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Expression of galanin receptor messenger RNAs in different regions of the rat gastrointestinal tract

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Abstract

Galanin effects are mediated by three G-protein-coupled receptors: galanin receptor 1 (GalR1), GalR2 and GalR3. We quantified mRNA levels of GalR1, GalR2 and GalR3 in the rat stomach, small and large intestine using real-time RT-PCR. All three GalR mRNAs were detected throughout the gut at different levels. GalR1 and GalR2 mRNA levels were higher in the large than in the small intestine. GalR2 mRNA was most abundant in the stomach. GalR3 mRNA levels were generally quite low. The differential regional distribution of GalRs suggests that the complex effects of galanin in the gut are the result of activating multiple receptor subtypes, whose density, subtype and signaling vary along the gastrointestinal tract.

Keywords

Real-time RT-PCR; Intestine; Stomach; Enteric nervous system

1. Introduction

Galanin is a neuropeptide of 29 amino acids in length (30 amino acids in humans) with a wide distribution in the central nervous system and periphery, where it is involved in numerous biological activities. In the gastrointestinal tract, galanin immunoreactivity is expressed by enteric neurons that are localized in the submucous and myenteric plexus, and in fibers projecting to the gut wall [5,9,28]. Galanin influences gastric emptying, inhibits acid secretion, regulates the release of numerous peptides [2], and affects mucosal epithelial cell absorption, ion transport [15,17] and gastrointestinal motility. The effects of galanin on gastrointestinal motility are both stimulatory and inhibitory, and are the result of a direct myogenic and a nerve-mediated effect involving the release of other transmitters. In general,

galanin actions vary depending on the tissue, the species and the experimental conditions [8,12,16,25].

Galanin activates three distinct receptor subtypes that have been cloned and characterized pharmacologically: galanin receptor 1 (GalR1), GalR2, and GalR3 [3,4]. Galanin receptor activation involves different signaling pathways. GalR1 and GalR3 activate intracellular effectors through pertussis-toxin sensitive Gi/o proteins [27] resulting in inhibition of adenylyl cyclase activity and activation of inward K⁺ current [23]. By contrast, GalR2 predominantly couples positively to phospholipase C through a Gq/11 protein to mediate inositol phospholipid turnover and Ca²⁺ mobilization with a pertussis-toxin independent mechanism, even though it also signals through Gi/Go proteins [22,27].

The expression of galanin receptors can be examined using various approaches that measure either protein or messenger RNA (mRNA). We have previously shown that GalR1 immunoreactivity is localized to enteric neurons and fibers innervating the gastrointestinal tract as well as to enterochromaffin cells of the stomach [18]. Unfortunately, specific and selective antibodies for the other galanin receptors are not currently available. Therefore, we chose to utilize quantitative real-time RT-PCR, which is currently the most accurate and sensitive method to detect and to quantify low abundance mRNA transcripts in various tissues [7], to investigate the distribution of the three GalR mRNAs in the gastrointestinal tract. The diverse actions of galanin in the gut are likely to be the result of the activation of multiple receptors with different signaling pathways. To better understand the function of individual galanin receptors, it is critical to determine their expression within specific tissues. The aim of the present study was to quantify and compare the distribution of rat GalR1, GalR2 and GalR3 mRNAs in the different regions of the gastrointestinal tract.

2. Materials and methods

2.1. Tissue samples

Adult Sprague-Dawley rats (200–250 g) of either sex (Hartley; Harlan Labs, San Diego, CA, USA) were used. Care and handling of the animals were in accordance with all National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Research Committees at UCLA, Los Angeles, CA, USA. All efforts were made to minimize the number of animals used and their suffering. Rats were anesthetized with halothane and decapitated before tissue removal. Tissue samples were frozen and stored in liquid nitrogen.

2.2. RNA extraction from tissues

Total RNA was extracted from stomach, duodenum (upper), jejunum, ileum (distal), proximal and distal colon of six rats using the Absolutely RNA[®] RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). The integrity of RNA was verified by presence of two distinct bands that correspond to 18S and 28S rRNA in 2% agarose gel upon electrophoresis. Spectrophotometric analysis of the sample consistently showed absorption ratio (OD) OD_{260 nm}/OD_{280 nm} > 1.9, indicating excellent purity of the ribonucleic acids.

2.3. Primers design

Specific primers for the three GalR genes were designed using Primer3 software (The Whitehead Institute, Boston, MA). The homology between different subtypes and the template secondary structure were carefully examined and avoided. Primer specificity was confirmed by a BLAST software-assisted search of a non-redundant nucleotide sequence database (National Library of Medicine, Bethesda, MD) and was tested in a RT-PCR reaction of 35 cycles at 94 °C for 30 s, 56 °C (57 °C for GalR3) for 30 s and 72 °C for 30 s.

In the consecutive agarose gel electrophoresis each sample produced a single band of the correct size corresponding to each amplicon. The sequences of the three GalR primers are as follows (see Table 1): GalR1, sense: 5'CCCCATCATGTCATCCACCT3', antisense: 5'A-TGGGGTTCACCGAGGAGTT3'; GalR2, sense: 5'CATCG-TGGCGGTGCTTTT3', antisense: 5'AGCGGGAAGCGA-CCAAAC3'; GalR3, sense: 5'AGCCAAGCAGTACCAC-AG3', antisense: 5'AGCACAGGATGAAGCAA3'. The sequence for the housekeeping gene, β -actin, is as follows: sense: 5'AGGGAAATCGTGCGTGACAT3', antisense: 5'TC-CAGGGAGGAAGAGGATGC3'.

2.4. Real-time quantitative RT-PCR

The levels of mRNA for the three GalRs in the different regions of the gastrointestinal tract were analyzed by real-time quantitative RT-PCR on a Mx3000P Real-Time PCR Detection System (Stratagene). We have established a reproducible qRT-PCR assay for measurement of the GalRs mRNA in various tissues. The efficiency of the qPCR reaction was examined over five orders of magnitude in fivefold serial dilutions of cDNA plasmids of the three GalRs. Melting curve analysis and agarose gel electrophoresis confirmed the specificity of the reactions. Total RNA (1 μ g) was reverse transcribed using oligo(dT) primers and SuperScript II reverse transcriptase (RT, Invitrogen Corp., Carlsbad, CA). Real-time PCR was performed using Platinum[®] SYBR[®] Green qPCR Super-Mix UDG (Invitrogen Corp.). Each cycle consisted of three steps: denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C (58 °C for GalR3), and 1 min of elongation at 72 °C. Data were collected in real-time during the elongation step of each cycle of the 45-cycle reactions. Each cDNA sample was analyzed in triplicates and a sample without cDNA template was included as a negative control in each experiment. Expression of β -actin was measured for every sample in parallel with GalRs. The data acquired from each sample assayed for GalRs were normalized to those of the β -actin. In addition to real-time and melting curve analyses of the reactions, amplified products were separated electrophoretically on 3% agarose gels containing ethidium bromide to confirm proper size of the amplicons. Selected amplified DNA samples were purified and sequenced at the UCLA DNA Sequencing Core Facility as an additional control. Finally, the results were analyzed using the comparative Ct method to derive relative quantities of each of the GalR mRNAs across different segments of the gastrointestinal tract (User Bulletin #2, ABI Prism 7700 Sequence Detection System, December 11, 1997). Values were expressed as the mean $\pm$ S.E.M. One-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was used for statistical analysis. *P* values < 0.05 were taken as significant.

3. Results

GalR1 mRNA is expressed in all regions of the gastrointestinal tract, with the highest levels detected in the proximal and distal colon and the lowest levels in the stomach (Fig. 1). There were no statistically significant differences among the levels of GalR1 mRNA in different regions of the gut. Fig. 2 shows that GalR2 mRNA was also expressed in all regions of the gastrointestinal tract with the highest levels in the stomach (1.46 ± 0.58) and the lowest in the duodenum and jejunum (0.10 ± 0.05 and 0.15 ± 0.06 , respectively; *P* < 0.05 versus stomach). There were no statistically significant differences between the levels of GalR2 mRNA in the stomach and those in the ileum, proximal or distal colon. Unlike GalR1 and GalR2, the expression of GalR3 mRNA was consistently low across all tissues examined. The expression in the jejunum, and proximal and distal colon was found to be slightly higher than in the stomach, duodenum and ileum (Fig. 3) without statistically significant differences among the different regions. The specificity of GalR1, GalR2 and GalR3 primer pairs for real-time RT-PCR was confirmed with conventional RT-PCR followed by agarose gel electrophoretic analysis (Fig. 4). A single PCR band of proper size, corresponding to

GalR1 or GalR2 was observed in each tissue with each primer set. A single band corresponding to GalR3 was also clearly detected in jejunum, proximal and distal colon, whereas a faint, indiscriminate band was observed in the stomach, duodenum and ileum.

4. Discussion

The aim of the present study was to determine the distribution of GalR mRNAs in different regions of the gastrointestinal tract, and to measure their relative levels using quantitative real-time RT-PCR. We found that mRNAs coding for all three GalRs were present in each segment of the gastrointestinal tract with different levels of expression. Both GalR1 and GalR2 mRNAs were more abundant in the large intestine than in the small intestine. GalR2 mRNA was the most abundant in the stomach. The levels of GalR3 mRNA were the lowest throughout the entire length of the gut. The highest levels of GalR3 mRNA were in the colon, and the lowest in the stomach and ileum. The availability of a highly sensitive technique like real-time RT-PCR, which allows the detection and quantification of very low levels of mRNAs, provides an important tool for measuring changes in individual GalR that might occur in pathological conditions.

The presence and distribution of GalR1 mRNA in the gastrointestinal tract are in agreement with the distribution of GalR1 immunoreactivity. We have previously shown that GalR1 immunoreactivity is localized to myenteric and sub-mucous neurons of the stomach, small intestine, and to enterochromaffin-like cells in the rat gastric mucosa [18]. GalR1 immunoreactivity is also found in enteric neurons of the rat colon (unpublished data) and guinea-pig distal ileum [24]. At present, the localization of GalR2 and GalR3 proteins is still unknown.

Galanin is widely expressed in the mammalian gastrointestinal system and it is involved in the modulation of secretion [2,13,19,21], epithelial ion transport and motility [14,19]. The direct effects of galanin differ depending on the regions of the gastrointestinal tract and the species, which might result from the activation of different receptor subtypes. For example, in the rat stomach, galanin has an inhibitory followed by an excitatory effect on gastric motility *in vivo*, which is partly explained by the activation of a GalR1 pathway. In contrast, *in vitro*, galanin has only an excitatory, tetrodotoxin-independent effect, indicating a non-neuronal, direct effect on smooth muscle. This effect is likely to be mediated by GalR2 [10], the only GalR that signals through a stimulatory Gq/11 pathway, thus its activation by galanin could result in smooth muscle contraction [6]. Participation of GalR2 in the effect of galanin on gastric motility is supported by our findings of high levels of GalR2 mRNA in the stomach and is consistent with functional studies indicating the GalR2 as the main GalR implicated in the excitatory mechanism for smooth muscle contraction [27]. Indeed, galanin induces contraction of the jejunum and colon by direct effect on longitudinal smooth muscle [1,26]. The presence of high levels of GalR2 mRNA in the jejunum and colon is in line with an involvement of this receptor in galanin contractile action in these regions.

Galanin also exerts indirect effects on gastrointestinal motility through the inhibition of transmitter release. In the guinea-pig ileum, galanin inhibits the excitatory neuroneuronal and neuromuscular transmission and affects peristalsis by reducing its efficiency and decreasing the compliance of the intestinal wall [24]. These inhibitory effects of galanin are induced via the activation of at least two receptors, one of which is the GalR1 that is predominantly Gi/Go-coupled, which is consistent with our finding of GalR1 mRNA in the small intestine (Fig. 1). GalR3, which is also Gi/Go-coupled, has been suggested as the second GalR to mediate neurogenic response inhibition [24], which is in agreement with the presence of small levels of GalR3 mRNA in the small intestine.

This study provides the first evidence for the presence and the differential levels of expression of the three known GalR mRNAs throughout the gastrointestinal tract. mRNA expression is not necessarily proportional to the level of transcribed protein, especially when the levels of mRNA transcripts are very low, and it should be kept in mind that weakly expressed transcripts might not result in functional proteins [11]. The presence of functional GalRs has been confirmed by galanin's ability to inhibit depolarization-evoked and electrically-stimulated Ca^{2+} increases in isolated myenteric neurons (Anselmi et al., unpublished; [20]), even though at present it is not known which of the GalRs mediates this response. Pharmacological studies using exogenous galanin as well as galanin antagonist or galanin desensitization approaches also indicate the existence of functional GalRs in the gut [24]. The differential distribution of mRNA transcripts for GalRs is in accord with previous functional studies suggesting the presence of multiple GalR subtypes in responsive tissues. The distinct pharmacological profiles, tissue distribution, and signal transduction pathways of GalRs support the concept that the different actions of galanin in the gastrointestinal tract reflect the interaction of galanin with multiple GalRs.

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Fig. 1.

Expression of GalR1 mRNA in the rat gastrointestinal tract. The mRNA levels in the stomach, duodenum, jejunum, ileum, proximal and distal colon were analyzed with quantitative real-time RT-PCR. The data acquired from each sample were normalized to those of β -actin in each tissue. Relative quantities (RQ) of mRNA were analyzed using the comparative Ct method. Each cDNA sample was amplified in triplicate and all data are expressed as the mean $\pm$ S.E.M.

Fig. 2.

Expression of GalR2 mRNA in different regions of the rat gastrointestinal tract. The levels of GalR2 mRNA were measured by real-time RT-PCR. The data acquired from each sample were normalized to that of β -actin. Relative quantities (RQ) of mRNA were analyzed using the comparative Ct method. Each cDNA sample was amplified in triplicate and all data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ vs. stomach.

Fig. 3.

Expression of GalR3 mRNA in different regions of the rat gastrointestinal tract. The levels of GalR3 mRNA were measured by real-time RT-PCR. The data acquired from each sample were normalized to that of β -actin. Relative quantities (RQ) of mRNA were analyzed using the comparative Ct method. Each cDNA sample was amplified in triplicate and all data are expressed as the mean $\pm$ S.E.M.

Fig. 4. Real-time RT-PCR specificity confirmed by traditional PCR. RT products from different regions of the gastrointestinal tract were used in PCR reactions with GalR1, GalR2 and GalR3 or β -actin primers.

Table 1

Sequences of primer pairs used for detection of GAL-R1, GalR2 and GalR3 mRNAs

Target gene	Primer sequence	Amplicon length (bp)	Accession no.
GalR1	Sense: 5'CCCCATCATGTCATCCACCT3' antisense: 5'ATGGGGTTCACCGAGGAGTT3'	119	U30290
GalR2	Sense: 5'/CATCGTGGCGGTGCTTTT3' antisense: 5'AGCGGGAAGCGACCAAAC3'	81	NM019172
GalR3	Sense: 5'AGCCAAGCAGTACCACAG3' antisense: 5'AGCACAGGATGAAGCAA3'	69	NM019173