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Site of action of galanin in the cholinergic transmission of guinea pig small intestine

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Abstract

The mode and site of action of galanin were examined in the guinea pig small intestine. Galanin (3×10^{-9}–10^{-7} M) inhibited the twitch contractions of longitudinally and circularly oriented muscle strips mediated by the stimulation of cholinergic neurons, but not the contractions mediated by direct stimulation of smooth muscle cells with carbachol. Galanin (3×10^{-9}–10^{-7} M) inhibited both the electrically stimulated and the tetrodotoxin-resistant high K^+ (40 mM)-induced increase of [^{3}H]acetylcholine outflow from the ileal strips preloaded with [^{3}H]choline, in a concentration dependent fashion. The inhibitory effect of galanin was antagonized by galantide and produced self-desensitization. The spontaneous and stimulated outflow of [^{3}H]noradrenaline and [^{3}H]γ-aminobutyric acid were not affected by galanin even at 10^{-7} M. Thus, galanin inhibits the motility of guinea pig ileum by inhibition of acetylcholine release from the enteric cholinergic neurons. Galanin may act on the specific receptor located on soma-dendritic regions and nerve terminals of cholinergic neurons.

Keywords: Acetylcholine release; Noradrenaline release; GABA release; Galantide;

1. Introduction

Galanin, a 29 amino acid peptide, is widely distributed in both the central and peripheral nervous systems (Tatemoto et al., 1983; Melander et al., 1985; Bishop et al., 1986; Rokäeus, 1987; Bartfai et al., 1992). Galanin-immunoreactive neurons are primarily intrinsic to the enteric nervous system (Ekblad et al., 1985a; Bishop et al., 1986). There are a few species and tissues differences in the distribution of galanin-containing neurons. In the guinea pig ileum, galanin immunoreactive nerve cell bodies are not seen in the myenteric plexus and immunoreactive nerve terminals are present in the myenteric plexus (Melander et al., 1985). Galanin binding sites have been demonstrated by receptor autoradiography to be present in the myenteric plexus and longitudinal muscle layers of the guinea pig, rat, rabbit and human (King et al., 1989). Receptor binding assays with membrane preparations showed the presence of galanin receptors in both the nerve and muscle membranes of canine small intestine (Chen et al., 1994a,b). The action of galanin on intestinal motility is species dependent (Rattan, 1991). Galanin produces contraction of small intestine of rat (Tatemoto et al., 1983; Ekblad et al., 1985b; Muramatsu and Yanaihara, 1988), but inhibition of intestinal motor activity of dog, guinea pig and human (Ekblad et al., 1985b; Fox et al., 1986; Bauer et al., 1989). It is possible that multiple sites of action and multiple receptors for galanin are involved in the differences in galanin action. Thus, the present study attempted to examine the site of action of galanin in the guinea pig ileum, especially in cholinergic neurons.

2. Materials and methods

Adult guinea pigs of either sex, weighing between 300 and 350 g, were killed by cervical dislocation. Strips of ileum were excised 10 cm proximal to the ileocaecal sphincter, and approximately 2 cm preparations were dissected.

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2.1. Measurements of the mechanical activity

The motility of longitudinally oriented and circularly oriented muscle was measured in strips prepared by cutting parallel to the oral-anal axis and by cutting at 90° to the oral-anal axis, respectively. The strips of ileum were placed in a 20 ml organ bath in the presence of Krebs solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and glucose 11, which was continuously gassed with 95% O_2 and 5% CO_2 and maintained at 34–36°C and pH 7.4. Mechanical activity was recorded by means of an isometric transducer (Nihon Kohden, Japan, SD-1T). Approximately 500 mg of resting tension was applied and was kept constant by re-adjustment during the equilibration period. Two parallel platinum electrodes were used to stimulate intramural nerves of the strip positioned between these two electrodes. The strips produced contractile responses to electrical transmural stimulation (1 ms pulse duration, 15 V intensity, at a frequency of 0.1 Hz). After stabilization of the stimulated contraction, galanin was added cumulatively to the organ bath during stimulation. Each concentration was added to the organ bath when the maximum effect of the previous concentration had been reached. The increase and decrease by agents of the stimulation-evoked contraction are presented as the % increase and % decrease relative to the stimulation-evoked contraction in the absence of agents, respectively.

2.2. Measurements of the outflow of [^{3}H]acetylcholine, [^{3}H]noradrenaline and [^{3}H]γ-aminobutyric acid

The methods used have been described previously (Nakayama et al., 1990; Taniyama et al., 1982). Briefly, the ileal strips were incubated with 2×10^{-7} M [^{3}H]choline, 5×10^{-8} M [^{3}H]noradrenaline or 5×10^{-8} M [^{3}H]γ-aminobutyric acid (GABA) for 60 min in Krebs solution. After being washed in fresh Krebs solution for 30 min, the preparations were mounted in the apparatus and superfused at 1.2 ml/min with the same solution gassed with 95% O_2 and 5% CO_2, maintained at 34–36°C and pH 7.4. Experiments were started 60 min after the spontaneous outflow of tritium had approached a plateau. The superfusates were collected every 1 min, and the radioactivity was counted in a liquid scintillation counter. For experiments on [^{3}H]acetylcholine outflow, Krebs solution containing hemicholinium-3, to prevent the uptake of choline formed from acetylcholine, was used for superfusion. For [^{3}H]noradrenaline outflow, the incubation and perfusion media were Krebs solution containing 10^{-5} M ascorbate and 10^{-4} M pargyline. For [^{3}H]GABA outflow, the incubation and perfusion media used were Krebs solution containing 10^{-5} M aminooxyacetic acid.

The proportion of unchanged [^{3}H]acetylcholine, [^{3}H]noradrenaline and [^{3}H]GABA to total tritium in the superfusates was estimated as follows: Superfusates were collected immediately before and during various stimulations at 60 min after the start of the superfusion. For measuring [^{3}H]acetylcholine, the extraction and separation of [^{3}H]choline and [^{3}H]acetylcholine were carried out by electrophoresis, according to Potter and Murphy (1967). Aliquots (1 ml) of the superfusates were collected in 1 ml of 3-heptanone-tetraphenylboron (10 mg/ml) on ice. [^{3}H]Choline and [^{3}H]acetylcholine were extracted with 1 N HCl, dried and dissolved in 1 N formic acid acetone (15:85 v/v). The samples were then subjected to electrophoresis at a constant voltage (200 V) for 1 h on Whatman-3 chromatography paper. Separated substances were stained by iodine vapor, the recovered radioactive compounds were extracted with 0.5 ml of ethanol, and the radioactivity was measured in a liquid scintillation spectrometer. When the recovery of added [^{3}H]-acetylcholine was measured after electrophoresis, at least 96% of applied [^{3}H]acetylcholine was present in the acetylcholine spot. Over 81.8% of the total radioactivity released from electrically and high K^+-stimulated preparations proved to be [^{3}H]acetylcholine. The superfusion medium used in the present study did not contain any cholinesterase inhibitor, and under such conditions the increase in the electrical stimulation-evoked release mainly represents the outflow of [^{3}H]acetylcholine (Vizi et al., 1984). The value of stimulated outflow of [^{3}H]acetylcholine is similar to that for electrical stimulation-evoked release in the presence of physostigmine from isolated ileal longitudinal muscle strips (Vizi et al., 1984). The total radioactivity in the superfusates from the stimulated preparation was considered to approximate the amount of [^{3}H]acetylcholine and therefore was denoted as [^{3}H]acetylcholine outflow. When electrical stimulation was applied successively four times to the preparation at 30 min intervals, the stimulation-induced increase of [^{3}H]acetylcholine outflow markedly decreased or increased from the first to second stimulation period, whereas there were no significant differences between the increase of outflow induced by the second to the fourth stimulations. Therefore, the increase of [^{3}H]acetylcholine outflow induced by the first stimulation was disregarded and the increase of [^{3}H] induced by the second stimulation served as control.

The proportion of unchanged [^{3}H]noradrenaline to total tritium in the superfusate was determined by the method of Fujiwara et al. (1984). As the carrier, ten nanograms of unlabeled noradrenaline was added to each sample, and noradrenaline in the sample was absorbed with activated alumina and identified by a high-performance liquid chromatograph (HPLC) equipped with an electrochemical detector (Yanaco).

Fractions containing noradrenaline were collected from the outflow of the HPLC, and the radioactivity in these fractions was determined by liquid scintillation spectrometry. The proportion of [^{3}H]noradrenaline to the total ^{3}H outflow in the superfusates exceeded 85.8% ($n = 6$). The total radioactivity in the superfusates was considered to approximate the amount of [^{3}H]noradrenaline and therefore was denoted as [^{3}H]noradrenaline outflow.

The proportion of unchanged GABA in the ^{3}H outflow of the superfusates was determined previously (Taniyama et al., 1982). Even 120 min later, over 93% of the total radioactivity in the superfusates was associated with unchanged GABA. Accordingly, the total radioactivity in the superfusate was denoted as [^{3}H]GABA outflow.

2.3. Calculations of the outflow of [^{3}H]acetylcholine, [^{3}H]noradrenaline and [^{3}H]GABA

At the end of release experiments, the radioactivity of the tissue dissolved in Soluene was counted in a liquid scintillation spectrometer. The outflow of tritium is presented as the fractional rate, obtained by dividing the amount of tritium in the superfusate by the respective amount of tritium in the tissue. The tritium content of the tissue at each period was calculated by adding cumulatively the amount of each fractional tritium outflow to the tritium content of the tissue at the end of experiment. From each of the outflow curves obtained by plotting the fractional outflow of tritium against time, the peak increase of tritium outflow induced by stimulation in each condition was calculated as the percentage increase over the basal release. Data were analyzed by Wilcoxon's signed rank test, and P value < 0.05 was considered statistically significant.

2.4. Drugs and chemicals

The substances used were as follows: [^{3}H]choline (60 Ci/mmol), [^{3}H]noradrenaline (40.8 Ci/mmol) and [^{3}H]γ-aminobutyric acid (94 Ci/mmol) (New England Nuclear, Boston, MA, USA), synthetic rat galanin and galantide (Peninsula, Belmonte, CA, USA), carbachol, pargyline, hemicholinium-3 and ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA) (Sigma Chemical Co., St. Louis, MO, USA), tetrodotoxin (Wako pure Chemicals, Osaka, Japan), Soluene (Packard, Downers Groves, IL, USA). Other chemicals used were of reagent grade.

3. Results

3.1. Effect of galanin on nerve-mediated twitch contractions and carbachol-induced contractions

Electrical transmural stimulation (1 ms, 30 V) at 0.1 Hz produced twitch contractions of the longitudinally oriented and circularly oriented muscle strips, and these contractions were prevented by treatment with 10^{-7} M atropine or 3×10^{-7} M tetrodotoxin (data not shown), thereby indicating that the contractions were mediated by the stimulation of cholinergic neurons. Galanin at 3×10^{-9}–10^{-7} M inhibited the twitch contractions of longitudinally oriented and circularly oriented muscle strips induced by electrical transmural stimulation, in a concentration-dependent manner (Fig. 1A). The inhibitory effect of galanin showed the same potency in the longitudinally oriented and in the circularly oriented muscle strips.

Carbachol at 5×10^{-5} M produced contractions of both longitudinally oriented and circularly oriented muscle strips, and these contractions were prevented by 10^{-6} M atropine, but not by 10^{-6} M tetrodotoxin, thereby indicating that the responses were mediated by muscarinic receptors located on the smooth muscles. When galanin was applied during the plateau phase of the carbachol-induced contractions, galanin at 10^{-8} M and 10^{-7} M failed to affect the contractions (Fig. 1B).

3.2. Effect of galanin on the increase of [^{3}H]acetylcholine outflow induced by electrical stimulation and high K^+ (40 mM)

The spontaneous outflow of [^{3}H]acetylcholine reached a steady state and a single exponential curve was obtained with a fractional rate of 0.00660 ± 0.00062/min ($n = 6$) 60 min after superfusion. Either electrical transmural stimulation (1 ms, 15 V, 180 pulses) at 3 Hz or high K^+ (40 mM) increased [^{3}H]-acetylcholine outflow from the ileal strips. The electrical stimulation-induced increase of [^{3}H]acetylcholine outflow was prevented either by removal of external Ca^{2+} or by treatment with 3×10^{-7} M tetrodotoxin. The high K^+-induced increase of [^{3}H]acetylcholine outflow was prevented by the removal of external Ca^{2+}, while it was reduced to approximately 75% by treatment with 3×10^{-7} M tetrodotoxin.

Pretreatment with galanin at 3×10^{-9}–10^{-7} M for 1 min significantly inhibited the electrical stimulation-induced increase of [^{3}H]acetylcholine outflow, in a concentration-dependent manner, while the tetrodotoxin-resistant high K^+-induced increase of [^{3}H]acetylcholine outflow was significantly inhibited by galanin at the concentration of 10^{-8} M and over (Fig. 2).

3.3. Effects of galantide and self-desensitization on galanin-induced inhibition of [^{3}H]acetylcholine outflow

Pretreatment with galantide at 10^{-8} M, a galanin receptor antagonist (Bartfai et al., 1992), for 8 min antagonized the galanin (3×10^{-8} M)-induced inhibition of [^{3}H]acetylcholine outflow evoked by both electrical stimulation and high K^+ in the presence of

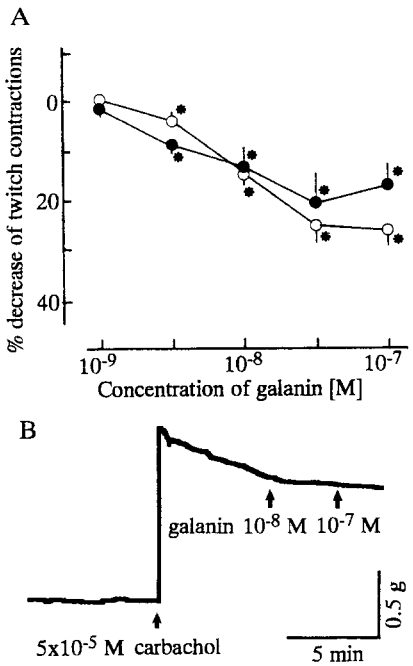

Fig. 1. Effect of galanin on electrical stimulation-induced twitch contractions and carbachol-induced contraction in the muscle strips isolated from guinea pig ileum. (A) Electrical stimulation was of 1-msec duration, 30 V intensity, and 0.1 Hz. (○) Longitudinally oriented muscle strips, (●) circularly oriented muscle strips. Each point represents the mean ± S.E.M. for six animals. (B) Typical pattern of galanin effect on carbachol-induced contraction in longitudinally oriented muscle strips. * Significantly different from value in the absence of galanin ($P < 0.05$).

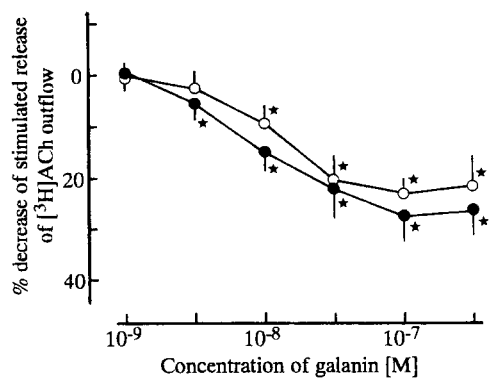

Fig. 2. Effect of galanin on the [^{3}H]acetylcholine (ACh) outflow induced by electrical transmural stimulation (ETS) and high K^+ (40 mM) from the ileal strips. Galanin was added 1 min before and during the application of ETS (1-ms duration, 15 V intensity, 180 pulses) at 3 Hz (●) or high K^+ (40 mM) in the presence of tetrodotoxin (3×10^{-7} M) (○). Each point is presented as the mean ± S.E.M. of the percent increase over spontaneous [^{3}H]ACh outflow from seven animals (in ETS-evoked release) and eight animals (in high K^+-evoked release). The percent increase of [^{3}H]ACh outflow over spontaneous outflow was calculated by dividing the fractional rate during stimulation by the spontaneous outflow. * Significantly different from value in the absence of galanin ($P < 0.05$).

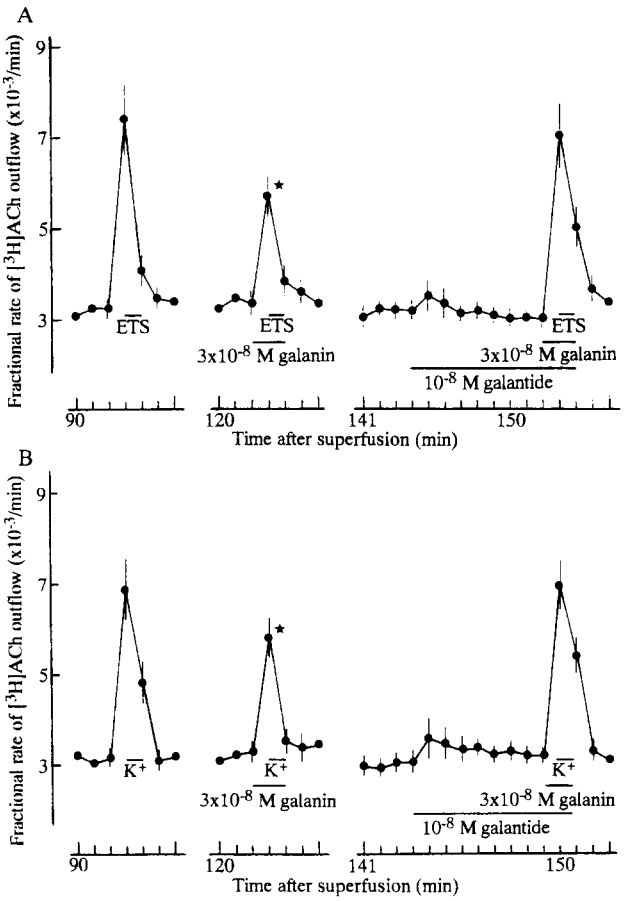

Fig. 3. Effect of galantide on galanin-induced inhibition of [^{3}H]acetylcholine (ACh) outflow evoked by electrical stimulation (A) and high K^+ in the presence of tetrodotoxin (B). Galanin (3×10^{-8} M) and galantide (10^{-8} M) were added 1 min and 9 min before and during the application of electrical transmural stimulation (ETS) (1-ms duration, 15 V intensity, 180 pulses) at 3 Hz or high K^+ (40 mM) in the presence of tetrodotoxin (3×10^{-7} M), respectively. Each point represents the mean fractional rate ± S.E.M. from five animals. * Significantly different from control (in the absence of galanin and galantide) ($P < 0.05$).

tetrodotoxin, with no effect on the spontaneous outflow (Fig. 3).

When galanin at 3×10^{-8} M was present in the superfusion medium for 30 min, a second addition of galanin even at 10^{-7} M failed to inhibit the electrical transmural stimulation (1 ms, 15 V, 180 pulses, 3 Hz)-induced increase of [^{3}H]acetylcholine outflow (Fig. 4), thereby indicating that galanin produces self-desensitization.

3.4. Effect of galanin on the [^{3}H]noradrenaline outflow and the [^{3}H]GABA outflow

The fractional rate of spontaneous outflow of [^{3}H]noradrenaline from the ileal strips preloaded with [^{3}H]noradrenaline was 0.00221 ± 0.00028/min ($n = 6$) 60 min after superfusion, a time when the spontaneous

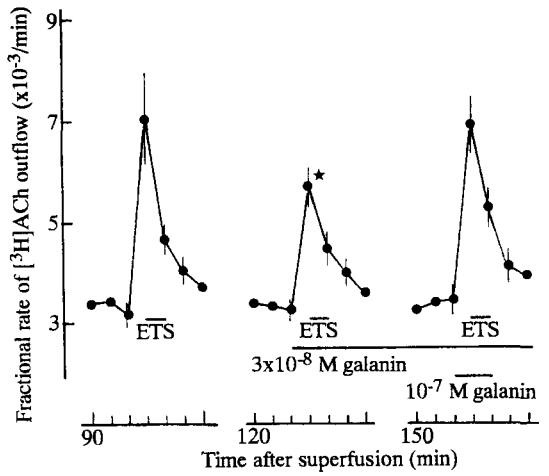

Fig. 4. Effect of desensitization on galanin-induced inhibition of electrically stimulated-[^{3}H]acetylcholine (ACh) outflow. In the presence of galanin at 3×10^{-8} M for 30 min, galanin at 10^{-7} M was further added 1 min before and during the application of electrical transmural stimulation (ETS) (1-ms duration, 15 V intensity, 180 pulses) at 3 Hz. Each point represents the mean fractional rate ± S.E.M. for four animals. *Significantly different from value in the absence of galanin ($P < 0.05$).

outflow had approached a plateau level. Galanin at 10^{-7} M did not affect the spontaneous outflow of [^{3}H]noradrenaline, and pretreatment with galanin at 10^{-7} M for 1 min also did not alter the high K^+-induced increase of [^{3}H]noradrenaline outflow (Fig. 5A).

The fractional rate of spontaneous outflow of [^{3}H]GABA from the ileal strips preloaded with [^{3}H]GABA was 0.01194 ± 0.00018/min ($n = 6$) 60 min after superfusion, a time when the spontaneous outflow had approached a plateau level. Galanin at 10^{-7} M did not affect the spontaneous outflow and high K^+-induced increased of [^{3}H]GABA outflow (Fig. 5B).

4. Discussion

Galanin was found to inhibit the cholinergic transmission but not to affect directly the smooth muscle cells of the guinea pig ileum. As the twitch contractions were prevented by either atropine or tetrodotoxin, the twitch response relates to stimulation of cholinergic neurons. The carbachol-induced contractions were inhibited by atropine, but not by tetrodotoxin, thereby indicating that the contractions are mediated by direct stimulation of smooth muscle cells. Galanin inhibited the twitch contractions of both longitudinally oriented and circularly oriented muscle strips evoked by electrical stimulation, but not the carbachol-induced contractions.

The inhibitory effects of galanin on the cholinergic transmission were confirmed by the release experiments. Galanin inhibited the nerve-stimulated increase of acetylcholine outflow from the ileal preparations, as noted by Yau et al. (1986). Both the electrical stimulation-induced increase and high K^+-induced tetrodotoxin-resistant increase of acetylcholine outflow were inhibited by galanin. The tetrodotoxin-sensitive release of neurotransmitter is considered to be induced by the stimulation of soma-dendritic regions of the neuron (Vizi et al., 1973; Gonella et al., 1980), while the tetrodotoxin-resistant release of neurotransmitter is assumed to be due to direct depolarization of nerve terminals (Starke, 1981; Alberts et al., 1982). Thus, the site of action of galanin may be the nerve terminals of cholinergic neurons as well as soma-dendritic regions, although the action of galanin at the nerve terminals appears to require a higher concentration of galanin than that needed at the soma-dendritic regions. The notion that the site of action of galanin is on nerve cell bodies within the myenteric plexus of guinea pig small intestine is supported by findings that galanin induces

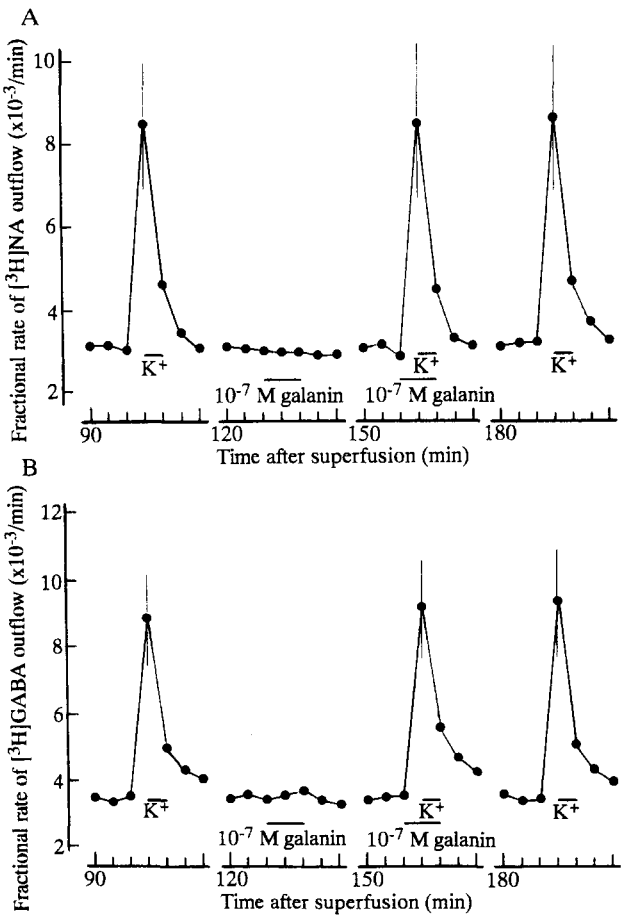

Fig. 5. Effect of galanin on the spontaneous outflow and high K^+-induced increase of [^{3}H]noradrenaline (NA) outflow (A) and [^{3}H]GABA outflow (B). Galanin (10^{-7} M) was added to 3×10^{-7} M tetrodotoxin-containing medium 1 min and 10 min before and during the application of high K^+ (40 mM) for 1 min, respectively. Each point represents the mean fractional rate ± S.E.M. from five animals (in [^{3}H]NA outflow) and from eight animals (in [^{3}H]GABA outflow).

a slow inhibitory postsynaptic potential-like response in myenteric neurons (Palmer et al., 1986; Tamura et al., 1988) and suppresses the fast excitatory postsynaptic potentials (Tamura et al., 1987; Tamura et al., 1988).

The inhibitory effect of galanin on acetylcholine outflow mediated by stimulation of both soma-dendritic regions and nerve terminals of cholinergic neurons was antagonized by galantide. Galantide is an antagonist at the galanin receptor (Bartfai et al., 1992) as well as an agonist at the substance P receptor. Substance P evokes the release of acetylcholine from the myenteric neurons (Pernow, 1983); however the concentration of galantide which antagonized the galanin effect did not affect the acetylcholine outflow. Furthermore, galanin produced self-desensitization. Thus, galanin may act on the specific galanin receptors located on the soma-dendritic regions and nerve terminals of cholinergic neurons. Autoradiographic study has shown that galanin binding sites are most numerous in the myenteric plexus and least numerous in the longitudinal and circular muscle layers in the guinea pig ileum (King et al., 1989). Since galanin failed to affect the contractions induced by direct stimulation of smooth muscle cells with carbachol, the galanin receptors in the muscle layers shown by autoradiography may correspond to the cholinergic nerve terminals innervating muscle layers in the guinea pig ileum, but not to the smooth muscle cells. In the canine small intestine, galanin receptors have been shown to be located on both neurons and muscle cells (Chen et al., 1994a,b).

It has been proposed that galanin induces contraction of the rat vas deferens due to stimulation of noradrenaline release from sympathetic nerve terminals (Ohhashi and Jacobowitz, 1985). Galanin has been reported to coexist with catecholamines and GABA in the brain tissue (Melander et al., 1986). In the gastrointestinal tract, it is well known that noradrenaline released from sympathetic neurons regulates the release of acetylcholine from cholinergic neurons via α_2-adrenoceptors located on cholinergic nerve terminals. This being the case, neuropeptide Y induces noradrenaline release from sympathetic neurons which, in turn, inhibits acetylcholine release by acting on presynaptic α_2-adrenoceptors located on the cholinergic nerves (Wiley and Owyang, 1987). In the present study, galanin did not affect the spontaneous and stimulated noradrenaline outflow from the guinea pig ileal preparations, therefore the inhibitory effect of galanin may not mediate the adrenergic nerve stimulation. GABA-containing neurons are present in the guinea pig ileum (Jessen et al., 1987; Erdö and Wolff, 1990; Tanaka and Taniyama, 1992) and regulate the release of acetylcholine via $GABA_B$ receptor (Kleinrok and Kilbinger, 1983). Galanin failed to affect the spontaneous and stimulated outflow of GABA, therefore GABA may not mediate the inhibitory effect of galanin.

Galanin immunoreactive neurons are present in the small intestines of humans, dogs, rats and guinea pigs (Tatemoto et al., 1983; Melander et al., 1985; Bishop et al., 1986; Rokäeus, 1987; Bartfai et al., 1992), while there are species differences in the mode and sites of action of galanin (Rattan, 1991). The small intestine of the rat contracts in response to direct stimulation of smooth muscle cells with galanin (Ekblad et al., 1985b), while canine intestine shows an inhibition of contractility due to a direct action of galanin on smooth muscle cells (Fox et al., 1986). In the canine intestine, different types of galanin receptors have been proposed on the basis of the results of receptor binding assays; the galanin receptors located on the enteric nerve are associated with pertussis toxin-sensitive G protein (Chen et al., 1994a) and the receptors located on the smooth muscle cells are associated with cholera toxin-sensitive G protein (Chen et al., 1994b). Electrophysiological studies indicate two mechanisms of action for galanin on the myenteric neurons (Tamura et al., 1988). Our results demonstrated that galanin has an inhibitory effect on the contractility of guinea pig small intestine by inhibiting cholinergic neuronal activity and has two sites of action.

Acknowledgements

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