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Actions of galanin on neurotransmission in the submucous plexus of guinea pig small intestine

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Abstract

Electrophysiologic recording methods were used to study the actions of galanin on synaptic transmission in the submucous plexus of guinea pig ileum. Exposure to galanin resulted in concentration-dependent suppression of slow noradrenergic inhibitory postsynaptic potentials and fast nicotinic excitatory postsynaptic potentials in the majority of neurons. Failure of galanin to suppress nicotinic depolarizing responses to micropressure pulses of acetylcholine and failure to suppress hyperpolarizing responses to micropressure pulses of norepinephrine suggested that galanin acted at presynaptic inhibitory receptors to suppress release of acetylcholine and norepinephrine. Galanin suppressed slow excitatory postsynaptic potentials in eight of eight neurons with AH (after-hyperpolarization) type electrical behavior and in none of 26 neurons with S (synaptic) type electrical behavior. Suppression of excitatory neurotransmission in AH neurons was always associated with membrane hyperpolarization. Excitatory responses caused by experimentally applied substance P were also inhibited by galanin. Galanin-(1–16) and galanin-like peptide mimicked the inhibitory actions of galanin on neurotransmission. The selective galanin GAL2 receptor agonist [D-Trp^2]galanin was inactive. The chimeric peptides, galanin-(1–13)-spantide I, galantide, galanin-(1–13)-neuropeptide Y(25–36) amide, galanin-(1–13)-bradykinin-(2–9)amide and galanin-(1–13)-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide all produced varying degrees of suppression of the synaptic potentials. The evidence suggests that the galanin GAL1 receptor, but not the galanin GAL2 receptor, mediated the presynaptic and postsynaptic inhibitory actions of galanin.

Keywords: Enteric nervous system; Submucous plexus; Small intestine; Synaptic transmission; Galanin

1. Introduction

Galanin is a 29 amino acid neuropeptide (30 amino acids in human) that was originally isolated from porcine intestine (Tatemoto et al., 1983) and found to be widely distributed in the mammalian central and peripheral nervous system including the enteric nervous system (Rokaeus, 1987; Rokaeus et al., 1984; Melander et al., 1985). Galanin-immunoreactive cell bodies in the guinea pig enteric nervous system are present in the myenteric and submucous plexuses of the small and large intestine and the myenteric plexus of the stomach (Melander et al., 1985; Furness et al., 1987). Galanin-immunoreactive nerve fibers are present in all layers of the gut wall and at all levels of the gastrointestinal tract (Ekblad et al., 1985; Melander et al., 1985; Furness et al., 1987). These fibers are primarily intrinsic to the enteric nervous system (Ekblad et al., 1985; Furness et al., 1987).

Numerous reports suggest roles for galanin in the enteric neural control and coordination of gastrointestinal motility and secretion. Exposure to galanin alters the contractile behavior of the gastrointestinal musculature in variable and species dependent manner. Application of galanin evokes contraction of longitudinal muscle strips from the rat gastric fundus, jejunum and colon (Korolkiewicz et al., 1997, 2000; Wang et al., 1998, 1999). Galanin suppressed spontaneous contractions of the circular muscle both in vivo and in vitro in canine small intestine (Fox et al., 1986). Contractions of the intestinal circular muscle coat and the release of acetylcholine evoked by transmural electrical field stimulation are suppressed with galanin present in the bathing solution in vitro studies (Akehira et al., 1995; Kakuyama et al., 1997). Kiyohara et al. (1992) reported that application of galanin

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increased short-circuit current in Ussing chamber studies with rat jejunal mucosa and proposed a role for neuronal release of galanin in the control of mucosal secretion. On the other hand, Brown et al. (1990) reported that galanin decreased short-circuit current across pig jejunal mucosa and Homaidan et al. (1994) found a similar action for rabbit ileal mucosa. The presence of galanin attenuated short-circuit current responses to transmucosal electrical stimulation in sheets of guinea pig intestinal mucosa in Ussing chambers in the absence of any change in baseline short-circuit current (McCulloch et al., 1987).

Results of intracellular electrophysiologic studies with "sharp" microelectrodes and "patch" pipettes, obtained for myenteric neurons of the guinea pig small intestine, revealed a hyperpolarizing action of galanin that was associated with decreased input resistance (i.e., increased conductance) and suppression of excitability (Tamura et al., 1987, 1988b; Ren et al., 2001). Exposure to galanin suppressed conductance in voltage-gated Ca^{2+} channels and activated an inwardly rectifying K^+ current in cultured myenteric neurons of the guinea pig small intestine (Ren et al., 2001). Fast excitatory postsynaptic potentials at nicotinic synapses in the guinea pig myenteric plexus were suppressed during exposure to galanin (Tamura et al., 1987).

Specific galanin receptor subtypes mediate the actions of galanin. Pharmacologic studies with peptide fragments of galanin or chimeric galanin peptides suggested the existence of more than one kind of galanin receptor in different tissues and cell lines (Bartfai et al., 1993; Ogren et al., 1993; Wynick et al., 1993; Chen et al., 1994; Gu et al., 1995). Three galanin receptors referred to as galanin GAL1 receptor, galanin GAL2 receptor and galanin GAL3 receptor have been cloned from human, rat and mouse (Branchek et al., 2000). Each of the receptor subtypes has seven putative transmembrane domains and belongs to the G protein-coupled receptor superfamily. The receptor subtypes are distinguished by their pharmacologic profiles in membrane binding assays and by their tissue distribution. Galanin-(1–16), an N-terminal fragment of galanin, displays high affinity for galanin GAL1 and galanin GAL2 receptors but low affinity for the galanin GAL3 receptor (Howard et al., 1997; Sullivan et al., 1997; Wang et al., 1998; Branchek et al., 2000). The modified galanin peptide [D-Trp^2]galanin has high affinity for the galanin GAL2 receptor and low affinity for galanin GAL1 and galanin GAL3 receptors (Smith et al., 1997; Branchek et al., 2000). A 60 amino acid galanin-like peptide from porcine hypothalamus is structurally related to galanin and appears to be more selective for the galanin GAL2 receptor than the galanin GAL1 receptor (Ohtaki et al., 1999).

Neurons of the submucous plexus are synaptically connected to form integrative microcircuits that exert minute-to-minute control of mucosal secretion and integrate secretion with mucosal blood flow (see reviews by Cooke, 2000; Wood, 1994a; Wood et al., 1999). Localization of immunoreactivity for galanin in neural elements of the submucous plexus, its electrophysiologic actions in myenteric neurons and its actions on intestinal mucosal ionic transport suggest that it may be an important mediator in the submucosal microcircuitry. In view of the absence of information on the electrophysiologic actions of galanin in the submucous plexus, the aim of the present study was to explore the pharmacology of its actions on electrical and synaptic behavior of the neurons that form the submucosal neuronal microcircuitry.

2. Materials and methods

Adult male Hartley-strain guinea pigs (300–350 g) were stunned by a blow to the head and exsanguinated from the cervical vessels according to procedures approved by the Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture Veterinary Inspectors. Segments of small intestine 3–5 cm in length were removed 20 cm proximal to the ileocecal junction. Preparations of the submucous plexus for electrophysiological recording were microdissected as described earlier (Zafirov et al., 1993). A 2.0 × 1.0 cm segment of the preparation was mounted in a 2.0 ml recording chamber that was perfused at a rate of 10–15 ml/min with Krebs solution warmed to 37 °C and gassed with 95% O_2/5% CO_2 to buffer at pH 7.3–7.4. The composition of the Krebs solution was (in mM) NaCl, 120.9; KCl, 5.9; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 14.4; $CaCl_2$, 2.5; and glucose, 11.5. Ganglia in the submucous plexus were visualized with differential interference contrast optics and epilumination. Ganglia selected for study were immobilized with L-shaped stainless-steel wires (100 μm in diameter) placed on either side of the ganglion.

Our methods for intracellular recording from the submucous plexus are described in detail elsewhere (Zafirov et al., 1993). Transmembrane electrical potentials were recorded with conventional "sharp" microelectrodes filled with 1% biocytin in 2 M KCl containing 0.05 M Tris buffer (pH 7.4). Resistances of the electrodes were 80–120 MΩ. The preamplifier (M767, World Precision Instruments, Sarasota, FL) was equipped with a bridge circuit for intraneuronal injection of electrical current. Constant rectangular current pulses were driven by a Grass SD9 stimulator (Grass instrument Division, Astro-Med, W. Warwick, RI). Electrometer output was amplified and observed on an oscilloscope (Tektronics 5113, Tektronics, Beaverton, OR). Fast excitatory postsynaptic potentials (EPSPs), slow EPSPs, and slow inhibitory postsynaptic potentials (IPSPs) were evoked by focal electrical stimulation of interganglionic fiber tracts with electrodes made from 20-μm diameter Teflon-insulated platinum wire and connected through stimulus-isolation units (Grass SIN5) to Grass S48 stimulators. The fast EPSPs at expanded time scale were displayed through a real-time oscilloscope (Tektronics TDS210, Tektronics) and output to a laserjet printer. All data were recorded on videotape for

later analysis. Chart records were made on Astro-Med thermal recorders. Amplitudes of action potentials in some of the figures were attenuated by the low-frequency response of the chart recorder.

At the end of each recording session, the marker dye biocytin was injected into the impaled neurons from the recording electrodes by the passage of hyperpolarizing current (0.5 nA for 10–30 min). The anal end of the preparations was marked and the tissue was transferred into a disposable chamber filled with fixative containing 4% formaldehyde plus 15% of a saturated solution of picric acid and kept at 4 °C overnight. The preparations were cleared in three changes of dimethyl sulfoxide and three 10-min washes with phosphate-buffered saline. The preparations were reacted with fluorescein streptavidin (1:100) for 1 h and examined with a Nikon Eclipse E600 fluorescent microscope and appropriate filter combinations.

Actions of galanin and related pharmacological agents were studied by pressure microejection or by application in the superfusion solution. Micropipettes (10-μm diameter) manipulated with the tip close to the impaled neurons were used to microeject the substances onto the neurons. Pressure pulses of nitrogen with predetermined force and duration were applied to the micropipettes through electronically controlled solenoid valves.

Pharmacologic agents used and their sources were as follows. Galanin (porcine), galanin-(1–13)-spantide I (C7), galanin-(1–13)-bradykinin (2–9)amide (M35), galanin-(1–13)-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide (M40), acetylcholine, substance P and hexamethonium were obtained from Sigma (St. Louis, MO). Galanin-(1–16), galantide (M15), galanin-(1–13)-neuropeptide Y-(25–36) amide (M32), [D-Trp^2]galanin (human) and galanin-like peptide (GALP) were from Bachem (Torrance, CA). Norepinephrine and phentolamine were from RBI (Matick, MA).

Data are expressed as means ± standard error; n value refers to the number of neurons. The continuous curves for the concentration–response relationships were constructed using the following least-squares fitting routine: $V = V_{Max}/[1+(IC_{50}/C)^{nH}]$, where V is the observed inhibitory response, IC_{50} is the concentration which induces the half-maximal inhibition, and nH is the apparent Hill coefficient. The graphs were drawn by averaging results from all experiments and fitting to a single concentration–response curve by using Sigma Plot software (SPSS, Chicago, IL). Student's t-test or one-way analysis of variance was used to determine the statistical significance. P values <0.05 were considered statistically significant.

3. Results

Results were analyzed for 131 submucous neurons from 61 guinea pigs with impalements lasting from 20 min to 8 h. All neurons had resting membrane potentials greater than −45 mV. The neurons were classified as either S- (synaptic) or AH- (after hyperpolarization) type according to the criteria described previously by Bornstein et al. (1994), Mihara (1993) and Wood (1994a,b). Of the 131 neurons, 115 were identified as S type and 16 as AH type.

3.1. Postsynaptic actions of galanin and related peptides

Application of galanin (1–1000 nM) did not significantly alter the membrane potential of any of 115 S-type neurons. Application of galanin (30–1000 nM) to AH-type neurons evoked hyperpolarizing responses ranging from 4 to 16 mV in 13 of 16 neurons. The hyperpolarizing responses were associated with decreased input resistance (i.e., increased ionic conductance). The hyperpolarizing action of galanin on AH neurons was reminiscent of the action on myenteric AH-type neurons reported by Tamura et al. (1988b). Exposure to galanin-(1–16) (300 nM), which is an N-terminal fragment of galanin, evoked mean hyperpolarizing responses of 9.5 ± 1.4 mV in six AH-type neurons. Application of galanin-(1–16) (300 nM) did not significantly alter the membrane potential in S-type neurons in the submucosal plexus. Application of 1-μM GALP, which is a newly identified galanin-like peptide, evoked membrane hyperpolarizing responses with a mean of 6.4 ± 1.2 mV in five AH-type neurons. Application of 1 μM GALP did not significantly change the membrane potential in 10 S-type neurons. Exposure to the galanin GAL2 receptor agonist [D-Trp^2]galanin (1 μM) did not significantly alter the membrane potential of either AH- or S-type submucous neurons.

3.2. Slow IPSPs

Repetitive focal electrical stimulation of interganglionic fiber tracts at 20 Hz for 200-ms evoked slow IPSPs in 102 of 115 S-type submucous neurons. Stimulus-evoked slow IPSPs were not found in any of 16 AH-type submucous plexus neurons. Total blockade of the IPSPs by 1-μM phentolamine identified the IPSPs as being mediated by the release of norepinephrine. The mean amplitude and duration of the noradrenergic IPSPs were, respectively, 29.53 ± 1.42 mV (range: 14–38 mV; $n = 30$) and 1.52 ± 0.12 s (range: 0.9–2.3 s; $n = 30$). The noradrenergic IPSPs were found exclusively in neurons with S-type electrophysiologic behavior and uniaxonal morphology. The non-adrenergic, non-cholinergic slow IPSPs in submucosal plexus neurons, which were reported by Shen and Surprenant (1993) to be mediated by the release of somatostatin from neurons intrinsic to the enteric nervous system, were found infrequently in the present study.

Application of galanin (1–1000 nM) in the superfusion solution suppressed the amplitude and shortened the duration of the slow IPSPs in a concentration-dependent manner in every submucosal neuron tested (Fig. 1A,C). Concentrations of 300 nM and higher abolished the slow IPSPs. Suppression of the IPSPs began within 1–1.5 min after

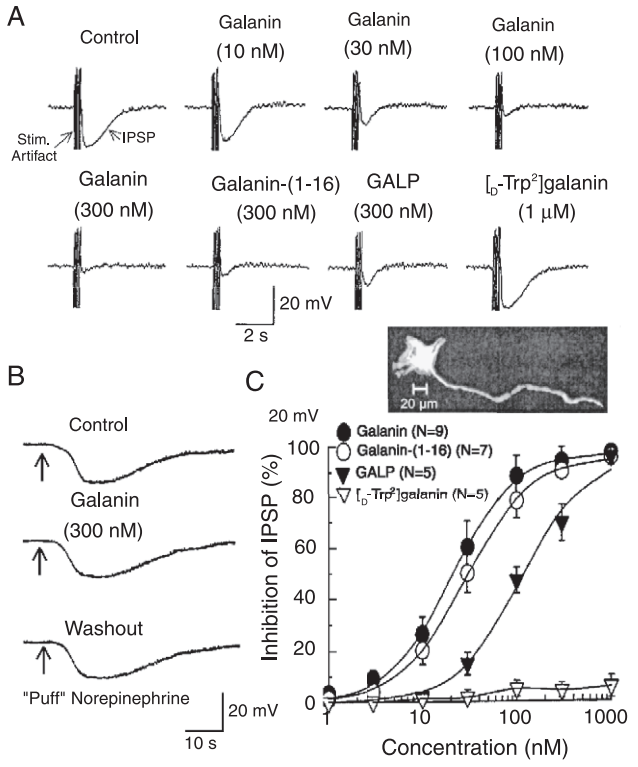

Fig. 1. Presynaptic inhibitory action of galanin and galanin analogs on noradrenergic slow IPSPs in submucous plexus neurons with uniaxonal morphology and S-type electrophysiologic behavior. (A) Noradrenergic slow IPSPs evoked by short-train stimulation (20 Hz, 0.2 s) applied to an interganglionic fiber tract were suppressed progressively by increasing concentrations of galanin from 10 to 300 nM. The inhibitory action of galanin was mimicked by galanin-(1–16) and GALP, but not [D-Trp^2]galanin. The neuron (inset) had uniaxonal morphology. (B) Microejection (“puff”) of 10 μM norepinephrine onto the cell soma evoked an IPSP-like hyperpolarizing response in the same neuron as A. Presence of 300 nM galanin in the bathing solution did not suppress hyperpolarizing responses to micropressure application of norepinephrine. (C) Concentration–response curves for suppression of noradrenergic slow IPSPs by galanin, galanin-(1–16), GALP and [D-Trp^2]galanin. The IC_{50}'s for galanin, galanin-(1–16) and GALP were 20.3 ± 0.7, 28.6 ± 0.7 and 111.6 ± 9.8 nM, respectively.

entry of galanin into the tissue chamber. Maximum inhibition was observed within 3–4 min and washout for periods of 8–10 min were sufficient for complete recovery. The IC_{50} value for the inhibitory action of galanin on the slow IPSPs was 20.3 ± 0.7 nM. Exposure to galanin (1–1000 nM) did not alter the membrane potential in any of the S-type neurons in which it suppressed the slow IPSPs.

Galanin analogs were used to investigate the kinds of galanin receptors responsible for inhibition of the slow IPSPs in S-type uniaxonal neurons in the submucous plexus. Application of galanin-(1–16) (1–1000 nM) resulted in concentration-dependent suppression of stimulus-evoked slow IPSPs with an IC_{50} of 28.6 ± 0.7 nM in seven neurons (Fig. 1A,C). GALP (30–1000 nM) suppressed the slow IPSPs in 9 of 13 neurons with an IC_{50} of 111.6 ± 9.8 nM, which made it less potent than galanin and galanin-(1–16) (Fig. 1A,C). Impalements were sustained in four neurons for periods of time sufficient for application of 300 nM galanin, 300 nM galanin-(1–16) and 300 nM GALP to each of the neurons. The rank order of potency was galanin>galanin-(1–16)>GALP. Galanin suppressed the IPSPs by 93.8 ± 7.2%; galanin-(1–16) by 89.2 ± 6.5%; GALP by 67.0 ± 9.9% (Fig. 1A, Fig. 2). Moreover, galanin and galanin-(1–16) suppressed the slow IPSPs in each of the four neurons that failed to respond to GALP (data not shown). Exposure to [D-Trp^2]galanin, which is a galanin GAL2 receptor agonist, did not alter the slow IPSPs when applied in concentrations up to 1 μM (Fig. 1A,C).

C7, M15, M32, M35 and M40 are substances reported to be antagonists for the actions of galanin in various regions in the brain and spinal cord and on pancreatic islet cells (Bartfai et al., 1991, 1993; Crawley et al., 1993; Gregersen et al., 1993; Xu et al., 1995). Nevertheless, others have reported that these chimeric peptides behaved as agonists rather than antagonists (e.g., Gu et al., 1993; Heuillet et al., 1994; Kinney et al., 1998).

We tested each of the putative antagonists and found that all behaved like galanin receptor agonists as reflected by suppression of slow IPSPs. Suppression of the IPSPs began within 1–2 min after entry of the chimeric peptide into the tissue chamber. Maximum inhibition was observed within 3–4 min and washout for periods of 8–10 min were sufficient for complete recovery prior to application of a different peptide. Application of C7 (300 nM) caused a reduction (60.8 ± 8.6%) of slow IPSPs in 10 of 12 cases examined, as did M15 (300 nM) (65.4 ± 7.7%; 8 of 11), M32 (300 nM) (82.6 ± 5.2%; 5 of 5), M35 (300 nM) (52.8 ± 4.0%; 8 of 9) and M40 (300 nM) (50.6 ± 6.3%; 6 of 8) (Fig. 2). Thus, in the majority of the experiments, application of these putative “antagonist” peptides resulted in a suppression of slow IPSPs, although the magnitude of suppression was less than that observed with galanin. The chimeric peptides therefore behaved like agonists rather than antagonists at receptors on submucosal neurons. They

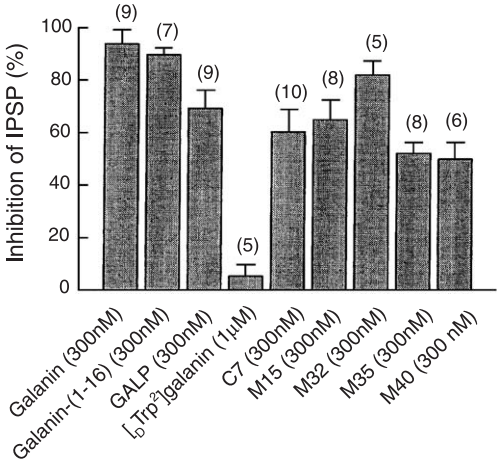

Fig. 2. Degree of inhibition of noradrenergic slow IPSPs by galanin analogs. The numbers of neurons from which data for each compound were obtained are given in parentheses.

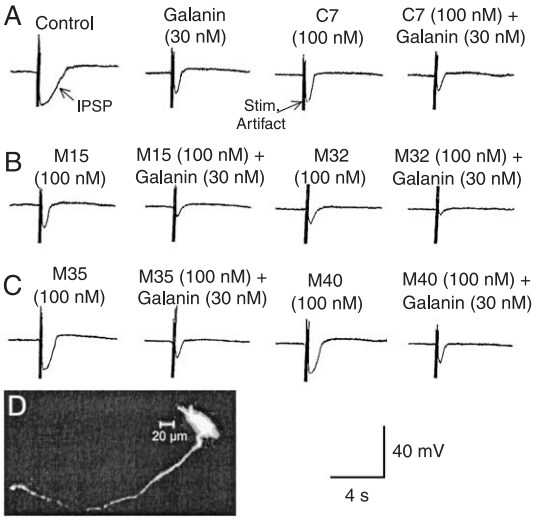

Fig. 3. Actions of chimeric peptides C7, M15, M32, M35 and M40 on stimulus-evoked slow noradrenergic IPSPs. (A) Application of galanin, C7 or C7 and galanin in combination suppressed the noradrenergic slow IPSPs. (B) M15 or M15 and galanin in combination suppressed the IPSPs. Likewise, M32 or M32 and galanin in combination suppressed the IPSPs. (C) M35 or M35 and galanin in combination suppressed the IPSPs. Likewise, M40 or M40 and galanin in combination suppressed the IPSPs. The action for each of the chimeric peptides and galanin were additive when applied in combination. (D) Morphology of the neuron from which the results in A–C were obtained.

never suppressed the actions of galanin on the slow IPSPs. Instead, application of galanin in the presence of one or the other of the chimeric peptides usually resulted in a further depression of slow IPSPs. Application of galanin (30 nM) alone produced 50.6 ± 10.3% depression of slow IPSPs ($n = 5$). In the presence of 100 nM C7, application of 30 nM galanin suppressed the slow IPSPs by 62.3 ± 9.5% in four of five neurons. Similar results were obtained with: (1) 100 nM M15 where the IPSPs were suppressed by 65.9 ± 12.4% in four of five neurons; (2) 100 nM M32 where the IPSPs were suppressed by 80.3 ± 11.6% in five of five neurons; (3) 100 nM M35 where the IPSPs were suppressed by 59.7 ± 13.5% in three of five neurons; (4) 100 nM M40 where the IPSPs were suppressed by 55.4 ± 12.7% in three of five neurons (Fig. 3).

Micropressure "puffs" of norepinephrine onto the cell somas evoked IPSP-like hyperpolarizing responses (Fig. 1B). Neither galanin nor galanin peptides altered the hyperpolarizing responses evoked by norepinephrine (Fig. 1B). In the absence of galanin, the mean amplitude of the hyperpolarizing responses evoked by 20-ms "puffs" of 1 μM norepinephrine was 28.4 ± 1.5 mV; in the presence of 300 nM galanin, the mean amplitude of the hyperpolarizing responses was 27.9 ± 1.2 mV ($P > 0.05$; $n = 5$).

3.3. Fast EPSPs

Electrical stimulation of interganglionic connectives with single stimulus pulses evoked fast EPSPs characteristic of well-documented nicotinic EPSPs known to occur in enteric neurons (Nishi and North, 1973; Hirst et al., 1974). Superfusion of galanin (3–1000 nM) reversibly suppressed the amplitude of the nicotinic fast EPSPs in 44 of 49 S-type and 2 of 2 AH-type submucous neurons. The inhibitory action of galanin was concentration dependent with an IC_{50} of 30.8 ± 1.6 nM (Fig. 4A,D). The time course of action was essentially the same as described above for the suppression of slow IPSPs. Galanin-(1–16) and GALP mimicked the inhibitory effects of galanin with IC_{50s} of 45.1 ± 3.4 nM and 131.3 ± 15.6 nM, respectively. No suppression of the fast EPSPs was seen with [D-Trp^2]galanin in a concentration range of 1–1000 nM (Fig. 4A,D).

The chimeric peptides C7, M15, M32, M35 and M40, with each one applied in the 300 nM concentration that suppressed stimulus-evoked slow IPSPs, also suppressed the

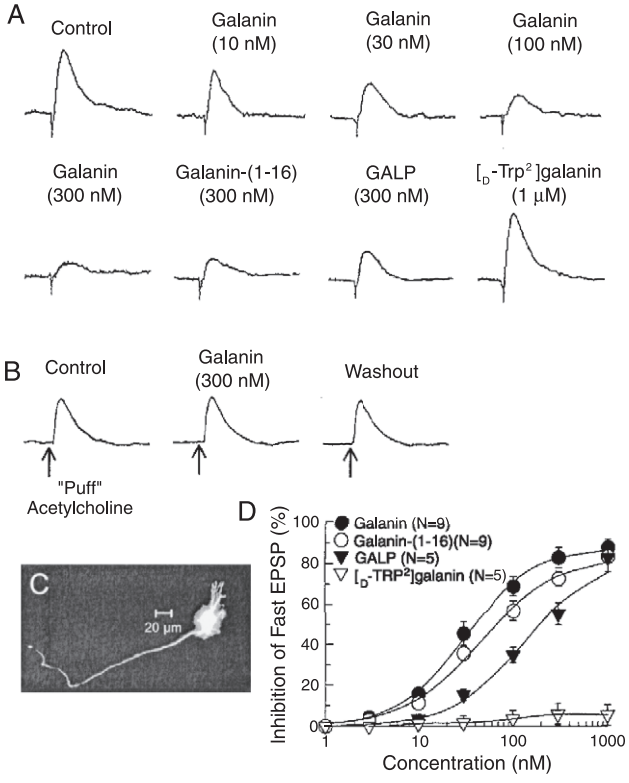

Fig. 4. Presynaptic inhibitory action of galanin and galanin analogs on nicotinic EPSPs in submucous plexus neurons with uniaxonal morphology and S-type electrophysiologic behavior. (A) Nicotinic EPSPs evoked by single-stimulus pulses applied to an interganglionic fiber tract were suppressed progressively by increasing concentrations of galanin from 10 to 300 nM. The inhibitory action of galanin was mimicked by galanin-(1–16) and GALP, but not [D-Trp^2]galanin. (B) Microejection ("puff") of 1 μM acetylcholine onto the cell soma evoked an EPSP-like depolarizing response in the same neuron as A. Presence of 300 nM galanin in the bathing solution did not suppress depolarizing responses to micropressure application of acetylcholine. (C) Morphology of the neuron from which the results in A–B were obtained. (D) Concentration–response curves for suppression of nicotinic EPSPs by galanin, galanin-(1–16), GALP and [D-Trp^2]galanin. The IC_{50}'s for galanin, galanin-(1–16) and GALP were 30.8 ± 1.6, 45.1 ± 3.4 and 131.3 ± 15.6 nM, respectively.

stimulus-evoked fast EPSPs. Application of C7 alone suppressed the mean amplitude of fast EPSPs by 58.1 ± 6.8% in 7 of 11 neurons. Likewise, M15 suppressed the fast EPSPs by 59.6 ± 5.6% in 7 of 9 neurons, M32 suppressed the fast EPSPs by 76.1 ± 5.8% in 8 of 8 neurons, M35 suppressed the fast EPSPs by 52.2 ± 7.9% in 6 of 8 neurons and M40 suppressed the EPSPs by 49.7 ± 5.3% in 6 of 10 neurons (Fig. 5). Co-application of galanin with one or the other of the chimeric peptides resulted in a further suppression of the fast EPSPs (Fig. 6). Application of 100 nM galanin alone suppressed the fast EPSPs by 60.8 ± 95% in 5 neurons. In the presence of 100 nM C7, application of 100 nM galanin suppressed the fast EPSPs by 69.3 ± 12% in 3 of 5 neurons. Similar results were obtained with: (1) 100 nM M15 where the EPSPs were suppressed by 71.2 ± 13.7% in 4 of 5 neurons; (2) 100 nM M32 where the IPSPs were suppressed by 79.6 ± 16.8% in 5 of 5 neurons; (3) 100 nM M35 where the EPSPs were suppressed by 68.3 ± 12.4% in 4 of 5 neurons; (4) 100 nM M40 where the EPSPs were suppressed by 65.6 ± 11.3% in 3 of 5 neurons (Fig. 6).

Micropressure "puffs" of acetylcholine onto the cell somas evoked hexamethonium-sensitive fast EPSP-like depolarizing responses (Fig. 4B). Neither galanin nor galanin peptides altered the depolarizing responses evoked by acetylcholine (Fig. 4B). In the absence of galanin, the mean amplitude of the depolarizing responses evoked by "puffs" of acetylcholine was 17.2 ± 1.5 mV; in the presence of 300 nM galanin, the mean amplitude of the depolarizing responses was 16.8 ± 2.3 mV ($P > 0.05$; $n = 6$ neurons). The results were essentially the same when 300 nM galanin-(1–16) or 1-μM GALP replaced galanin in studies with 4 neurons.

3.4. Slow EPSPs

Characteristic enteric slow EPSPs (Katayama and North, 1978; Wood and Mayer, 1978) were recorded in 26 of 35 S-type and 8 of 14 AH-type submucosal neurons. The EPSPs ranged in amplitude from 5 to 16 mV and were 5 to 50 s in duration. Application of galanin, galanin-(1–16) or GALP in concentrations as high as 1 μM failed to change the amplitude or duration of the stimulus-evoked slow EPSPs in any of 26 S-type neurons (Fig. 7A,B). In situations where both a slow IPSP and a slow EPSP occurred in the same S-type neuron, galanin and galanin peptides suppressed only the slow IPSP (Fig. 7B). Exposure to 100 nM galanin suppressed or abolished the stimulus-evoked slow EPSPs in each of the eight AH-type neurons studied. The mean slow EPSP amplitude was reduced by 84.2 ± 8.1% coincident with membrane hyperpolarization amounting to 10.6 ± 1.3 mV (Fig. 7C). Galanin-(1–16) and GALP mimicked the actions of galanin to suppress the slow EPSPs and evoke hyperpolarization of the membrane potential in the AH-type neurons. Galanin-(1–16) (100 nM) suppressed slow EPSP amplitude by 82.7 ± 10.5% coincident with membrane hyperpolarization of 9.5 ± 1.4 mV in five AH-type neurons. GALP (1 μM) suppressed slow EPSP amplitude by 62.5 ± 9.9% coincident with membrane hyperpolarization of 6.4 ± 1.2 mV in five AH-type neurons. [D-Trp^2]galanin (1 μM) did not suppress the slow EPSPs in AH-type neurons nor did it hyperpolarize the membrane potential (not shown).

Substance P is a putative mediator of slow EPSPs in the enteric nervous system (Katayama and North, 1978; Surprenant, 1984). Application of 30 nM substance P in the superfusion solution evoked membrane depolarization amounting to 10.3 ± 1.9 mV in five AH-type neurons.

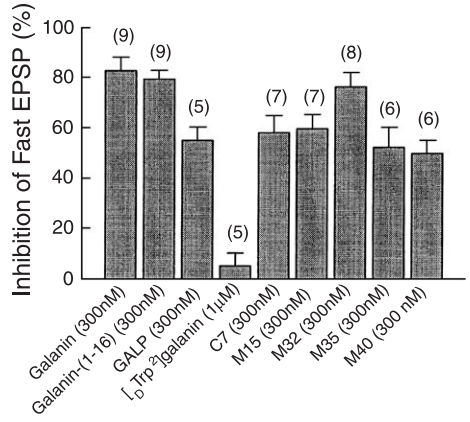

Fig. 5. Degree of inhibition of nicotinic EPSPs by galanin analogs. The numbers of neurons from which data for each compound were obtained are given in parentheses.

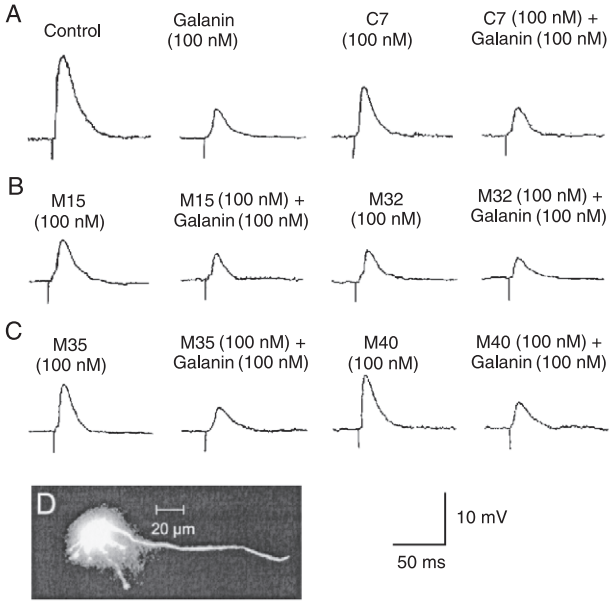

Fig. 6. Actions of chimeric peptides C7, M15, M32, M35 and M40 on stimulus-evoked nicotinic EPSPs. (A) Application of galanin, C7 or C7 and galanin in combination suppressed the EPSPs. (B) M15 or M15 and galanin in combination suppressed the EPSPs. Likewise, M32 or M32 and galanin in combination suppressed the EPSPs. (C) M35 or M35 and galanin in combination suppressed the EPSPs. Likewise, M40 or M40 and galanin in combination suppressed the EPSPs. The action for each of the chimeric peptides and galanin were additive when applied in combination. (D) Morphology of the neuron from which the results in A–C were obtained.

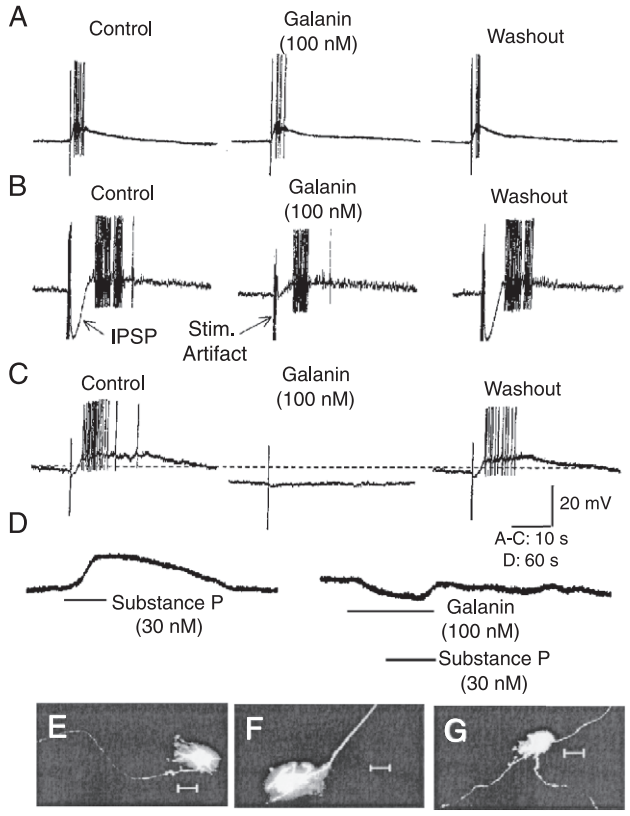

Fig. 7. Action of galanin on slow EPSPs. (A) Repetitive electrical stimulation applied to an interganglionic fiber tract evoked a slow EPSP following fast EPSPs (fast EPSPs not readily apparent due to slowed time base) in an S-type submucous neuron. The slow EPSP was not altered during exposure to galanin. (B) Repetitive electrical stimulation applied to an interganglionic fiber tract evoked a typical triphasic response consisting of initial fast EPSPs followed by a slow IPSP and a slow EPSP in an S-type submucous neuron. Introduction of galanin into the bathing solution reversibly abolished the slow IPSP while the slow EPSP remained unaffected. (C) Repetitive electrical stimulation applied to interganglionic fiber tract evoked a slow EPSP in an AH-type submucous neuron. The membrane potential was reversibly hyperpolarized and the slow EPSP was abolished reversibly during exposure to galanin. (D) Application of the slow EPSP mimetic substance P evoked a slow EPSP-like response in the same neuron as in C. The slow EPSP-like response to substance P was also suppressed in the presence of galanin. (E) Morphology of the S-type neuron from which results in A were obtained. (F) Morphology of the S-type neuron from which results in B were obtained. (G) Morphology of the AH neuron from which data in C and D were obtained.

Pre- or co-application of 100 nM galanin with 30 nM substance P reduced the depolarizing response to substance P to 2.4 ± 0.4 mV in the five neurons (Fig. 7D).

4. Discussion

4.1. Presynaptic inhibition

Slow IPSPs evoked by release of norepinephrine from sympathetic postganglionic axons in the submucous plexus are a unique property of secretomotor neurons that display S-type electrophysiologic behavior and uniaxonal morphology (Bornstein et al., 1988). Firing of secretomotor neurons releases vasoactive intestinal peptide and/or acetylcholine, which act on epithelial cells of the crypts of Lieberkühn to stimulate mucosal secretion (Cooke, 2000). Activation of sympathetic input to the bowel during physical exercise shunts blood from the splanchnic to systemic circulation during which time intestinal secretion is not advantageous. Noradrenergic inhibitory input to secretomotor neurons is a compensatory mechanism that suppresses secretion in concert with reduction in intestinal blood flow.

Presynaptic inhibition of norepinephrine release from sympathetic postganglionic fibers in the submucous plexus removes sympathetic braking action from the secretomotor neurons. Presynaptic inhibitory functions of this nature are common in the enteric nervous system. For example, histamine released from enteric mast cells during allergic responses to sensitizing antigens acts at presynaptic histamine H_3 receptors to suppress the release of norepinephrine and thereby suppress slow IPSPs in the neurons (Liu et al., 2003). This in concert with stimulation of histamine H_2 receptors expressed by the cell bodies of secretomotor neurons leads to secretory diarrhea. Our results suggest that galanin can be added to the list of enteric mediators that act presynaptically to suppress the release of norepinephrine at inhibitory synapses on secretomotor neurons in the submucosal plexus.

Criteria for presynaptic inhibition in the enteric nervous system are firmly established (see North et al., 1980; Tamura et al., 1987, 1988a). To satisfy the criteria, the mediator in question must suppress stimulus evoked synaptic potentials and at the same time not alter mimicry of the synaptic potential by application of the neurotransmitter responsible for the synaptic potential in the same neuron. Galanin met the criteria by suppressing stimulus-evoked noradrenergic IPSPs while slow IPSP-like responses to norepinephrine were unaffected by the presence of galanin.

Presynaptic inhibition of acetylcholine release by galanin at nicotinic synapses in the myenteric plexus of guinea pig small intestine was reported earlier (Tamura et al., 1987). We found that galanin action on nicotinic neurotransmission was the same in the submucous plexus as in the myenteric plexus. The physiologic significance of this braking action of galanin on fast nicotinic transmission in the integrative microcircuits of the enteric nervous system is not entirely clear. Nevertheless, suppression of transmission at the multitude of fast nicotinic synapses that make-up enteric neural networks would be expected to deenergize the network and thereby alter its output to the intestinal effector systems (i.e., secretory glands, blood vasculature and the musculature).

Lack of availability of effective pharmacologic tools prevented unequivocal identification of the galanin receptor subtype responsible for presynaptic inhibition of the release of acetylcholine and norepinephrine. One or more of the three cloned galanin receptors (i.e., GAL1, GAL2 and GAL3) could possibly be expressed at the presynaptic terminals. Unfortunately, all available antagonists for the

galanin receptor are peptide derivatives of galanin itself and not subtype selective. During the course of the study, we never learned of availability of any selective nonpeptide galanin receptor antagonists. Nevertheless, data on the relative potency of galanin and its analogs can offer clues to the identity of the galanin receptor subtypes that may be involved. Others found that galanin had high affinity for all three receptor subtypes and galanin-(1–16) had high affinity for galanin GAL1 and GAL receptors, but low affinity for galanin GAL3 receptors (Branchek et al., 2000; Howard et al., 1997; Sullivan et al., 1997). The modified galanin peptide [D-Trp^2]galanin was reported to have significant selectivity for galanin GAL2 over GAL1 and GAL3 receptors (Branchek et al., 2000; Smith et al., 1997). GALP, a recently isolated 60 amino acid galanin-like peptide, also displayed high affinity for the galanin GAL2 receptor over the galinin GAL1 receptor (Ohtaki et al., 1999). Binding and pharmacological studies reported by Ohtaki et al. (1999) found high affinity binding of galanin at the GAL1 receptor with binding of GALP being 44-fold less potent. GALP and galanin were similar in having high affinity for the galinin GAL2 receptor in the Ohtaki et al. (1999) study. We found that galanin and galanin-(1–16) suppressed slow noradrenergic IPSPs and fast nicotinic EPSPs with similar IC_{50s}, while [D-Trp^2]galanin was inactive. GALP mimicked the inhibitory actions of galanin and galanin-(1–16); nevertheless, with significantly lower potency relative to that of galanin and galanin-(1–16). Two observations support the galanin GAL1 rather than the galanin GAL2 receptor subtype as the mediator for presynaptic inhibition at sympathetic and cholinergic nerve terminals in the submucous plexus. One observation was the absence of action of [D-Trp^2]galanin, which is an acknowledged galanin GAL2 receptor agonist. The second was an apparent rank order of potency galanin = galanin-(1–16)>GALP. On the other hand, our results cannot exclude the possibility for expression of the galinin GAL3 receptor by noradrenergic and cholinergic axons in the submucous plexus. Availability of effective pharmacologic tools in the form of a selective galanin GAL3 receptor agonist and antagonist will be necessary for resolution of this question.

The chimeric peptides C7, M15, M32, M35 and M40 held promise as effective tools for identification of the galanin receptor subtype/s involved in the presynaptic actions of galanin in the submucous plexus based on earlier reports that they were effective antagonists at galanin receptors in other systems (Leibowitz and Kim, 1992; Corwin et al., 1993; Crawley et al., 1993; Bartfai et al., 1991, 1993). Nevertheless, our experience with the use of chimeric peptide fragments as galanin receptor antagonists was similar to an earlier experience in which peptide fragments of substance P were reported to be selective antagonists at substance P receptors on enteric neurons. In neither case did the peptide fragments behave as selective antagonists. They behaved instead as agonists (Nemeth et al., 1983; Surprenant et al., 1987). The agonist activity of the chimeric peptides was notably weaker than galanin, which was similar to reports of others (e.g., Gu et al., 1993; Heuillet et al., 1994; Kinney et al., 1998).

4.2. Postsynaptic inhibition

Postsynaptic actions of galanin at the cell somas of submucous neurons were inhibitory and occurred in two forms. One form was hyperpolarization of the membrane potential coincident with an increase in membrane ionic conductance that was reflected by decreased neuronal input resistance. The second was suppression of stimulus-evoked slow EPSPs. Both postsynaptic inhibitory actions of galanin were restricted to neurons with AH-type electrophysiologic behavior and multipolar Dogiel Type II morphology. Receptors for galanin appeared not to be expressed on the surfaces of the cell somas of submucous neurons with S-type electrophysiologic behavior and uniaxonal morphology, many of which are secretomotor neurons. This is another example of the complexities inherent in the enteric nervous system.

Two lines of evidence suggested that the inhibitory action of galanin on the slow EPSP was mediated by receptors located postsynaptically on the somal membranes of the neurons. The first was that suppression of the slow EPSPs by galanin was always associated with membrane hyperpolarization and decreased input resistance. A second line of evidence for a postsynaptic site of action was suppression of the slow EPSP-like responses to experimental application of substance P, which is one of the putative mediators for the slow EPSPs in the submucous plexus (Surprenant, 1984).

Our study did not address the underlying mechanisms responsible for the postsynaptic actions. Nevertheless, decreased conductance for K^+ is known to underlie the depolarization phase of the slow EPSP in AH-type neurons (for a review, see Wood, 1994a). Opening of inwardly rectifying K^+ channels, which clamps the membrane potential near the K^+ equilibrium potential of $\simeq$ 90 mV, appears to underlie the hyperpolarizing action of galanin on AH-type enteric neurons (Ren et al., 2001). This change in K^+ conductance is opposite to the change associated with slow EPSPs and the actions of substance P in AH neurons. By counteracting the conductance changes necessary for the slow EPSP, they may account for galanin-mediated postsynaptic inhibition in AH neurons.

The previously known scarcity of AH-type neurons in the submucous plexus (Mihara, 1993) added to the complications mentioned above for pharmacologic efforts to identify the receptor subtype responsible for galanin-mediated postsynaptic inhibition. The small numbers of AH neurons encountered in the submucous plexus prohibited acquisition of potency profiles for galanin and its analogs. Nevertheless, by evaluating the responses to single concentrations in the same neurons, we found that galanin and galanin-(1–16) were essentially equipotent in suppression of slow EPSPs and their hyperpolarizing actions, while GALP was less

potent. This resembles the pharmacologic profile obtained for the presynaptic actions of galanin and implicates the galanin GAL1 receptor as the mediator of postsynaptic inhibitory actions. Absence of any action for [D-Trp^2] galanin appeared to rule out a role for the galanin GAL2 receptor. Whether the galanin GAL3 receptor is involved in the postsynaptic inhibitory actions of galanin remains to be clarified.

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