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Functional CRF receptors in BON cells stimulate serotonin release

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ABSTRACT

BON cells are human, pancreatic carcinoid-derived, endocrine-like cells that share functional similarities with intestinal enterochromaffin (EC) cells. We investigated the presence of corticotropin-releasing factor (CRF) receptors, their signalling pathways and the functional effects of their stimulation in BON cells (clone #7). Expression analysis showed that BON cells contain mRNA for the CRF receptor types 1 and 2 ($CRF_{1/2}$), although CRF_2 mRNA levels were 23-fold higher than those of CRF_1 mRNA. The $CRF_{1/2}$ ligand, rat/human (r/h)CRF (EC_{50} = 233 nM), and the selective CRF_2 ligand, human urocortin 3 (Ucn 3) (EC_{50} = 48 nM), induced a dose-dependent increase in cAMP formation. Effects of r/hCRF were blocked by 44% with the selective CRF_1 antagonist DMP-696, while the selective CRF_2 antagonist antisauvagine-30 had only marginal effects. Both ligands (100 nM) stimulated the release of serotonin with similar efficacy (3-fold increase over basal). Effects of r/hCRF, but not Ucn 3, were blocked by pre-incubation with antisauvagine-30. These observations demonstrate that the EC cell-related BON cells express functional CRF_2 receptors linked to the release of serotonin. This suggests that EC cells may be a target for CRF and/or Ucn 3 in the intestine during stress-related responses. Actions of CRF/Ucn 3 and EC cell-derived mediators, such as serotonin, might underlie several motor, secretory and/or sensory disorders of the gastrointestinal (GI) tract which may play a role in the pathophysiology of functional GI disorders, such as irritable bowel syndrome.

1. Introduction

The serotonergic system is a key component of the regulatory mechanisms modulating motility, secretion and sensitivity in the gastrointestinal (GI) tract. In mammals, over 95% of the body's serotonin (5-hydroxytryptamine, 5-HT) is produced and stored in the GI tract, while only about 5% is localized in the brain. In the gut, 5-HT is mostly synthesized and stored in mucosal enterochromaffin (EC) cells, although it can also be localized in other cell types, including neurons of the enteric nervous system and some immune cells [1,2].

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); ASV-30, antisauvagine-30; cAMP, cyclic AMP; CGA, chromogranin A; CRF, corticotropin-releasing factor; CRF_1, CRF receptor type 1; CRF_2, CRF receptor type 2; EC, enterochromaffin; GI, gastrointestinal; IBS, irritable bowel syndrome; TPH, tryptophan hydroxylase; Ucn, urocortin

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From animal and human studies, changes in the density of EC cells and the release and turnover of 5-HT have been suggested as part of the pathophysiological mechanisms underlying motor, secretory and/or sensory disorders of the GI tract [3–5]. Therefore, it is of interest to isolate a pure and viable population of these cells for physiological/pharmacological studies, however, EC cells are only sparsely distributed in the intestinal mucosa difficulting such a process [6–9]. Alternatively, the BON cell line may be an appropriate model to study EC cell regulation in vitro. BON cells are a human carcinoid cell line derived from a metastasis of a pancreatic carcinoid tumor of EC cell origin [10,11]. BON cells retain chemo- and mechanosensitive properties, along with the capability to synthesize, store and release 5-HT, features that are characteristic of non-transformed EC cells [11–13].

Corticotropin-releasing factor (CRF) and CRF receptors (types 1 and 2, CRF_1 and CRF_2) are present in the GI tract [14–20]. In vivo and in vitro studies showed that peripherally administered CRF stimulates colonic motility and secretion and shortens colonic transit time, resulting in increased defecation and in some cases leading to the development of diarrhea [21,22]. These effects are similar to those observed after stimulation of the gut serotonergic system, which also results in increased colonic motor and secretory activities [23–25]. In addition, both CRF and 5-HT, seem to be implicated in the modulation of local inflammatory responses [1,26]. Early studies showed that normal human colonic mucosa EC cells co-store CRF and 5-HT [27]. Moreover, CRF receptors have been localized in the colonic enteric nervous system and in epithelial and immune cells, which is similar to the expression pattern of 5-HT within the gut [16,20,28–32]. Together with these morphological observations, recent reports suggest a functional interaction between the serotonergic and the CRF systems modulating colonic motility [33,34]. However, the exact mechanisms mediating these interactions have not been characterized. Although part of the potential interaction between CRF and 5-HT could be explained by independent actions of these two systems acting in parallel, and thus having simultaneous direct effects on enteric excitatory neuronal pathways, an in-series effect cannot be ruled out. Altogether, these observations suggest a potential interaction between the serotonergic and the CRF system within the gut as part of the neuro-immune-endocrine mechanisms regulating GI functions.

The general objective of the present study was to further explore the basis for a potential interaction between serotonergic- and CRF-dependent mechanisms in the GI tract using BON cells as a model of EC cells. The subclone #7 of BON cells was used. First, we assessed the validity of this clone as a model of non-transformed EC cells by evaluating its capacity to synthesize and store 5-HT. Thereafter, we characterized the presence of CRF receptors in the same cells by determining CRF receptor mRNA expression levels and the presence of the protein. Functionality of CRF receptors was determined by assessing activation of signal transduction mechanisms (production of cAMP) and the capability to elicit the release of 5-HT. In addition, to better characterize these mechanisms, the effect of the CRF_1 selective antagonist, DMP-696 [35,36], and the CRF_2 selective antagonist, antisauvagine-30 [37], was also determined.

2. Materials and methods

2.1. Chemicals

Forskolin, rat/human (r/h)CRF, human urocortin 3 (Ucn 3) and antisauvagine-30 [(D-Phe^{11},His^{12})-Sauvagine, ASV-30] (all from Sigma–Aldrich, St. Louis, MO, USA) were dissolved in DMSO. DMP-696 (AstraZeneca R&D) was dissolved in ethanol.

2.2. Cell culture

Monolayers of BON cells (subclone #7; provided by C.M. Townsend, Jr., University of Texas, Galveston, Texas, USA) were maintained at 37 °C in DMEM with glutamax-I:Ham's F12K (Kaighns modification) (1:1) media, supplemented with 10% FCS and 1% PEST (penicillin and streptomycin) in a humidified atmosphere of 95% air and 5% CO_2. Cells were passed at 90–100% confluence. Passage numbers were 8–15. For functional studies, the culture medium was removed, the cells were washed once with PBS w/o Ca^{2+} and the cell monolayer was overlaid with trypsin/EDTA. After 3–5 min incubation at 37 °C, the cell suspension was centrifuged (5 min × 900 rpm) and the pellet was resuspended with fresh culture medium. Cells were seeded at a cell density of 4–6 × 10^4 cells per well (cpw) in 96-well culture plates or at a cell density of approximately 10^6 cpw in 6-well culture plates, depending upon the experiments considered. All experiments were performed 24–48 h after cell seeding, when an ~80% confluence was achieved.

2.3. Immunocytochemistry

BON cells were resuspended in PBS (10^6 cells/ml), placed on glass slides, air dried and fixed with formalin for 10 min at room temperature. After three washes in PBS the cells were incubated with normal donkey serum (1:10; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 30 min, followed by the incubation with a goat anti-5-HT antibody (1:100; Europa Bioproducts Ltd., Ely, UK) or a rabbit anti-chromogranin A (CGA) antibody (1:500; Euro-Diagnostica, Malmö, Sweden) in a humidity chamber at 4–8 °C, overnight. The secondary antibody, FITC-labeled donkey anti-goat or donkey anti-rabbit, as appropriate (1:50; Jackson Immuno Research Laboratories Inc.) was added for 60 min at room temperature. As negative control, in some slides, PBS was used to replace the primary antibody. Between steps and at the end of the procedure, the slides were washed with PBS (pH 7.4, three times, 5 min each). At the end of the procedure the slides were air-dried and coversliped with antifading fluorescent mounting medium and visualized in a fluorescence microscope (Carl Zeiss Inc., Germany).

In some cases, for nuclear staining, the Vectashield® Mounting Mediun containing 4′,6-diamidino-2-phenylindole (DAPI) was used (Vector Laboratories, Burlingame, California, USA).

2.4. Real time quantitative PCR (qRT-PCR, Taqman)

Human CRF receptors 1 and 2 and tryptophan hydroxylase 1 and 2 (TPH-1 and TPH-2) transcripts were amplified from BON cell cDNA by gene-specific oligonucleotide primers. Specific

Table 1 – Sequence of PCR primers and predicted size of PCR products

Gene	Forward	Reverse	Product size (bp)
CRF_1	TCCGCATCCTCATGACCAA	GACGAAGAACAGCATGTAGGTGAT	125
CRF_2	CACCTACATGCTCTTCTTCGTCAAT	CAGACACGAAGAAACCCTGGAA	107
TPH-1	TGTGGAGTTTGGTCTATGTAAACAAGAT	GCCAGCACCAAAGACTCTTAGC	55
TPH-2	ACAAGGGAAGCAGCAAACGT	CTGCTGTCTTGCCACTTTCG	51
36B4	CCATTCTATCATCAACGGGTACAA	AGCAAGTGGGAAGGTGTAATCC	73

primers were selected with the Primer Express software (v2.0.0; Applied Biosystems, Foster City, CA, USA) (Table 1). Aliquots of pooled RNA samples (1 μg) were used as templates for synthesis of cDNA. The cDNA obtained (10 ng) was added to 25 μl of reaction mixtures containing 12.5 μl of 2× SYBR Green PCR master mix (Applied Biosystems) and 12.5 μl of a 400 nM solution of gene-specific primers. Assays were performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems). For relative quantification of expression levels all data were normalized to 36B4 mRNA levels.

For relative comparisons, the expression of CRF_1 and CRF_2 receptors was also determined, following the same experimental protocol as described above, in stable cultures of T84 and Caco2 cells.

2.5. Western blot

BON cells were harvested with lysis buffer (0.1 M Tris–HCl, pH 8.0 and 1% SDS) containing the Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland). The samples were heated at 95 °C for 5 min and cleared by centrifugation. The supernatant was collected and the protein concentration determined using the Protein DC assay (Bio-rad, Hercules, CA, USA). For each sample 10 μg of protein was loaded onto a 10% polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). After separation, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with a selective rabbit HRP-conjugated polyclonal antibody against the CRF_2 receptor (1:500 dilution; AgriSera, Umeå, Sweden). Finally, the membranes were rinsed with tris-buffered saline tween-20 (TBST) and bound HRP-conjugates were visualized using the Supersignal West Pico chemiluminiscent substrate kit (Pierce, Rockford, IL, USA). CHO cells stably transfected with CRF_1 and CRF_2 receptors were processed in parallel and served as positive controls for antibody specificity.

2.6. cAMP assay

Cells, seeded in 96-well culture plates (4–6 × 10^4 cpw), were pre-incubated in serum-free 0.1% BSA-containing medium for 1 h and then incubated for 15 min (37 °C) in serum-free medium supplemented with 100 μM isobutylmethylanthine (IBMX) in the absence or presence of r/hCRF (1–1000 nM), Ucn 3 (1–1000 nM) or forskolin (1 μM). In some cases, cells were pre-incubated for 15 min with the selective CRF_1 antagonist, DMP-696 (1 μM) [35,36], or the selective CRF_2 antagonist, ASV-30 (1 μM) [37], before the addition of r/hCRF (1–1000 nM or a single 100 nM concentration) or Ucn 3 (1–1000 nM or a single 100 nM concentration). Following the appropriate incubation periods, the incubation medium was aspirated and lysis reagent (200 μl/well) was added. The plate was shaked on a microtitre plate shaker for at least 10 min to facilitate cell lysis. Resulting cell lysates were transferred to microtubes and stored at −80 °C until cAMP assay.

Cyclic AMP levels were determined with a competitive enzyme immunoassay kit (Biotrak™; Amersham Pharmacia Biotech, Braunschweig, Germany). The assay was carried out according to the manufactor's instructions. Cyclic AMP production values (fmol/well/15 min) were calculated by direct read-off from the standard curve.

2.7. Measurement of 5-HT release from BON cells

To assess 5-HT release, cells were seeded in 6-well culture plates (10^6 cpw) and 24 h later the culture medium was changed to fresh medium containing vehicle or drug (2 ml/well). The effects of r/hCRF (100 nM), Ucn 3 (100 nM) and forskolin (3 μM), used as a positive control, were determined. In some experiments, cells were pre-incubated (15 min) with ASV-30 (1 μM) before the incubation with either r/hCRF (100 nM) or Ucn 3 (100 nM) for 60 min. In all cases, after the incubation period, the medium was aspirated and stored at −80 °C until the 5-HT assay.

Quantification of 5-HT released into the culture medium was performed as described by Vatassery et al. [38]. Briefly, 1 ml aliquots of culture medium or standard, consisting of 5-HT dissolved in culture medium, were freeze dried and the resulting pellet was dissolved in 220 μl of ascorbic acid (5 mg/ml). Concentrated HCl (60 μl) was then added to each sample and the fluorescence activity determined with activation set at 295 nm and emission at 540 nm.

2.8. Statistical analysis

All data are expressed as mean ± S.E.M. Comparisons within multiple groups were performed using one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls multiple comparison test, whenever appropriate. EC_{50} values were estimated by non-linear regression to a sigmoidal equation (Prism, GraphPad, San Diego, CA, USA). P-values <0.05 were considered statistically significant.

3. Results

3.1. Immunocytochemistry

As expected, BON cells (subclone #7) maintained in stable culture stained positive for 5-HT. Comparison of nuclear and 5-HT stainings showed virtually a complete coincidence (>98%; Fig. 1C). In addition, almost all cells were positive for

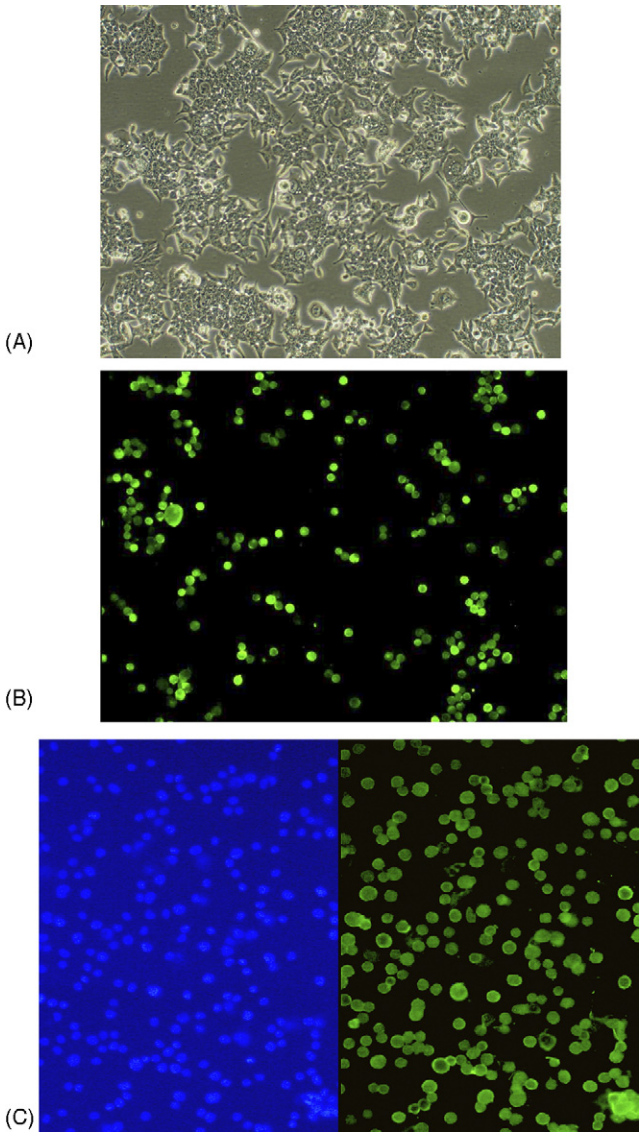

Fig. 1 – (A) Microphotograph showing BON cells, subclone #7, in a culture with 80% confluence (×20). (B) Microphotograph of a culture of BON cells showing chromogranin A-like immunoreactivity, indicating the endocrine nature of the cells (×20). (C) Microphotograph showing the same population of BON cells visualized by nuclear staining (DAPI, left panel) and by fluoresence staining for 5-HT (right panel) (×20). Note the almost complete coincidence between the two images, indicating the cellular localization of 5-HT.

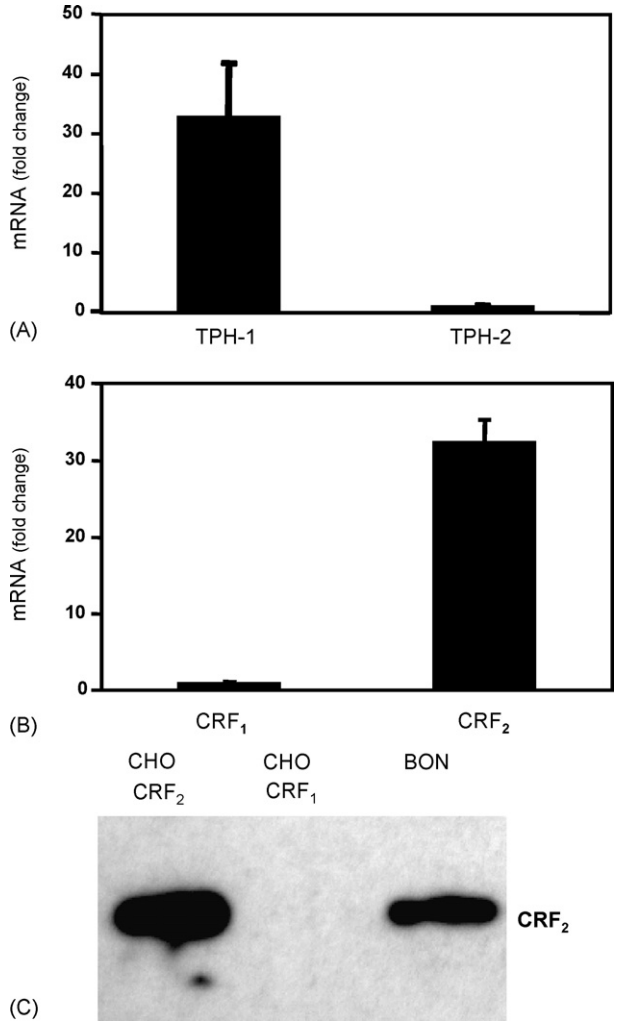

Fig. 2 – (A) Relative expression of TPH-1 and TPH-2 in the clone #7 of BON cells maintained in culture. Data represent relative levels of mRNA (fold change) normalized to those of 36B4. (B) Relative expression of CRF_1 and CRF_2 receptors in the clone #7 of BON cells maintained in culture. Data represent relative levels of mRNA (fold change) normalized to those of 36B4. See Table 1 for primers used to detect THP-1, TPH-2, CRF_1 and CRF_2 expression. (C) Western blotting of CRF_2 receptors in BON cells (clone #7) and in CHO cells, stably transfected with either CRF_1 or CRF_2 receptors, using a selective CRF_2 antibody. Specific bands corresponding to the size of the CRF_2 receptor were observed in extracts from BON cells and CRF_2-transfected CHO cells, but not in CRF_1-transfected CHO cells.

CGA, considered as a valid marker for endocrine cells of the GI tract [39], indicating their endocrine characteristics (Fig. 1B). Substitution of the primary antibodies by PBS resulted in the complete lost of immunoreactivity, demonstrating the specificity of the antibodies (data not shown).

3.2. *Expression of CRF receptors in BON cells*

BON cells maintained in stable culture expressed high levels of TPH-1, but not TPH-2 (Fig. 2A). Moreover, they also contained mRNA for CRF_1 and CRF_2 receptors (Fig. 2B and C), although the expression levels were 23-fold higher for CRF_2 than for CRF_1 (Fig. 2B). For both CRF_1 or CRF_2 receptors, mRNA levels was undetectable in T84 or Caco2 cells, when maintained in culture in similar conditions (data not shown). Western blotting confirmed the presence of CRF_2 receptors in BON cell extracts, revealed as a single band of a molecular weight between 50 and 64 kDa. A band of identical size was also detected in protein preparations obtained from CHO cells stably transfected with the CRF_2 receptor. Protein extracts

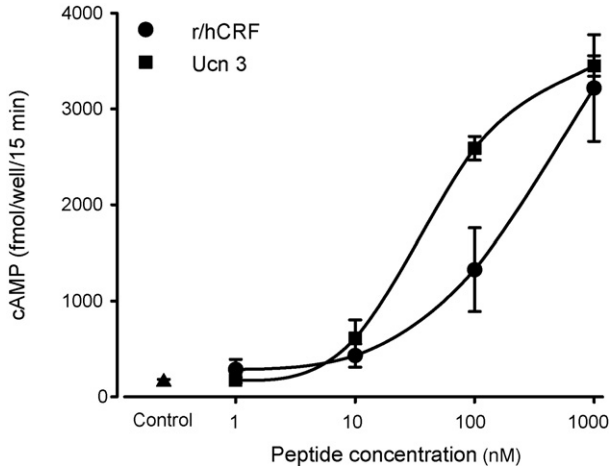

Fig. 3 – Effects of r/hCRF and Ucn 3 (1–1000 nM) on cAMP production in BON cells (subclone #7). Control values correspond to cAMP levels in BON cells incubated only with medium. Data represent the mean ± S.E.M. of 5 (r/hCRF) and 3 (Ucn 3) experiments.

from CHO cells stably transfected with the CRF_1 receptor failed to bind the CRF_2 antibody (Fig. 2C). CRF_1 protein was not assessed because of the low levels of expression.

3.3. *r/hCRF- and Ucn 3-stimulated cAMP formation in BON cells*

Basal cAMP production in BON cells, was 127 ± 28 fmol/well/15 min ($n = 5$). Incubation with forskolin (1 μM) for 15 min increased cAMP production 2241 ± 490% over basal levels. Incubation with r/hCRF (1–1000 nM) induced a dose-dependent cAMP production, with an estimated EC_{50} of 233 nM (95% confidence interval: 128–423 nM, $r^2 = 0.94$, $n = 5$; Fig. 3). Similar, but more pronounced effects were observed when the cells were incubated with Ucn 3 ($EC_{50} = 48$ nM, 95% confidence interval: 37–61 nM, $r^2 = 0.99$, $n = 3$; Fig. 3).

When BON cells were pre-incubated with the selective CRF_1 antagonist, DMP-696 (1000 nM), before the addition of r/hCRF (100 nM), cAMP production was inhibited by 44 ± 9% ($n = 3$), compared to the response evoked, in parallel experiments, by r/hCRF alone (Fig. 4A). DMP-696 (1000 nM) also attenuated the dose-related cAMP production induced by r/hCRF at 1, 10, 100 and 1000 nM (Fig. 4C). Under the same conditions, the selective CRF_2 antagonist, ASV-30 (1000 nM), reduced the r/hCRF (100 nM)-evoked response by only 19 ± 17% ($n = 3$). In one experiment, the response to r/hCRF was not affected by the antagonist (14% increase in cAMP production), while in 2 additional experiments the antagonist reduced cAMP formation by 36 and 34% (Fig. 4B). In parallel experiments, the effects of ASV-30 (1000 nM) on r/hCRF- (1–1000 nM) evoked cAMP production were also minimal (Fig. 4C). None of the antagonists affected, per se, cAMP production in control conditions.

In similar experiments, Ucn 3 (100 nM)-induced cAMP production was not affected by pre-incubation with ASV-30 (1000 nM) (cAMP levels: Ucn 3, 1835 ± 411 fmol/well/15 min; Ucn 3 + ASV-30, 2019 ± 497 fmol/well/15 min; $n = 6$ for each; Fig. 5A). Likewise, in parallel experiments, ASV-30 (1000 nM) did not affect the dose-related cAMP production evoked by Ucn 3 at 1–1000 nM ($n = 3$; Fig. 5B). The activity of DMP-696 on Ucn 3-evoked cAMP production was not tested.

3.4. *r/hCRF- and Ucn 3-stimulated 5-HT release from BON cells*

BON cells spontaneously released 5-HT to the culture medium (3.6 ± 0.1 μg/ml/60 min). Incubation with forskolin (3 μM) for 60 min increased the release of 5-HT 3-fold (10.3 ± 1.6 μg/ml/60 min, $n = 3$; $P < 0.05$ versus basal 5-HT release; Fig. 6). Incubation with r/hCRF (100 nM) or Ucn 3 (100 nM) increased the release of 5-HT with similar efficacy. The concentration of 5-HT in the medium was of similar magnitude as that observed after stimulation with forskolin (r/hCRF: 10.1 ± 0.9 μg/ml/60 min, $n = 3$; Ucn 3: 10.0 ± 1.1 μg/ml/60 min, $n = 3$; both $P < 0.05$ versus basal 5-HT release; Fig. 6).

r/hCRF (100 nM)-induced 5-HT release was completely blocked by pre-incubation of the cells with ASV-30 (1000 nM). However, pre-incubation with ASV-30 did not modify Ucn 3 (100 nM)-induced 5-HT release (Fig. 6).

4. Discussion

Several reports have established BON cells as a valid model to study the neuroendocrine regulation of 5-HT secretion from EC cells [12,13,40]. The present study further expands these findings and suggests that CRF and/or CRF-related peptides are involved in the modulation of EC cell activity, establishing a potential functional link between the serotonergic and the CRF systems. Results obtained in the present study show that the subclone #7 of BON cells, maintained in stable culture, contained high levels of the transcript for the isoform 1 of the enzyme TPH (TPH-1), while the levels of expression of the TPH-2 isoform, which is mainly expressed in neuronal cells [41,42], were comparatively very low. In addition, BON cells contained 5-HT-like immunoreactivity and spontaneously released 5-HT to the media. These results agree with previous reports highlighting subclone #7 of BON cells as having the highest content of releasable 5-HT [12]. Altogether, these observations show that BON cells of subclone #7 have characteristics of endocrine cells, as shown by the presence of CGA immunoreactivity, and can synthesize, store and release 5-HT; further supporting their similarities to non-transformed EC cells and their validity as a model to study EC cell functionality.

Transcripts encoding CRF_1 and CRF_2 receptors were found in BON cells. Expression levels for the CRF_2 transcript were about 23-fold higher than for CRF_1, thus suggesting that this subclone (#7) of BON cells preferentially expresses CRF_2 receptors. From the present results, we cannot determine which of the splice variant(s) ($CRF_{2\alpha}$, $CRF_{2\beta}$ or $CRF_{2\gamma}$) is present since the PCR primers used did not differentiate among them. The presence of CRF_2 receptors at the protein level was confirmed by Western blotting using a selective CRF_2 antibody. Specificity of the antibody was demonstrated by the fact that a band corresponding to an idential molecular weight to that detected in BON cells was also detected in CHO cells stably transfected with the CRF_2 receptor, while was completely

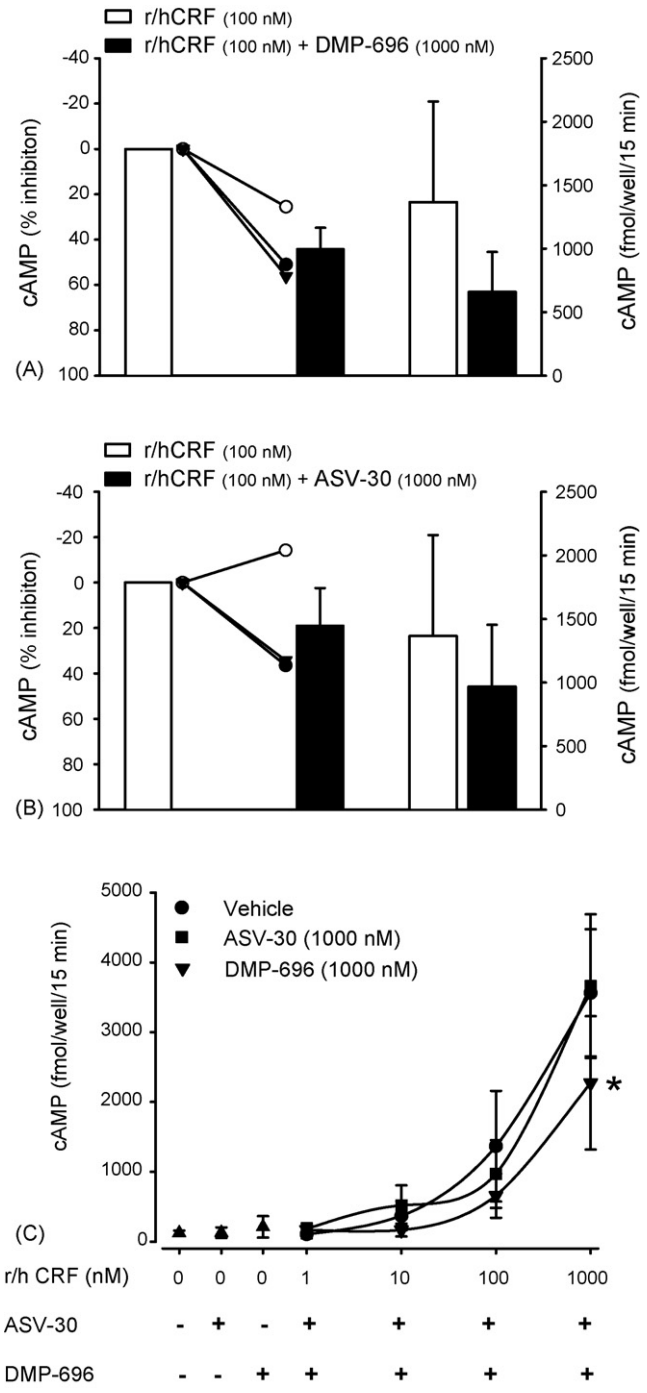

Fig. 4 – Effects of the CRF receptor antagonists antisauvagine-30 and DMP-696 on r/hCRF-induced production of cAMP in BON cells (clone #7). (A) Effects of the selective CRF_1 antagonist, DMP-696, (1000 nM) on r/hCRF (100 nM)-induced cAMP production. The percent inhibition of cAMP levels, compared to those in the r/hCRF-treated group, taken as 0%, is shown for the 3 individual experiments (left panels). The bars to the right show the absolute change in cAMP levels for the same experiments. (B) Effects of the selective CRF_2 antagonist, ASV-30 (1000 nM), on r/hCRF (100 nM)-indcued cAMP production. The percent inhibition of cAMP levels, compared to those in the r/hCRF group, taken as 0%, is shown for the 3 individual experiments (left panels). The bars to the right show the absolute change in cAMP levels for the same experiments. (C) Effects of DMP-696 and ASV-30 (1000 nM) on r/hCRF (1–1000 nM)-induced dose-related changes in cAMP production. For the 3 panels, data are mean ± S.E.M. of 3 independent experiments. $^{*}P < 0.05$ vs. r/h CRF alone.

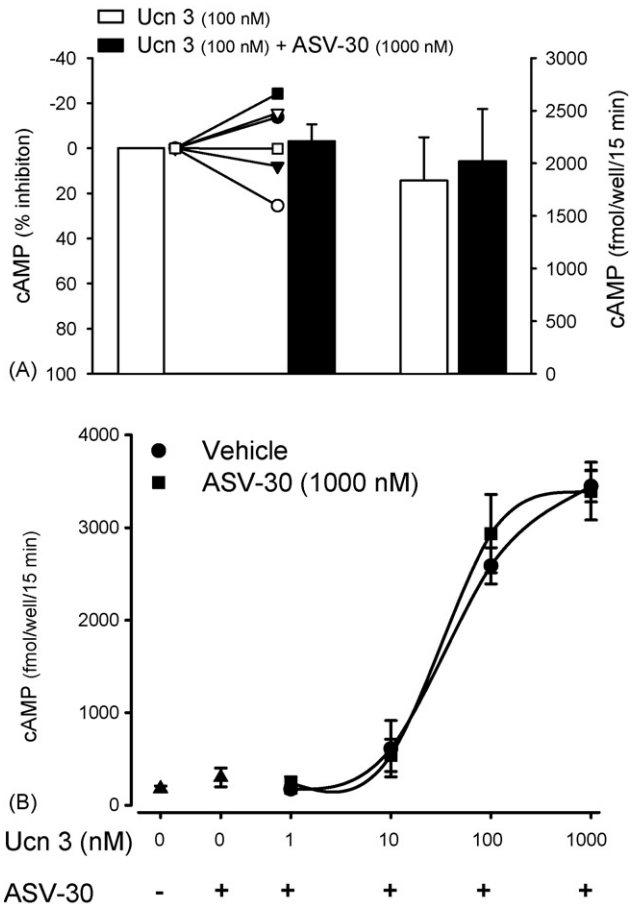

Fig. 5 – Effects of the selective CRF_2 antagonists, antisauvagine-30, on Ucn 3-induced production of cAMP in BON cells (clone #7). (A) Effects of ASV-30 (1000 nM), on Ucn 3 (100 nM)-indcued cAMP production. The percent inhibition of cAMP levels, compared to those in the Ucn 3-treated group, taken as 0%, is shown for 6 individual experiments (left panels). The bars to the right show the absolute change in cAMP levels for the same experiments (mean ± S.E.M.). (B) Effects of ASV-30 (1000 nM) on Ucn 3 (1–1000 nM)-induced dose-related changes in cAMP production. Data are mean ± S.E.M. of 3 independent experiments.

absent in protein extracts of CHO cells stably transfected with the CRF_1 receptor. Specificity of the antibody was also established in immunohistochemical studies and confirmed by preabsorption with a specific peptide (data not shown). These data agree with recent preliminary observations showing expression of CRF_1 and CRF_2 receptors in the subclone 1 of BON cells [43]. This suggests that the expression of CRF receptors is probably not subclone-specific but rather a

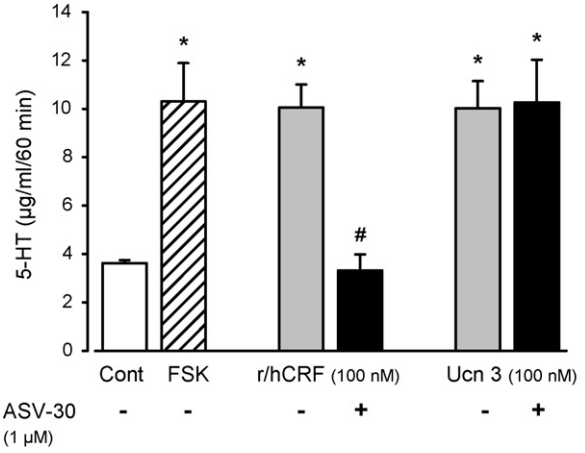

Fig. 6 – Effects of r/hCRF and Ucn 3 (0.1 μM), with or without addition of the selective CRF_2 antagonist, antisauvagine 30 (ASV-30), on 5-HT release from BON cells (subclone #7). Forskolin (FSK, 3 μM) served as positive control. Data represent the mean ± S.E.M. of 3 independent experiments. $^{*}P < 0.05$ vs. 5-HT release in control (Cont) conditions; $^{\#}P < 0.05$ vs. r/hCRF-evoked 5-HT release in the absence of ASV-30 (ANOVA).

general feature of BON cells, although this, together with differences in the relative expression levels of CRF_1 versus CRF_2 receptors, needs to be further assessed in different subclones of BON cells. Immunohistochemical studies have shown the presence of CRF_1 and CRF_2 receptors in epithelial cells of the colonic mucosa [16]. However, the exact nature of these cells has not been fully characterized and the presence of $CRF_{1/2}$ immunoreactivity in EC cells has not been confirmed. Transcripts of CRF receptors were undetectable in Caco-2 or T84 cells, two epithelial cells lines frequently used as a model of the colonic/intestinal epithelial barrier [44,45]. Therefore, in normal conditions, expression of CRF receptors in the colonic/intestinal mucosa might not be a feature of colonocytes/enterocytes but rather a characteristic of other epithelial cell types, such as endocrine cells.

Incubation of BON cells with r/hCRF resulted in a dose-dependent increase in cAMP formation, suggesting that the receptors expressed are functional. Similar results were obtained with the CRF-related peptide, Ucn 3, although it was more potent than r/hCRF at stimulating cAMP formation (EC_{50}; CRF: 233 nM; Ucn 3: 48 nM). Maximal CRF- and Ucn 3-induced cAMP production was relatively high, reaching the levels elicited by 3 μM forskolin, suggesting a tight functional coupling of these receptors in the BON cells. Rat/human CRF is a non-selective $CRF_{1/2}$ ligand, with higher affinity (10- to 40-fold) for CRF_1 than CRF_2, while Ucn 3 is considered a selective CRF_2 ligand [46–48]. The relative higher potency of Ucn 3 at stimulating cAMP formation when compared with r/hCRF agrees with the predominant presence of CRF_2-type receptors in the subclone #7 of BON cells. Effects of r/hCRF on cAMP formation were blocked by 44% by pre-incubation with the selective CRF_1 antagonist, DMP-696. In the same experimental conditions, pre-incubation with the selective CRF_2 antagonist, ASV-30, had minor effects on r/hCRF-evoked cAMP responses. These differences agree with the higher affinity of r/hCRF for CRF_1 versus CRF_2 receptors [46] and suggest that, despite their relatively low level of expression, CRF_1 receptors significantly contribute to the effects of CRF on cAMP formation. The lower efficacy of ASV-30 at inhibiting CRF effects is probably not due to an insufficient concentration of the antagonist, since at the same antagonist:agonist ratio (10:1) ASV-30 was effective at preventing CRF-induced release of 5-HT (see below). Similar antagonist:agonist ratios (1:1 to 10:1) were also effective at preventing the CRF_2-mediated effects of Urocortin 2 in isolated rabbit cardiomyocytes [49]. Surprisingly, pre-incubation with ASV-30 did not affect Ucn 3-induced cAMP formation. Lack of effects of ASV-30 are likely not due to a different blocking capacity of the antagonist between the identified splice variants of the CRF_2 receptor. Although ASV-30 was initially designed as a $CRF_{2\beta}$ antagonist [37], experimental data, so far, do not show significant pharmacological differences between mammalian $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors [50]. Therefore, it should be expected that the antagonist is active on both splice variants. From our observations, the possibility that Ucn 3 acts through alternative, ASV-30 non-sensitive, CRF_2 splice variant(s) or through a novel CRF receptor subtype, yet to be characterized, cannot be excluded. Supporting this view, recent preliminary observations suggest that BON-1 cells might express several variants of the CRF_1 and CRF_2 receptors, which might include functional receptors with unique affinities for ligands and antagonists [43].

Functionality of the CRF receptors present in the subclone #7 of BON cells is further supported by the observation that r/hCRF and Ucn 3 stimulated the release of 5-HT. Several studies have previously shown that different BON cell clones release 5-HT [12,13,40]. Pre-incubation with ASV-30 at an antagonist:agonist ratio of 10:1, completely prevented the secretory effects of r/hCRF, while not affecting Ucn 3-induced 5-HT release. Thus suggesting a predominant role of CRF_2 receptors mediating r/hCRF-dependent release of 5-HT from BON cells. The effects of Ucn 3 on 5-HT secretion were not blocked by ASV-30, providing further evidence that ASV-30 insensitive CRF receptors mediate the effects of Ucn 3.

In summary, the present study shows that the subclone #7 of BON cells expresses functional CRF receptors, linked to the formation of cAMP and the release of 5-HT. In addition to the expression data, showing a predominant presence of CRF_2 over CRF_1, the functional data suggest the presence of alternative binding sites (either novel CRF receptor(s) or novel CRF_2 splice variants) that can be activated by Ucn 3 but are ASV-30 insensitive.

Although BON cells have been suggested as a model to study the physiology and pharmacology of EC cells [12,13,40], the physiological/pathophysiological translation of the present observations to non-transformed EC cells needs to be further studied. Enterochromaffin cells contain and secrete CRF [27] and Ucn 1 and Ucn 3 are present in colonic tissues [51,52]. This, together with the possible existence of CRF receptors in EC cells, suggest that, by paracrine and/or autocrine mechanisms, CRF and/or CRF-related peptides might modulate EC cell activity. It can be speculated that the local release of these peptides might mediate functional responses from EC cells similar to those observed in vitro in BON cells, including the release of 5-HT, and probably other

cellular mediators. This might initiate a series of local neuro-immune responses important for the physiology and pathophysiology of the gut. These mechanisms may be particularly significant for the pathophysiology of the functional GI disorder, irritable bowel syndrome (IBS). A significant proportion of IBS patients have EC cell hyperplasia [4], an altered 5-HT metabolism [3] and altered gut immune responses [53–55]. Moreover, altered responses to stress and a hyperreactive CRF system have also been implicated in the pathophysiology of the disease [21,22,56,57]. In these conditions, the interaction between the CRF system and EC cells might trigger a self-maintaining loop that generates neural and immune responses that contribute to the development and/or maintenance of the colonic sensory, motor and secretory alterations that characterize IBS.

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