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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