skin, gastrointestinal tract (GIT), pancreas, and adrenal glands, where they are proposed to play a role in tissue homeostasis (Slominski *et al.* 2001, Li *et al.* 2007, Tsatsanis *et al.* 2007, Larauche *et al.* 2009, Squillacioti *et al.* 2011). It is now well established that CRF-related peptide receptors can activate a plethora of signal transduction pathways, including PKA, PKC, PKB/AKT, ERK, and p38 MAPK (Grammatopoulos 2012), which enables them to mediate diverse and sometimes opposing functions. For example, in human hepatocytes CRFR1 and CRFR2 mediate opposing pro- and anti-proliferative actions respectively (Paschos *et al.* 2010). In the GIT, CRFR1 drives gastric contractility while CRFR2 activation downregulates the activity (Nozu *et al.* 2013).

In both humans and animals, physical and emotional stress appears to play a role in visceral dysfunction, such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), overactive bladder (OAB), and interstitial cystitis/painful bladder syndrome (IC/PBS) (Lutgendorf *et al.* 2001, Klausner & Steers 2004, Robbins & Ness 2008, Mayer 2010, Stengel & Tache 2010, Smith *et al.* 2011, Stella *et al.* 2011). There is now convincing evidence that local CRF-related peptide signaling within the intestinal tissues plays an important role in both the onset and exacerbation of IBS and IBD, involving changes in the permeability of the intestinal epithelial lining, impacting the barrier function (Larauche *et al.* 2009, Chatzaki *et al.* 2013).

Although the etiology and pathogenesis of IC/PBS are incompletely understood, patients have an abnormal, leaky bladder epithelial lining, proposed to be a key event that initiates the cascade of nerve upregulation, muscle reactions, and tissue injury in IC/PBS (Parsons 2011). Very little is known as to whether there is a functioning CRF-related peptide signaling system in the urinary bladder and its potential role in the well-described correlation between stress and symptom exacerbation in bladder diseases, such as IC/PBS. IC/PBS is characterized by urinary frequency, urgency, and pelvic pain (Hanno *et al.* 2010) and patients are reported to display symptom exacerbation and a heightened sensitivity to pain after exposure to stressful situations (Rothrock *et al.* 2001).

The mammalian urinary bladder is composed of the urothelium (a specialized stratified epithelial lining) and an outer layer of smooth muscle, called the detrusor muscle (Fig. 1). It is innervated by sensory afferents and efferent/motor nerves of the somatic and autonomic nervous system (Yoshimura & de Groat 1997). The urothelium is a vital blood–urine barrier, deficiency of which would allow the passage of water, urea, and toxic substances into the underlying bladder tissue, affecting neural and/or muscle layers, resulting in symptoms of urgency and frequency as seen in IC/PBS (Lavelle *et al.* 2000, Birder & de Groat 2007). The urothelium expresses a wide range of receptors and signaling molecules such as nitric oxide, ATP (Birder 2010), and acetylcholine (ACh) (Hanna-Mitchell *et al.* 2007, Yoshida *et al.* 2008), suggesting the potential for chemical dialog with underlying sensory nerve endings (Burnstock 2001, Vlaskovska *et al.* 2001, Birder 2010). Changes in this chemical dialog could precipitate nociceptive signaling from the bladder and also play a role in IC/PBS.

We undertook this study to investigate for bladder urothelial (UT) CRFR1 and CRFR2 receptor expression and functional activity, as well as UT release of endogenous ligands, which might shed some light on potential mechanistic pathways linking stress to symptoms of IC/PBS. We chose to use tissue isolated from the bladders of normal healthy domestic cats and cats diagnosed with feline interstitial cystitis (FIC), a clinically recognized naturally occurring animal model of IC/PBS (Buffington 2011,

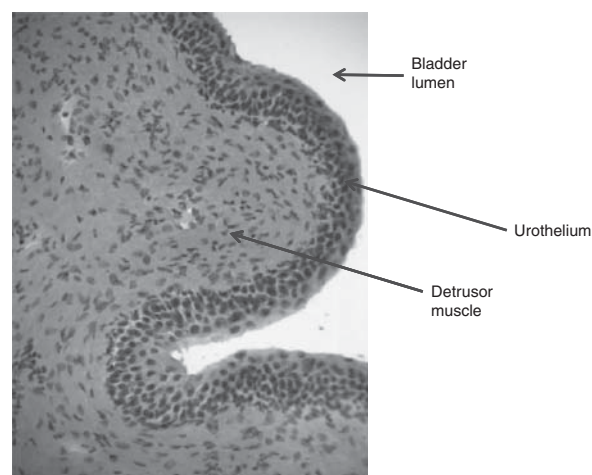


Figure 1 Hematoxylin and eosin (H&E)-stained cross-section of the feline urinary bladder. Arrows indicate i) the urothelium which faces ii) the bladder lumen and the iii) underlying detrusor muscle.

Malykhina & Hanno 2014), in order to gain potential insights into alterations in this system under conditions of pathophysiology. We chose the following methodological approaches to assess for evidence of functional responses in UT cells to CRF-receptor-selective pharmacological ligands: i) measurement of ATP release from UT cell using the luciferin-luciferase assay (Schwiebert & Zsembery 2003) and ii) measurement of UT cell exocytotic/secretory responses in real time (by microscopy), using the membrane-impermeant fluorescent dye FM1-43 (Gaffield & Betz 2006). In addition, molecular biological techniques were used to assess for: i) endogenous release on CRF-related peptides by UT cells (*in vitro*; Elisa) and ii) mucosal expression of CRFR1 and R2 (western blot analysis).

Materials and methods

Animals

All procedures were conducted in accordance with Ohio State University and University of Pittsburgh Institutional Animal Care and Use Committee policies. Adult male and female domestic cats (*Felis catus*) were used for this study. The diagnosis of FIC was based on compatible history and on consideration of standard National Institutes of Health inclusion and exclusion criteria. Healthy, age-matched cats obtained from commercial vendors, which were determined to be free of disease and signs referable to the lower urinary tract according to the same diagnostic criteria as cats with FIC, were used as controls. The animals were housed in stainless steel cages and allowed to acclimatize to their environment for at least 3 months before the study (Buffington *et al.* 1999).

Primary cell culture

Urinary bladders were removed under anesthesia (isoflurane; 4%), after which the animals were killed. Excised bladders were cut open, gently stretched, and pinned on a Sylgard-coated plate, with the mucosal side up. Following overnight incubation in minimum essential medium (MEM; Invitrogen, Life Technologies) containing 2.5 mg/ml dispase, the urothelium was gently scraped from underlying tissue, treated briefly with 0.25% trypsin, and triturated. Dissociated UT cells were plated (30–40 000 cells) onto either six-well collagen-coated dishes (Enzyme immunoassay) or onto collagen-coated glass cover slips (functional experiments). The cells were grown in protein-free CnT-16 culture medium

(CELLnTEC, Zenbio, NC, USA) in an incubator with an atmosphere of 5% CO₂ at 37 °C.

Molecular physiology

Western blotting analysis Bladder mucosa was carefully dissected away from the underlying smooth muscle and homogenized in Hanks' Balanced Salt Solution (HBSS: 5 mM KCl, 0.3 mM KH₂PO₄, 138 mM NaCl, 4 mM NaHCO₃, 0.3 mM Na₂HPO₄, 5.6 mM glucose, and 10 mM HEPES, pH 7.4) containing complete protease inhibitor cocktail (one tablet/10 ml; Roche) and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). The homogenate was centrifuged (13 000 g; 15 min). Whole-cell protein lysates were obtained and proteins were analyzed for relative expression levels using a standard immunoblotting protocol as follows. Proteins were separated on a 10–20% PageR gel (Lonza, Walkersville, MD, USA) and subsequently transferred to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked with 5% milk in Tris-buffered saline Tween-20 (TBS-T) for 1 h. After a brief rinse in TBS-T, the membranes were incubated 48–72 h at 4 °C with goat anti-CRFR1 antibody (1:1000, C-20, Santa Cruz) and rabbit anti-CRFR2 (1:2000, Abcam, Cambridge, MA, USA) with and without pre-incubation with the respective blocking peptide as control. Santa Cruz anti-CRFR1 antibody (C-20) was developed against the C-terminus of CRFR1 of human origin and produces a single immunoreactive band at 66 kDa, which is blocked by the peptide. The validity of this antibody has been tested in HEK-293 and CHO-1 cells transfected with full-length cDNA encoding rat *Crf1* or *Crf2b* and in a rat GH4 cell line that endogenously expresses *Crf1* by western blot and immunohistochemistry (Dr Y Tache, personal communication). C-20 preferentially reacted with CRFR1 and with CRFR2 with a very weak cross-reaction. Abcam CRFR2 antibody was developed against the N-terminus of CRFR2a/b (synthetic peptide). Peptide experiments showed a single immunoreactive band at 48 kDa in addition to a non-specific band at 70 kDa, which is not blocked by the peptide. Following extensive washing, the membranes were incubated in secondary antibody (rabbit anti-goat HRP, Everest, Ramona, CA, USA and goat anti-rabbit poly HRP, Pierce, Rockford, IL, USA respectively) for 1 h in TBS-T and subsequently washed. The membranes were developed with enhanced chemiluminescence plus (ECL Plus; GE Healthcare, Piscataway, NJ, USA) and exposed to film. The membranes were stripped (membrane recycling kit from Alpha Diagnostic International, San Antonio, TX, USA) and re-probed with anti β-actin

(Abcam) in 5% milk TBS-T as a loading control. Receptor expression was quantified by densitometry (Personal Densitometer SI; Molecular Probes, Grand Island, NY, USA) normalized by β -actin.

Enzyme immunoassay Primary cultured UT cells isolated from normal and FIC cat bladders ($n=3$ per group) were assessed for evidence of endogenous release of stress-related peptides CRF and Ucn. The plated cells were allowed to stabilize and grow for 2 days *in vitro* (2DIV) before commencement. The culture medium was carefully removed from the culture wells, replaced with sterile filtered HBSS, and the dishes replaced in the incubator. At 30 min, the medium was removed to investigate the presence of CRF and UCN. Enzyme Immunoassay (EIA) Kits from Phoenix Pharmaceuticals (Burlingame, CA, USA; EK-019-06 and EK-019-15, detect CRF and non-selectively Ucn I, II, and III respectively) were used following the manufacturer's instructions. The linear detection range of both kits was 0.1–4 ng/ml. CRF Immunoassay has been validated against prepro-CRF (125–151), PACAP-38, LH-RH, ACTH, [Arg⁸] Vasopressin, and BNP45. UCN Immunoassay is 100% cross-reactive with UCN I, II, and III and has been validated against cortistatin-14, CRF, MCH, LH-RH, NPY, and somatostatin. Before assay, peptides were extracted from the cell culture supernatant using C-18 SEP Columns (Phoenix Pharmaceuticals) following the manufacturer's instructions.

Functional studies

ATP release Normal and FIC UT cells, grown on collagen-coated cover slips (2–3DIV), were transferred into a perfusion chamber and superfused with an oxygenated physiological saline solution (flow rate = 0.5 ml/min) until a stable baseline level of ATP release was measured (Control Phase). The perfusate was modified by the addition of the chemical agents (Test Phase). Perfusate was collected (100 μ l) in the Control and Test phases at 30–60 s intervals. The agents used were CRF (CRFR1 agonist; 0.01 μ M; Tocris, Minneapolis, MN, USA) and UCN3 (CRFR2 receptor agonist; 0.1 μ M; Sigma–Aldrich). CRFR1 and CRFR2 antagonists used were antalarmin (0.1 μ M) and astressin-2B (Ast2B; 1.0 μ M) respectively (Tocris). ATP levels were quantified using a luciferin-luciferase reagent (ATP Assay Kit; Sigma–Aldrich) and ATP concentrations were extrapolated from a standard-curve graph (ATP assay, Sigma–Aldrich). Data are expressed as mean percentage change in ligand-evoked ATP release with respect to basal, taking basal as 100%.

Fluorescence microscopy (live-cell imaging) The fluorescent membrane-impermeant dye FM1–43 (Molecular Probes, Life Technologies) was used as an 'activity marker' in order to track membrane exocytosis from the cytoplasm to the UT cell plasma membrane in response CRF receptor-selective pharmacological ligands. FM dyes are non-toxic water soluble, styryl pyridinium dyes, which fluoresce in a lipid environment but not an aqueous one. They reversibly stain but do not penetrate the plasma membranes of cells and have been used to study vesicle movement in a number of cell types (Gaffield & Betz 2006). When cells are incubated with FM dye, the dye partitions into the outer leaflet of the plasma membrane and the cells become visible when excited at the appropriate excitation wavelength (465 nm) (Gaffield & Betz 2006). Membrane exocytosis is detected by an overall increase in the measured fluorescence intensity with dye in the bath. This is due to the addition of untagged (naïve) membrane from newly exocytosed vesicles (i.e., the FM dye in the bath now has more membrane to tag and the fluorescence intensity of the cell membrane increases). The dye can be internalized by endocytosis, evidenced by the presence of fluorescent punctae in the cytoplasm.

Coverslips of normal and FIC UT cells at 2–3DIV were placed into a flow chamber specifically designed to fit the stage of an inverted epifluorescence microscope (Olympus IX70) equipped with a 40 \times oil immersion lens and connected to a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland). All solutions were added to the dish via a gravity-fed perfusion system (flow rate: 1.5 ml/min). Following perfusion with HBSS alone for 10 min to allow for equilibration, the perfusate was changed to FM1–43 (5 μ M) in HBSS for 10 min, to allow the dye to partition into the plasma membranes of the cells. Images were taken of the cells at 30 s intervals during the partition-equilibration phase, and then at 10 s intervals upon change of the perfusate to one containing both FM1–43 and the chemical agents. The agonists were CRF (CRFR1 selective; 0.01 μ M) and UCN3 (CRFR2 selective; 0.1 μ M). An average of three coverslips per culture ($n=3$) per group were used for each experimental protocol. Post-analysis data (corrected for background fluorescence) were quantified as mean intensity (arbitrary units; AU) per experimental phase using Simple PCI Imaging software (Hamamatsu, Sewickley, PA, USA). Results are expressed as percentage change in fluorescence following the addition of agonist, relative to basal intensity – a measurement of exocytotic activity.

Statistical analysis

Data are expressed as mean \pm S.E.M. and analyzed using Student's unpaired *t*-test; statistical significance was accepted when $P \leq 0.05$.

Results

UT cells express CRFR1 and CRFR2

Normal and FIC mucosa express positive immunoreactive bands for both CRFR1 (66 kDa) and CRFR2 (48 kDa) (Fig. 2a); this was eliminated in the presence of respective blocking peptide (data not shown). In both normal and FIC tissues, CRFR2 protein expression was significantly higher than CRFR1 (3 \times); $P < 0.005$; paired *t*-test, $n = 8$ (Fig. 2b).

UT expression of endogenous ligands

Normal and FIC UT cells cultured for 2 days exhibited endogenous release of CRF and UCN (non-selective Ucn I,II, II) into the bathing medium of a similar magnitude ($P > 0.05$; $n = 3$) (Fig. 2c).

CRFR1- and CRFR2-evoked ATP release

We examined for functional evidence of the presence of CRFR1 and CRFR2 in cultured UT cells by assessing receptor selective, agonist-evoked release of ATP, a major UT cell-signaling molecule. ATP release in response to CRF (CRFR1 agonist; 0.01 μ M) and UCN3 (CRFR2 agonist; 0.1 μ M) occurred in both normal and FIC UT. In normal UT, CRF induced a larger, though not statistically significant, ATP-release compared with UCN3 (Fig. 3a; $P > 0.05$; $n = 3$). By contrast, in FIC UT, UCN3 induced a larger (though not statistically significant) ATP-release compared with CRF, which in turn inhibited basal/constitutive ATP release (Fig. 3a; $P > 0.05$, $n = 3$). The release of ATP occurred with a consistent time lag of 3–5 min following the addition of agent to bath in all experiments. Selectivity of CRF for CRFR1 was confirmed using the selective CRFR1 antagonist antalarmin (0.1 μ M), which significantly inhibited ($P < 0.05$, $n = 3$) CRF-evoked ATP release in normal UT (data not shown). Astressin-2B/Ast2B (CRFR2 antagonist; 1.0 μ M) alone evoked ATP release in both normal and FIC UT, which did not differ significantly (Fig. 3b; $P > 0.05$; $n = 3$).

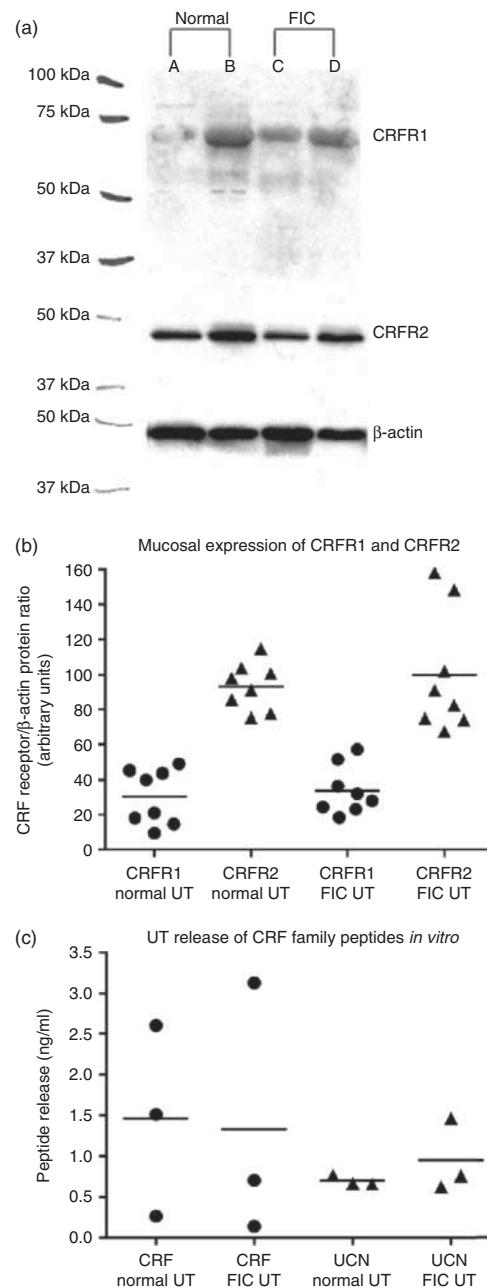
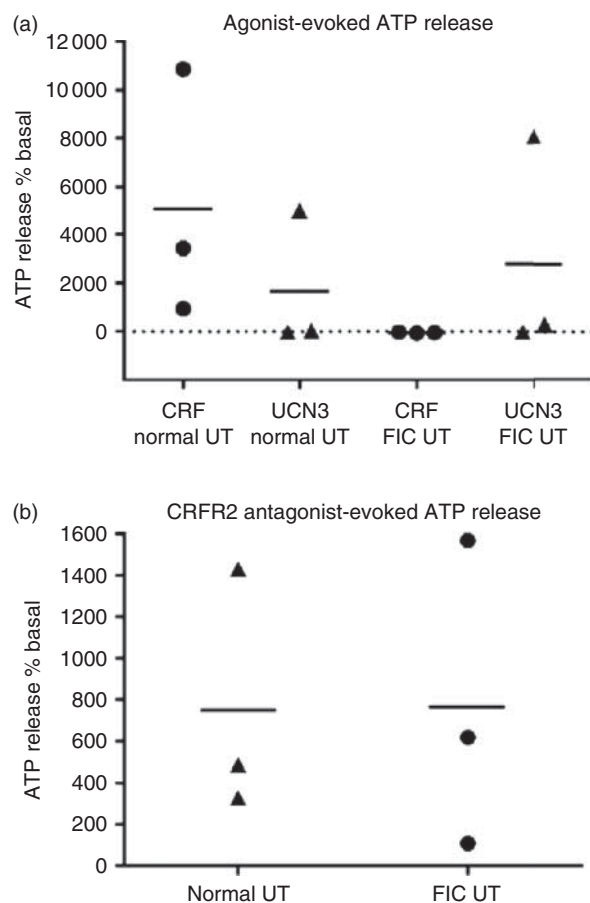


Figure 2

Mucosal expression of CRF receptors and *in vitro* UT release of endogenous ligands. (a) Representative immunoblot of CRFR1 and CRFR2 in normal and FIC cat mucosa lysates. When pre-incubated with competing peptide, the CRFR1 immunoreactive band (66 kDa) was greatly reduced and the CRFR2 immunoreactive band (48 kDa) was completely blocked. (b) In both normal and FIC mucosae, CRFR2 (closed triangles) protein expression is significantly higher than CRFR1 (closed circles; $P < 0.005$; $n = 8$). Mean expression is indicated by horizontal bars. (c) Normal and FIC UT cells exhibited i) endogenous release of CRF into the bathing medium of a similar magnitude (closed circles; $P > 0.5$; $n = 3$) and ii) while endogenous release of UCN (nonselective Ucn I, II, II) appeared greater, no significant difference (closed triangles; $P > 0.05$; $n = 3$) was found between the two groups. Mean release is indicated by horizontal bars.

**Figure 3**

Selective CRFR1 and CRFR2 agonist-evoked ATP release by UT cells *in vitro*. (a) In normal UT, CRFR1 activation (CRF; 0.01 μM; closed circles) evoked a larger ATP release (albeit not significant) compared with CRFR2 (UCN3; 0.1 μM; closed triangles)-evoked release ($P > 0.05$; $n = 3$). By contrast, in FIC UT, CRFR2 activation evoked a larger ATP release relative to CRFR1 activation (albeit not significant; $P > 0.05$; $n = 3$). (b) Astressin-2B/Ast2B (CRF-R2 antagonist) alone evoked ATP release in both normal and FIC UT, of a similar magnitude ($P > 0.05$; $n = 3$). Mean release is indicated by horizontal bars.

CRFR1 and CRFR2 agonists evoke UT membrane traffic (vesicular movement)

In normal UT, both CRF and UCN3 evoked membrane exocytosis of a similar magnitude (Fig. 4a; $P > 0.05$; $n = 3$). By contrast, in FIC UT, CRF-evoked membrane exocytosis was significantly larger compared with responses to UCN3 (Fig. 4a; $P < 0.05$; $n = 3$). All membrane exocytotic responses were typically slow, indicative of GPCR (metabotropic) type response (Saini & Gautam 2010), with a characteristic temporal signature of a 3–5 min time lag between entry of agent to the bath and onset of membrane trafficking, evidenced by an increase in fluorescence intensity (exocytosis) and the appearance of fluorescent

punctae in the cytoplasm (endocytosis). Characteristic image stills (shown from FIC UT) from real-time experiments are depicted in Fig. 4b and c.

Discussion

While peripheral CRF-related peptide signaling is reported to contribute to pathological symptoms in tissues such as the GIT (Teitelbaum *et al.* 2008), esophagus (Cho *et al.* 2011), and skin (Slominski 2007), very little is known about this system in the bladder and whether it plays a role in stress-related exacerbation of lower urinary tract disorders such as IC/PBS (Rothrock *et al.* 2001). Studies using animal models of chemically induced cystitis report the presence and upregulation of CRF and CRFR2, but no evidence of CRFR1 in adult rat bladder (LaBerge *et al.* 2006). In contrast, CRFR1 presence and upregulation are reported in the mouse bladder (Saban *et al.* 2002). These studies focused on gene expression in whole bladder and not specifically within the urothelium. The differential findings may be due to experimental approach and/or species differences.

Although the etiology and pathogenesis of IC/PBS are incompletely understood, deficiency in the epithelial lining of the bladder is proposed to be a causative factor (LaBerge *et al.* 2006, Hanno *et al.* 2011). Due to the strong evidence of CRF-related peptide signaling locally impacting epithelial cell function (e.g. GIT and skin), we focused on investigation for the expression and functional activity of this system in the bladder urothelium and whether there was evidence of change in IC/PBS. As FIC is a naturally occurring animal model of IC and an important translational model in the study of IC/PBS (Buffington *et al.* 1999, Stella *et al.* 2011), we chose to examine this in feline bladder urothelium. Evidence of CRFR1 and CRFR2 presence was investigated directly by assessing protein expression in isolated bladder mucosa (western blot). As the urothelium releases a wide array of signaling molecules, exocytotic membrane movement in response to CRFR1 and CRFR2 selective agents was chosen as an indirect indicator of CRF receptor presence and activity. In addition, as ATP is well established to be an UT signaling molecule, released in response to both mechanical and chemical stimuli with changes in pathology (Birder 2010), ATP release in response to these agents was also measured.

To our knowledge, this is the first study to report a functioning CRF-related peptide signaling system involving both CRFR1/CRFR2 receptors in the epithelial lining of the mammalian urinary bladder. In this study, we report our findings of mucosal expression of both CRFR1 and

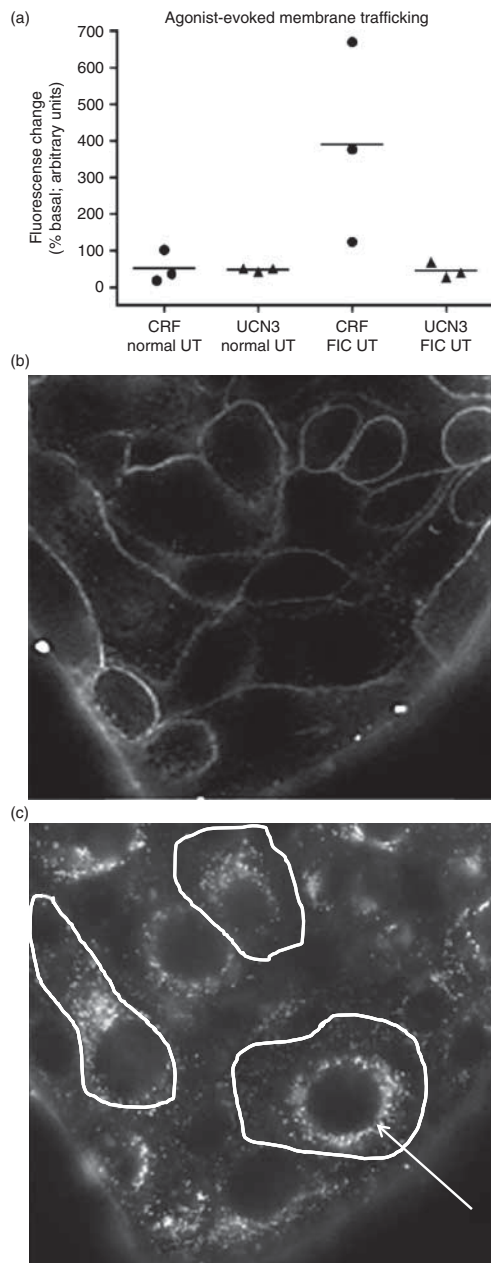


Figure 4

Selective CRFR1 and CRFR2 agonist-evoked UT membrane trafficking *in vitro*. (a) In normal UT, CRFR1 and CRFR2 activation (CRF 0.01 μM ; closed circles and UCN3 0.1 μM ; closed triangles respectively) evoked membrane exocytosis of a similar magnitude ($P > 0.05$; $n = 3$). By contrast in FIC UT, membrane exocytosis in response to CRFR1 activation was significantly greater than that evoked by CRFR2 activation ($P < 0.05$; $n = 3$). Mean release is indicated by horizontal bars. (b and c) Characteristic image stills from time-lapse experiments using FM1-43 (5 μM). (b) The cells are bathed in HBSS + FM-1-43 (5 μM) and unstimulated; (c) the UT cells exhibited a large membrane exocytosis in response to CRFR1 receptor activation (CRF 0.01 μM) as shown by the large number of fluorescent punctae in the cytoplasm. Following washout of dye from the bath, the plasma membrane is no longer visible, white lines delineate its presence. Endocytosed dye-containing vesicles gather around the nuclei in almost all cells, shown by the arrow.

CRFR2 receptors, which are functionally responsive to selective ligands in *in vitro* studies using primary cultures of UT cells. We find that functional activity of these receptors is altered by pathology, in contrast to relative protein expression, which remained unchanged. In addition, we report the release of endogenous ligands CRF and Ucn by UT cells.

Our finding that CRFR2 antagonism (Ast2B, in the absence of exogenous (bath) agonist) evoked ATP release in both normal and FIC UT cells (Fig. 3b), suggests an endogenous, possibly constitutive activation of CRFR2 receptors to allow persistent dampening of constitutive (basal) ATP release by the urothelium. Our finding that UT cells release the endogenous ligands CRF and Ucn is supportive of this theory. A similar counter-regulatory input mediated by CRFR2 on CRFR1 has been reported in the amygdala (Fu & Neugebauer 2008). Overstimulation by increased levels of local or systemic (serum) CRF may account for the observed hyposensitivity of CRFR1-ATP pathway in FIC UT compared with normal UT (Fig. 3a).

The use of FM dyes for real-time imaging of membrane trafficking responses in bladder UT cells, as reported in this study, is novel. Existing information on the roles and dynamics of membrane-bound cytoplasmic vesicles in UT cell physiology is from (real time) capacitance studies and microscopical analysis of fixed tissue and cells (electron and light microscopy) (Wang *et al.* 2005, Khandelwal *et al.* 2009). Our real-time experiments revealed not only that membrane exocytosis was evoked by both CRFR1 and CRFR2-selective ligands indicating functional activity of both CRF receptor types in UT cells but also that ATP release under these circumstances may include a non-vesicular component, as membrane exocytosis did not always correlate with ATP release. For example, CRF caused a much larger membrane exocytotic event in FIC UT compared with normal UT (Fig. 4a), while in contrast; CRF-evoked ATP release was larger in normal UT (Fig. 3a). One possible non-vesicular route of exit for ATP is via the membrane channel pannexin1 (Pannx1). *PANX1* has recently emerged as a candidate ATP release channel (Sandilos & Bayliss 2012) and GPCR-mediated activation of *PANX1* has been reported (Billaud *et al.* 2011). *PANX1* is expressed by bladder urothelium and reported to play a role in bladder dysfunction (Negoro *et al.* 2013, Timoteo *et al.* 2014).

Conclusion

CRF-related peptides and receptors are phylogenetically ancient and well preserved across species; they exert a wide

spectrum of actions in the CNS and the periphery that underpin their critical role in integrating and coordinating the activity of diverse physiological systems (Hillhouse & Grammatopoulos 2006). The wide range of activity of this group of peptides is reflected in the complexity of their physiology and pathophysiology. The pattern of G-protein activation by CRFRs appears to be unique for each tissue and controlled by as yet undefined mechanism (Hillhouse & Grammatopoulos 2006). This study is an initial investigation into the presence of CRF-related peptide signaling in the epithelial lining of the urinary bladder, which in turn is known to play an important role in bladder function and dysfunction. Our findings show that both receptor types and in addition, their endogenous ligands, CRF and Ucn, are expressed by the urothelium and show differences in function in health and disease. Differential functional responses of CRFR1 and CRFR2 to pharmacological ligands both in normal and FIC UT indicate a similar level of complexity reported in other tissues. Further studies will explore signal transduction pathways involved in CRF receptor signaling in order to gain a better understanding of the physiological role for CRF-related peptides within the bladder mucosa and whether they play a role in bladder pathophysiology. IC/PBS affects both women and men (~1.5:1) (Berry *et al.* 2011, Suskind *et al.* 2013); the associated symptoms seriously interfere with daily work and activities with a devastating impact on quality of life. The aim is to uncover novel therapeutic strategies for the treatment of this debilitating disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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