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Galanin Inhibition Of Voltage Dependent Ca^{2+} Influx In Rat Cultured Myenteric Neurons Is Mediated By Galanin Receptor 1

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Abstract

Galanin activates three receptors, the galanin receptor 1 (GalR1), GalR2 and GalR3. In the gastrointestinal tract, GalR1 mediates the galanin inhibition of cholinergic transmission to the longitudinal muscle and reduction of peristalsis efficiency in the small intestine. Galanin has also been shown to inhibit depolarization-evoked Ca^{2+} increases in cultured myenteric neurons. Since GalR1 immunoreactivity is localized to cholinergic myenteric neurons, we hypothesized that this inhibitory action of galanin on myenteric neurons is mediated by GalR1. We investigated the effect of galanin 1–16, which has high affinity for GalR1 and GalR2, in the presence or absence of the selective GalR1 antagonist, RWJ-57408, and of galanin 2–11, which has high affinity for GalR2 and GalR3, on Ca^{2+} influx through voltage-dependent Ca^{2+} channels in cultured myenteric neurons. Myenteric neurons were loaded with fluo-4 and depolarized by high K^{+} concentration to activate voltage-dependent Ca^{2+} channels. Intracellular Ca^{2+} levels were quantified with confocal microscopy. Galanin 1–16 (0.01–1 μM) inhibited the depolarization-evoked Ca^{2+} increase in a dose-dependent manner with an EC_{50} of 0.172 μM . The selective GalR1 antagonist, RWJ-57408 (10 μM) blocked the galanin 1–16 (1 μM) mediated inhibition of voltage-dependent Ca^{2+} channel. By contrast, the GalR2/GalR3 agonist, galanin 2–11 did not affect the K^{+} -evoked Ca^{2+} influx in myenteric neurons. GalR1 immunoreactivity was localized solely to myenteric neurons in culture as previously observed in intact tissue. These findings indicate that the inhibition of depolarization-evoked Ca^{2+} influx in myenteric neurons in culture is mediated by GalR1 and confirm the presence of functional GalR1 in the myenteric plexus. This is consonant with the hypothesis that GalR1 mediates galanin inhibition of transmitter release from myenteric neurons.

Keywords

G protein coupled receptors; enteric nervous system; gastrointestinal motility; transmitter release

INTRODUCTION

Galanin is a neuropeptide widely distributed in the nervous system, which modulates a variety of biological functions (Bedecs et al. 1995; Gundlach 2002; Kinney et al. 2002; Liu and Hokfelt 2002). In the gastrointestinal tract, galanin is localized to nerve cell bodies in

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both myenteric and submucosal plexuses and neuronal processes projecting to the smooth muscle and mucosa (Ekblad et al. 1985b; Furness et al. 1987). The regional and cellular distribution of galanin is consistent with its involvement in the regulation of neurotransmitter and hormone release, acid secretion, motility, and ion transport (Bauer et al. 1989; Ekblad et al. 1985a; Homaidan et al. 1994; Kisfalvi et al. 2000; McCulloch et al. 1987).

Galanin effects are mediated via the activation of three distinct galanin receptors (GalRs), termed GalR1, GalR2 and GalR3, which belong to the G-protein-coupled receptor superfamily (Branchek et al. 2000; Waters and Krause 2000). GalRs differ in their distribution, effectors and downstream signaling (Floren et al. 2000; Lang et al. 2007). GalR1 couples negatively through pertussis toxin (PTX)-sensitive G-protein to inhibit adenylyl cyclase and the formation of cyclic adenosine monophosphate (Branchek et al. 2000). In addition, GalR1 activation has been shown to open a G-protein-regulated inwardly rectifying K^+ channel (Smith et al. 1998), and stimulate mitogen-activated protein kinase activity (Wang et al. 1998b). The predominant pathway for GalR2 involves coupling through a G_q -type G-protein, stimulating phospholipase C activation and resulting in an increase of inositol triphosphate formation, which mediates the release of Ca^{2+} from intracellular stores (Borowsky et al. 1998; Pang et al. 1998; Wang et al. 1998b). GalR3 signaling is poorly defined; in *Xenopus* oocytes, GalR3 has been shown to couple to a $G_{i/o}$ -type G-protein to activate an inward K^+ current (Smith et al. 1998).

All three GalR mRNAs are expressed in the gut (Anselmi et al. 2005b). GalR1 and GalR2 mRNAs are more abundant than GalR3 mRNA. GalR2 mRNA is highly expressed in the stomach, and GalR1 mRNA is present throughout the gastrointestinal tract with higher levels in the intestine than the stomach. GalR1 immunoreactivity is localized to myenteric and submucous neurons in the intestine, the vast majority of which are cholinergic (Pham et al. 2002; Sternini et al. 2004). We have shown that activation of GalR1 mediates galanin inhibition of cholinergic transmission to the longitudinal muscle and reduction of peristalsis efficiency in the small intestine (Anselmi et al. 2005a; Sternini et al. 2004). These findings are consistent with the observation that galanin inhibits acetylcholine release from cholinergic neurons, through a PTX-sensitive pathway (Mulholland et al. 1992; Sarnelli et al. 2004), further supporting the role of GalR1 in mediating the inhibitory effects of galanin in the gastrointestinal tract.

Galanin has been shown to inhibit electrically evoked Ca^{2+} increase and voltage-dependent Ca^{2+} current in cultured myenteric neurons (Ren et al. 2001; Sarnelli et al. 2004). However, the GalR subtype that mediates these effects is not known. The aim of the present study was to test the hypothesis that galanin alters Ca^{2+} influx through voltage-dependent Ca^{2+} channels in myenteric neurons via the activation of GalR1. We tested the effects of 1) galanin 1–16, a high affinity agonist for GalR1 and GalR2, in the presence or absence of the GalR1 antagonist, RWJ-57408, and of 2) galanin 2–11, an agonist with high affinity for GalR2 and GalR3, on depolarization-evoked intracellular Ca^{2+} increases in rat cultured myenteric neurons. We also used immunohistochemistry with selective markers for neuronal and non-neuronal cells to characterize the cell populations in our cell culture preparation and for GalR1 to determine whether GalR1 immunoreactivity is expressed in neurons in primary cell culture as in intact tissue. Our results showed that the GalR1/GalR2 agonist, galanin 1–16, but not the GalR2/GalR3 agonist, galanin 2–11, inhibits the depolarization-evoked Ca^{2+} influx in a concentration-dependent manner and that this effect was antagonized by the GalR1 antagonist, RWJ-57408, indicating that this inhibitory effect is mediated by GalR1. Preliminary results of this study have been published in the Proceedings of the 3rd International Symposium on Galanin and its Receptors (Anselmi et al. 2005c).

MATERIALS AND METHODS

Culture of Myenteric Neurons using the Sandwich Method

Primary cultures of myenteric neurons were prepared from postnatal 8–10 day-old Sprague-Dawley rats (Hartley; Harlan Labs, San Diego, CA) of either sex. Care and handling of the animals were in accordance with all National Institute of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the Animal Research Committee at UCLA. The pups were anesthetized with halothane and were euthanized by decapitation. The small intestine was removed, and divided into 6 cm long pieces and stored in chilled Hank's balance salt solution (HBSS) (Mediatech, Manassas, VA). Each segment of small intestine was stretched over a glass Pasteur pipette whose tip had been fire-polished. The longitudinal muscle layer with attached myenteric plexus was separated from the underlying circular muscle by stroking tangentially away from the mesenteric attachment with the pressure of the fingers. The tissue was then transferred to a flask containing 10 ml collagenase type IA (1 mg/ml) and deoxyribonuclease I (DNaseI) (1 mg/ml) (Sigma-Aldrich, St Louis, MO) in HBSS, and digested in a CO₂ incubator at 37°C on an orbital shaker for 45 min. The tissue was then transferred to a Ca²⁺ and Mg²⁺ free HBSS trypsin solution (0.25%) containing EDTA (2.21 mM) (Cellgro/Mediatech, Manassas, VA) and incubated for another 30 min. Following trypsin digestion, tissue was transferred to a 2 ml vial and spun down for 2 min at 800 rpm. The pellet was washed twice with Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and then triturated 4–6 times through a 25-gauge needle. The cells were counted and plated at a density of 3.0×10^5 cells/ml on 15 mm glass coverslips coated with poly-L-lysine (0.1 mg/ml) (Sigma-Aldrich, St Louis, MO) in DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen, Carlsbad, CA), DNaseI (1 mg/ml) and 10% FBS. Cells were allowed to settle on coverslips at 37°C in a humidified CO₂ incubator for 2–4 hours in serum-based media. Following this incubation, coverslips were flipped cell side facing down into new wells, containing defined media, which consisted of DMEM supplemented with N1 supplement (Sigma-Aldrich, St Louis, MO), insulin-transferrin-Se (1X) (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 U/ml streptomycin. In this configuration, termed sandwich cultures by Brewer and Cotman (Brewer and Cotman 1989), cells survived for long periods of time without medium changes. Proliferation of non-neuronal cells was reduced by low oxygen tension environment, absence of FBS and by adding 10 μ M arabinose C furanoside (Sigma-Aldrich, St Louis, MO), an anti-mitotic inhibitor, in the defined media. Neurons were used for the Ca²⁺ imaging and immunohistochemistry after 3–6 days *in vitro*.

Solutions

Solutions were applied by a single-pass, gravity-fed perfusion system, which delivered medium to the chamber (chamber volume: ~ 0.5 ml) at a rate of 1.0 ml/min. Experiments were performed in a HEPES buffered mammalian superfusate containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid (HEPES), and 10 glucose. The elevated K⁺ solution contained (in mM): 72 NaCl, 72 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose. The pH of all solutions was adjusted to 7.4 with NaOH. The osmolarity was 319 ± 5 mOsm/kgH₂O.

Measurements of [Ca²⁺]_i transients

Myenteric neurons were loaded for 20 min in the dark with 1 ml of 2 μ M fluo-4/AM (Molecular Probes, Eugene, OR). Neurons were then washed with HEPES buffered mammalian superfusate and transferred to a recording chamber.

Images were collected with Zeiss 510 META LSM or LSM 5 Pascal mounted to an upright microscope (Zeiss Axioplan 2 or Axioskop FS2, Thornwood, NY) equipped with an Axoplan 63X (NA 0.95) water-immersion objective. A 488 nm laser line from an argon laser provided excitation of the sample and the emission was collected through a 505 nm LP filter and collected on a photomultiplier tube. Additional magnification, time series, and background subtraction were controlled by Zeiss LSM acquisition software (ver. 3.2). All images were acquired as 12-bit images.

To activate voltage-dependent Ca^{2+} channels, cells were depolarized by increasing extracellular potassium ($[\text{K}^+]_o$) from 2.5 to 72 mM for 1 min. Cadmium chloride (500 μM ; Sigma-Aldrich, St Louis, MO), a blocker of voltage dependent Ca^{2+} channels in neurons (Correia-de-Sa et al. 2000), was used to determine whether the increase in $[\text{Ca}^{2+}]_i$ induced by high concentration of K^+ was due to Ca^{2+} influx through voltage dependent Ca^{2+} channels. For analysis, a region of interest was drawn over the soma, and the change in fluorescence was interpreted as reflecting changes in $[\text{Ca}^{2+}]_i$. The change produced in the presence of the test solution was compared with the averaged baseline measurements, which consist of three measurements prior to the application of the drug or stimulus. Values for control conditions were determined by averaging the response obtained before the drug administration and after the drug was washed out. Typically, 4 or 5 cells could be analyzed on each coverslip, and all experiments were performed on at least three different preparations. To determine the EC_{50} , the concentration required to produce a half-maximal inhibition of the K^+ -evoked Ca^{2+} increase in the presence of galanin 1–16, the data were fit to a theoretical sigmoidal binding equation: $B/B_{\text{max}} = [1 + (c/\text{EC}_{50})^h]^{-1}$, where c is the concentration of galanin 1–16 tested, h is the Hill coefficient, and B/B_{max} is the fractional normalized response or percentage inhibition of galanin 1–16 at the specific concentration tested. Statistical analysis was performed using the Student's t -test (GraphPad Prism 4.0, San Diego, CA). Significance was chosen as $P < 0.05$ and variance is reported as $\pm$ SE.

Drug solutions

Galanin 1–16 was obtained from Bio-Synthesis Inc. (Lewisville, TX). Galanin 1–16 has high affinity for GalR1 and GalR2 (4.8 ± 1.5 nM and 5.66 ± 3.7 nM K_i , respectively), and low affinity for GalR3 (49.6 ± 15.3 nM K_i) (Wang et al. 1997). Galanin 2–11, which has 500-fold selectivity for GalR2 versus GalR1 and which exhibits comparable affinity for GalR2 and GalR3 (Liu et al. 2001; Lu et al. 2005), was obtained from Sigma-Aldrich (St Louis, MO). RWJ-57408, a non-peptide, GAL-R1 antagonist was a generous gift from Johnson Pharmaceutical Institute (Spring House, PA). The binding affinity IC_{50} of RWJ-57408 ranged between 190 and 2700 nmol L⁻¹ in human Bowes melanoma cells (Scott et al. 2000). Galanin 1–16 and galanin 2–11 were dissolved in distilled water with bovine serum albumin (0.1%) as 1 mM stock solution. RWJ-57408 was dissolved in distilled water as 10 mM stock solution. All the dilutions were prepared using the HEPES buffered mammalian superfusate (pH 7.4).

Immunohistochemistry

Cultured myenteric neurons were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 10 min, and then stored in PB with 0.1 % sodium azide at 4°C before processing for immunohistochemistry. The cells were incubated for 1 hour in a 0.1 M PB solution containing: 0.5% Triton X-100 (Sigma-Aldrich, St Louis, MO), and 10% goat serum (Invitrogen, Carlsbad, CA). Primary antisera were diluted in this blocking solution, and preparations were incubated overnight. For these experiments, we used a specific antibody raised against GalR1 [rabbit GalR1_{Y225–238} antiserum (1:1000)] that has been previously characterized (Pham et al. 2002); a chicken antibody raised against microtubule-associated proteins 2 (MAP2) (1:3000; Neuromics, Edina, MN) as a neuronal marker

(Scheuermann et al. 1991); a monoclonal antibody raised against human smooth muscle actin (clone 1A4) (1:100; Dako, Carpinteria, CA) as smooth muscle marker (Ennes et al. 1997); or a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:1000; Neuromics, Edina, MN) as marker for glial cells (Sitmo et al. 2007). After incubation in the primary antibody, coverslips were rinsed with 0.1 M PB solution and incubated for 1 hour at room temperature in affinity purified goat anti-rabbit IgG Alexa 568 (1:1000), goat anti-mouse IgG Alexa 568 (1:1000), or goat anti chicken IgG Alexa 488 (1:1000) (Invitrogen, Carlsbad, CA). The fluorophores were visualized using a Zeiss 510 META laser scanning upright compound confocal microscope (Axioplan 2) equipped with two helium-neon lasers (543nm and 633nm), and an argon laser (488nm). All images were acquired with a PlanApo 63× 1.4 NA objective. Typically, 4–6 optical sections were taken with a z-axis of 0.50 μm . Images were processed and labeled using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

RESULTS

Characterization of the cells in the sandwich-method culture system

The low density used for plating the cells and their morphology under differential interference contrast optics permitted easy identification of the neuronal cell bodies that were used for our experiments (Fig. 1A). The morphological identification of different cell types was confirmed using single and double labeling fluorescent immunohistochemistry with specific markers for neurons (MAP2, Fig. 1B, C and D), glial cells (GFAP, Fig. 1B) and smooth muscle (smooth muscle-actin, Fig. 1C). Myenteric neurons in sandwich culture were characterized by a round soma with multi-polar processes originating from the soma and forming extensive branching and arborizations (Fig. 1A). Generally, the neurite outgrowth occurred within the first 24 hrs, and neurons began to extensively arborize after that time. The actual size and shape of the somas varied ranging from 10–15 microns, which is likely due to different neuronal populations and some flattening that may occur in the sandwich culture preparation. These morphological characteristics are in agreement with previous reports of myenteric neurons in culture (Nishi and Willard 1985; Vanden Berghe et al. 2000). The combination of serum-free, chemically defined growth media and the sandwich culture technique adapted from Brewer and Cotman (1989) optimized neuronal survival and neurite outgrowth by allowing greater than 80% survival of myenteric neurons for at least 10 days *in vitro*. This culture method allowed to obtain highly enriched cultures of myenteric neurons with reduced numbers of non-neuronal cells, such as smooth muscle cells, glial cells and fibroblasts.

GalR 1 immunoreactivity in myenteric neurons

To demonstrate that the cultured myenteric neurons possess GalR1, we performed indirect immunofluorescence labeling with specific antibodies for GalR1 and MAP2 (Fig. 1 D, E), a cellular marker for neurons. GalR1 immunolabeling occurred predominately at the cell surface of the cell body (Fig. 1E, F). GalR1 labeling always colocalized with MAP2 (Fig. 1F), supporting that GalR1 was restricted to neurons in culture as observed previously in intact tissue preparations (Pham et al. 2002).

Galanin 1–16 inhibits depolarization-evoked $[\text{Ca}^{2+}]_i$ increases in cultured myenteric neurons

Ca^{2+} -imaging experiments were performed on myenteric neurons to test whether the high affinity GalR1/GalR2 agonist, galanin 1–16, altered depolarization-evoked Ca^{2+} influx in cultured myenteric neurons. Cells plated on coverslips were incubated with fluo-4 to measure intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) changes. To activate voltage-dependent Ca^{2+} channels expressed by myenteric neurons we bath applied elevated $[\text{K}^+]_o$ (72 mM),

which consistently evoked $[Ca^{2+}]_i$ increases from neurons. Myenteric neurons were depolarized by elevating $[K^+]_o$ from 2.5 to 72 mM for 1 min. Smooth muscle cells also responded to elevated $[K^+]_o$ applications, however, glial cells and other non-neuronal cells rarely showed any significant change in basal Ca^{2+} level during elevated $[K^+]_o$ applications (data not shown). The depolarization-evoked $[Ca^{2+}]_i$ increase was abolished in the presence of 500 μM $CdCl_2$, a blocker of voltage dependent Ca^{2+} channels in neurons, indicating that all $[K^+]_o$ -evoked $[Ca^{2+}]_i$ changes observed in neurons in our preparation were likely due to Ca^{2+} entry through voltage-gated Ca^{2+} channels ($n=5$) (data not shown). Figure 2A shows pseudocolor images of fluorescence illustrating an example of cultured myenteric neuron in normal (2.5 mM), elevated (72 mM) $[K^+]_o$ mammalian superfusate, and elevated (72 mM) $[K^+]_o$ mammalian superfusate in the presence of galanin 1–16 (1 μM). Figure 2B shows a plot of the $[Ca^{2+}]_i$ changes measured before, during and after the application of galanin 1–16 (1 μM) from the same cell as in Figure 2A. Galanin 1–16 produced a reversible inhibition of the depolarization-evoked $[Ca^{2+}]_i$ increase in 60 out of 100 neurons. Consecutive administration of galanin 1–16 induced similar inhibition of the depolarization-evoked $[Ca^{2+}]_i$ increase indicating that this effect was reproducible (not shown). On average, galanin 1–16 (1 μM) caused a $53.55 \pm 5.95\%$ ($n = 17$, $P < 0.05$) reduction in the K^+ -evoked fluorescence change in myenteric neurons. Significant inhibition of the K^+ -evoked $[Ca^{2+}]_i$ increase was seen at concentrations as low as 10 nM ($24.10 \pm 6.37\%$, $n = 8$, $P < 0.05$), and inhibition increased in a concentration-dependent manner with concentrations $\leq 1 \mu M$ (Fig. 3), with an EC_{50} value of 0.172 μM . Bath application of galanin 1–16 alone did not evoke any change in basal $[Ca^{2+}]_i$ levels (not shown), ruling out any effect on Ca^{2+} stores or receptor operated channels.

Galanin inhibition of the depolarization-evoked $[Ca^{2+}]_i$ increase in myenteric neurons is mediated by GalR1

To test whether the effects of galanin 1–16 are mediated by GalR1, we used a selective GalR1 antagonist RWJ-57408. Consistent with the hypothesis that GalR1 activation inhibits the K^+ -evoked $[Ca^{2+}]_i$ increase in myenteric neurons, co-application of RWJ-57408 (10 μM) with galanin 1–16 (1 μM) prevented galanin 1–16 from inhibiting the K^+ -evoked $[Ca^{2+}]_i$ increase in cultured myenteric neurons ($P < 0.0001$) (Fig. 4). After washout of both RWJ-57408 and galanin 1–16, application of galanin 1–16 (1 μM) inhibited the K^+ -evoked $[Ca^{2+}]_i$ increase of $65.23 \pm 9.69\%$ (Fig. 4, $n = 5$, $P < 0.0001$). Together, these results suggest that the galanin inhibition of voltage-dependent Ca^{2+} channels in myenteric neurons is mediated by GalR1. To determine whether another GalR subtype might be involved in the inhibition of voltage dependent Ca^{2+} influx in myenteric neurons, we tested the effect of galanin 2–11 (1 μM), which has high affinity for GalR2 and GalR3, on depolarization-evoked $[Ca^{2+}]_i$ increase. Bath application of galanin 2–11 alone evoked a very small change in basal $[Ca^{2+}]_i$ levels (data not shown), but had no significant effect on the K^+ -evoked $[Ca^{2+}]_i$ increase in myenteric neurons ($n = 5$) (Fig. 4) supporting the hypothesis that GalR1 mediates the galanin inhibitory effect on voltage-dependent calcium channels.

DISCUSSION

This study demonstrates that galanin 1–16, which has high affinity for GalR1 and GalR2, inhibited the depolarization-evoked Ca^{2+} influx in a concentration-dependent manner. The blockade of this effect by RWJ-57408, a selective antagonist for GalR1, and the lack of effect of the GalR2/GalR3 agonist, galanin 2–11, provided evidence for the involvement of GalR1. This hypothesis is further supported by the localization of GalR1 immunoreactivity in myenteric neurons and not in other types of cells in culture, which is consistent with our previous localization in intact tissue (Pham et al. 2002). The increase in $[Ca^{2+}]_i$ induced by K^+ depolarization was primarily due to Ca^{2+} influx through voltage-dependent Ca^{2+}

channels as indicated by its abolition by CdCl_2 , a blocker of voltage-dependent Ca^{2+} channels in neurons. These findings support the hypothesis that galanin inhibits voltage-dependent Ca^{2+} channels in myenteric neurons via the activation of GalR1.

Galanin has a wide range of effects in the gastrointestinal tract that are mediated by the activation of different GalR subtypes (Branchek et al. 2000; Lang et al. 2007). The existence of multiple GalRs within the gastrointestinal tract has been indicated by receptor binding experiments (King et al. 1989), and studies using galanin peptide fragments with different affinities for GalR subtypes (Bartfai et al. 1993; Gu et al. 1995). The presence of multiple GalRs has been confirmed by quantitative RT-PCR analysis showing that all three GalR mRNAs are expressed at different levels throughout the gut (Anselmi et al. 2005b). The actions of galanin on gastrointestinal functions are complex, ranging from stimulatory to inhibitory. Galanin has been shown to modulate gastrointestinal motility by acting prejunctionally through a nerve-mediated effect involving the release of other transmitters/modulators (Mulholland et al. 1992), and postjunctionally by acting directly on the muscle (Botella et al. 1992; Umer et al. 2005). We have shown that the prejunctional effect of galanin is mediated at least in part by GalR1. This conclusion is supported by morphological and functional studies showing the localization of GalR1 to cholinergic myenteric neurons, and the reduction of galanin inhibition of electrically-induced contractions in a longitudinal myenteric plexus preparation by the GalR1 antagonist, RWJ-57408 (Sternini et al. 2004). GalR1 antagonist also prevented the galanin 1–16-mediated inhibition of K^+ -evoked Ca^{2+} increase in myenteric neurons shown in this study. Taken together, these findings suggest that galanin modulates the excitatory cholinergic transmission in myenteric plexus via the activation of GalR1. By contrast, the direct excitatory effect of galanin on smooth muscle (Wang et al. 1998a) is likely to be mediated by GalR2 that is predominantly coupled to $\text{G}_{q/11}$. Galanin 2–11, induced a small release of Ca^{2+} from few myenteric neurons suggesting that the $\text{G}_{q/11}$ pathway, the main signaling pathway of GalR2, can be activated in these cells (data not shown). However, galanin 2–11 did not inhibit the K^+ -evoked Ca^{2+} increase in cultured myenteric neurons (Fig. 4). In addition, GalR2 activation with galanin 2–11 results in significant $[\text{Ca}^{2+}]_i$ increase in cultured smooth muscle cells, suggesting that GalR2 mediates the excitatory effect of galanin on smooth muscle cells by post-junctional mechanism (Anselmi and Sternini, unpublished), but not the pre-junctional galanin inhibition on enteric neurons.

Primary cultures have been extensively used for functional studies of myenteric neurons since they maintain their ability to express functional receptors for different transmitters (Kimball and Mulholland 1995; Vanden Berghe et al. 2001; Vanden Berghe et al. 2000). There are several studies demonstrating galanin inhibitory effect on cultured myenteric neurons (Palmer et al. 1986; Ren et al. 2001; Sarnelli et al. 2004; Tamura et al. 1988). Palmer et al. (1986) showed that galanin inhibits the excitability of cultured myenteric neurons by inducing membrane hyperpolarization and decreasing input resistance. Additional studies showed that galanin suppresses a voltage-dependant Ca^{2+} conductance in myenteric neurons of guinea pig (Ren et al. 2001; Tamura et al. 1988). Galanin inhibition of Ca^{2+} current was blocked by PTX, suggesting that this effect is mediated by a $\text{G}_{i/o}$ -coupled receptor (Horn and Marty 1988; Ren et al. 2001). In addition, Sarnelli et al (2004) have shown that galanin reduced the Ca^{2+} responses evoked by different excitatory stimuli, including electrical stimulation, substance P and 5-HT stimulation. However, this study did not examine the subtype of GalR mediating this effect. Our study extends these investigations by showing that galanin 1–16-mediated inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels in cultured myenteric neurons is blocked by the GalR1 antagonist, RWJ-57408, and that GalR1 immunoreactivity is localized to neuronal cells in culture as we previously showed in tissue (Anselmi et al. 2005a; Pham et al. 2002; Sternini et al. 2004).

In summary, our study provides strong evidence that GalR1 mediates galanin inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels in myenteric neurons and confirms the presence of functional GalR1 in the myenteric plexus. These findings are in agreement with the hypothesis that GalR1, a receptor coupled to $\text{G}_{i/o}$ inhibitory proteins, mediates galanin inhibition of transmitter release from cholinergic motor neurons. Overall, this study contributes to further our understanding of circuitry and mechanisms underlying neurotransmission regulating gut motility.

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Fig. 1.

Primary cell culture preparation from rat longitudinal muscle-myenteric plexus (5 days *in vitro*). A: DIC image of primary cultured myenteric neurons showing cells with long processes. B–C: Simultaneous visualization of MAP2 (green, B and C, neuronal marker) and GFAP (red, B, marker of glial) or actin (red, C, marker of smooth muscle cells) immunoreactivities showing the presence of different types of cells in the preparation. D–F: Primary cultured rat myenteric neurons immunolabeled with MAP2 and GalR1. The same cell is visualized with MAP2 immunoreactivity (D), and GalR1 immunoreactivity (E). F: Simultaneous visualization of MAP2 and GalR1 immunoreactivity. GalR1 immunoreactivity

is predominantly localized at the cell surface, whereas MAP2 immunoreactivity is in the cytoplasm. Scale bars: 10 microns.

Fig. 2.

Galanin 1–16 inhibition of depolarization-evoked Ca^{2+} increases in a cultured myenteric neuron. A: 1–6 are pseudocolor images corresponding to fluorescence peaks from the same cell plotted in B. 1, control $[\text{K}^+]_o$ (5 mM) mammalian superfusate; 2, elevated $[\text{K}^+]_o$ (72 mM) mammalian superfusate; 3, galanin 1–16 (1 μM) containing mammalian superfusate; 4, galanin 1–16 (1 μM) and elevated $[\text{K}^+]_o$ (72 mM) mammalian superfusate; 5, control $[\text{K}^+]_o$ (5 mM) mammalian superfusate; 6, elevated $[\text{K}^+]_o$ (72 mM) mammalian superfusate. B. A plot of the $[\text{Ca}^{2+}]_i$ changes in the soma of a cultured myenteric neuron loaded with fluo-4/AM. Each number represents a time-point when an image was captured during the experiment. All measurements were made from regions of interest drawn over the soma (see Materials and Methods). The stimulus for depolarization was a 1 min application of mammalian superfusate containing 72 mM KCl. Scale bar is 10 microns.

Fig. 3.

Concentration-dependent inhibition of K⁺-evoked [Ca²⁺]_i increase in cultured myenteric neurons by galanin 1–16. Fluo-4-loaded myenteric neurons were stimulated with elevated [K⁺]_o in the presence of different concentration of galanin 1–16 (0.01–1 μM). Inhibition of K⁺-evoked change produced by galanin 1–16 was normalized to the average of the initial control and subsequent recovery following the test solution. Each point represents the mean ± SE (n=8–17). *P<0.05 compared with control.

Fig. 4.

GalR1 antagonist blocks galanin 1–16 inhibition of K^+ -evoked $[Ca^{2+}]_i$ increases in rat cultured myenteric neurons. Depolarization-evoked $[Ca^{2+}]_i$ increases in myenteric neurons were stimulated by 1-min applications of 72 mM $[K^+]_o$ mammalian superfusate with galanin 1–16 (1 μ M), and galanin 1–16 (1 μ M) plus RWJ-57408 (10 μ M) or galanin 2–11 (1 μ M). The fractional response represents the K^+ -evoked $[Ca^{2+}]_i$ increase induced by galanin 1–16 with or without RWJ-57408 or by galanin 2–11 divided by the average of the K^+ -evoked $[Ca^{2+}]_i$ increases obtained in basal conditions before and after application of the test substances. Galanin 1–16 alone inhibited the K^+ -evoked $[Ca^{2+}]_i$ increase (0.35 ± 0.10 , $*P=0.0025$, $n=5$). Galanin 1–16 (1 μ M) plus the GalR1 antagonist, RWJ-57408 (10 μ M) did not inhibit the K^+ -evoked $[Ca^{2+}]_i$ increase (1.12 ± 0.10 , $n=11$; $**$, $P < 0.0001$, unpaired t -test). Galanin 2–11 (1 μ M) did not modify the depolarization-evoked $[Ca^{2+}]_i$ increases ($n=5$). Each bar represents the mean $\pm$ SE.