Galanin inhibits glucagon-like peptide-1 secretion through pertussis toxin-sensitive G protein and ATP-dependent potassium channels in rat ileal L-cells

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Abstract

The neuropeptide galanin is widely distributed in the gastrointestinal tract and exerts several inhibitory effects, especially on intestinal motility and on insulin release from pancreatic β -cells. The presence of galanin fibres not only in the myenteric and submucosal plexus but also in the mucosa, prompted us to investigate the regulatory role of galanin, and its mechanism of action, on the secretion of the insulinotropic hormone glucagon-like peptide-1 (GLP-1). Rat ileal cells were dispersed through mechanical vibration followed by moderate exposure to hyaluronidase, DNase I and EDTA, and enriched for L-cells by counterflow elutriation. A 6- to 7-fold enrichment in GLP-1 cell content was registered after elutriation, as compared with the crude cell preparation (929 ± 81 vs 138 ± 14 fmol/ 10^6 cells). L-cells then accounted for 4–5% of the total cell population. Bombesin induced a time- (15–240 min) and dose- (0.1 nM–1 μ M) dependent release of GLP-1. Glucose-dependent insulinotropic

peptide (GIP, 100 nM), forskolin (10 μ M) and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 μ M) each stimulated GLP-1 secretion over a 1-h incubation period. Galanin (0.01–100 nM) induced a dose-dependent inhibition of bombesin- and of GIP-stimulated GLP-1 release (mean inhibition of 90% with 100 nM galanin). Galanin also dose-dependently inhibited forskolin-induced GLP-1 secretion (74% of inhibition with 100 nM galanin), but not TPA-stimulated hormone release. Pre-treatment of cells with 200 ng/ml pertussis toxin for 3 h, or incubation with the ATP-sensitive K⁺ channel blocker disopyramide (200 μ M), prevented the inhibition by galanin of bombesin- and GIP-stimulated GLP-1 secretion. These studies indicate that intestinal secretion of GLP-1 is negatively controlled by galanin, that acts through receptors coupled to pertussis toxin-sensitive G protein and involves ATP-dependent K⁺ channels.

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Introduction

Endocrine intestinal L-cells, mainly located in the distal small intestine and in the colon, synthesize proglucagon-derived peptides including glicentin, oxyntomodulin, glucagon-like peptide-1(7–36)NH₂ (GLP-1) and GLP-2. Glicentin and oxyntomodulin both constitute the so-called enteroglucagon-like immunoreactivity (GLI). GLP-1 is the most potent known incretin, stimulating glucose-dependent insulin secretion *in vivo* and in a variety of experimental systems (for reviews, see Holst 1994, Thorens 1995). Furthermore, GLP-1 has been shown to suppress glucagon release from the pancreas, to inhibit gastric secretion, to enhance the sensitivity of pancreatic B-cells to glucose and to participate in the central regulation of feeding. Due to its glucose-dependent insulinot-

tropic effects, GLP-1 has been proposed as a potential therapeutic agent in the treatment of Type II diabetes (Gutniak *et al.* 1994, 1996).

Various experimental approaches have been used to examine the regulation of intestinal L-cells. On the one hand, several luminal factors including glucose, bile salts and dietary fibres are potent stimulants of GLP-1 secretion (Roberge & Brubaker 1991, Reimer & McBurney 1996). On the other hand, several regulatory peptides including glucose-dependent insulinotropic peptide (GIP), peptides of the bombesin/gastrin-releasing peptide (GRP) family, calcitonin-gene related peptide (CGRP), as well as cholinergic agonists were shown to elicit GLP-1 secretion *in vivo* and *ex vivo* in a model of isolated vascularly perfused intestinal loops (Plaisancié *et al.* 1994, Dumoulin *et al.* 1995, Herrmann-Rinke *et al.* 1995). The establishment of

in vitro models, based on the use of short-term cultures of dispersed foetal rat intestinal cells or of rat dispersed intestinal cells enriched in L-cells, led to results supporting these observations (Brubaker 1988, Brubaker & Vranic 1987, Saïfia *et al.* 1994, Huang & Brubaker 1995).

Apart from the reported action of somatostatin on GLI peptide secretion (Sagor *et al.* 1985, Brubaker 1991), little is known about the intestinal inhibitory influences that reduce the postprandial discharge of GLP-1. Galanin, a 29-amino acid neuropeptide originally isolated from porcine small intestine, is widely distributed in the gastrointestinal tract. Galanin-like immunoreactivity is found in nerve cell bodies and fibres of the myenteric plexus and submucosal plexus (Rattan 1991). Galanin participates in the regulation of intestinal motility and inhibits pancreatic exocrine secretion and insulin release from B-cells (Crawley 1995). It was also shown, *in vivo* and *ex vivo* with the isolated vascularly perfused rat ileal model, that galanin reduced the secretory activity of L-cells (Bauer *et al.* 1989, Herrmann-Rinke *et al.* 1996). Since intestinal endocrine cells are under the influence of mediators released from the enteric nervous system, a model of endocrine cells, uncoupled from the underlying lamina propria, is required for further identification of signal transduction pathways put into play by regulatory factors that directly modulate hormone release.

For this purpose, we developed an *in vitro* model of rat dispersed ileal cells enriched in L-cells by counterflow elutriation, to examine the effect of galanin on the secretion of GLP-1. We here report that galanin potently inhibits forskolin-, bombesin- and GIP-stimulated GLP-1 secretion. These effects involve a pertussis toxin-sensitive G protein and ATP-dependent K^+ channels.

Materials and Methods

Materials

The following solutions were used: Hank's balanced salt solution (HBSS, Sigma Chemical Co., Saint Quentin Fallavier, France) supplemented with NaHCO_3 4 mM, Hepes 10 mM, streptomycin sulphate (1 g/l), penicillin G (160 000 IU/l) and 0.1% bovine serum albumin (BSA), pH 7.4; basal medium Eagle (BME, Life Technologies, Cergy Pontoise, France) supplemented with NaHCO_3 26 mM, Hepes 10 mM, glutamine 2 mM, BSA 0.1% and containing 100 mg/l hyaluronidase and 10 mg/l DNase I (Boehringer, Mannheim, Germany). The pH was adjusted to 7.4; Earle's balanced salt solution (EBSS, Life Technologies) supplemented with NaHCO_3 26 mM, Hepes 10 mM, glutamine 2 mM, BSA 0.1% and ethylene diaminetetraacetic acid (EDTA) 1 mM, pH 7.4; HAM/F-12 medium (Life Technologies) supplemented with NaHCO_3 12 mM, Hepes 20 mM, glutamine 2 mM and BSA 0.1%, pH 7.4.

Bombesin, GIP, rat galanin, GLP-1(1–36)amide, GLP-1(7–36)amide, GLP-1(1–37), GLP-1(7–37), forskolin, 12–O-tetradecanoylphorbol-13-acetate (TPA), 4- α phorbol, pertussis toxin and disopyramide were obtained from Sigma. Fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G was obtained from Nordic (Tilburg, The Netherlands). All other reagents were of analytical grade.

Cell isolation

Male Wistar rats (200–250 g) were killed by cervical dislocation and the abdomens were quickly opened. The distal part of the small intestine, about 30 cm long, was removed, washed first in saline at 4 °C then in HBSS (without BSA), and incubated at 37 °C for 15 min under a continuous 95% O_2 –5% CO_2 flow. After this equilibrium period, the ileum was everted on a glass spiral (diameter 3 mm) and connected to a vibro-mixer (Vibro Mischer, Chemap, Switzerland). The intestinal segment was immersed in 120 ml HBSS buffer and vertically vibrated for 5 min (60 vibrations/s, 2 mm amplitude). The resulting cell medium, containing mainly villous cells and mucus, was discarded, and the ileum was again vibrated for 15 min at 120 vibrations/s (amplitude 2 mm). The cell suspension (aggregates of 5–15 cells) was centrifuged (100 g for 5 min at 20 °C) and incubated in a shaker for 5 min at 20 °C with EBSS. After washing, the cells were again incubated for 5 min at 20 °C in BME containing 100 mg/l hyaluronidase and 10 mg/l DNase I. After two washing steps in HBSS and filtration through 250 and 75 μm nylon meshes, the final cell suspension mainly consisted of single cells. Cell number was estimated with a haematometer and cell viability was assessed by 0.05% trypan blue exclusion.

Cell fractionation

Dispersed ileal cells were separated by counterflow elutriation according to their sedimentation velocities with a Beckman elutriator rotor (model JE-5-0, Beckman Instruments, Los Angeles, CA, USA) run in a centrifuge (model J-6M/E, Beckman). A peristaltic pump (Masterflex, Chicago, IL, USA) was used to load the cell suspension (200×10^6 cells/elutriation) into the separation chamber. Five different cell fractions (100 ml each) were collected at the following combinations of flow rate (ml/min) and rotor velocities (revolutions/min): 1:25 and 2500; 2:30 and 2200; 3:45 and 2200; 4:55 and 2000; 5:100 and 2000. Elutriated fractions were centrifuged (100 g for 5 min at 20 °C) and the cell pellets were homogenized in 1 ml extraction medium (2 M acetic acid+20 mM HCl) at 4 °C and then sonicated. The homogenate was placed in a heating block for 10 min at 100 °C, neutralized with 2.5 M NH_4OH and stored at –20 °C until radioimmunoassay (RIA). The elutriated fractions containing

maximum GLP-1 concentrations were pooled, centrifuged (100 *g* for 5 min at 20 °C), resuspended in complete HAM/F12 at a concentration of 10⁶ cells/ml and immediately used for release studies.

Peptide release

Test tubes containing 0.9 × 10⁶ native cells were incubated at 37 °C under a continuous 95% O₂–5% CO₂ flow in an orbital shaker for the indicated time, with or without the tested agents. The cell suspensions were then centrifuged and the supernatants were immediately frozen at –20 °C for RIA. Cell pellets from both unstimulated and stimulated conditions were treated as described above for GLP-1 determination.

Peptide assays

GLP-1 concentrations were measured by RIA as previously described (Abello *et al.* 1994, Saïfia *et al.* 1994). Gel filtration on a 5 × 100 cm Sephadex G-50 column revealed in elutriated cells and in the release medium the presence of a single peak corresponding to the position of synthetic GLP-1(7–36)amide and GLP-1(1–36)amide, which were indistinguishable in the present system. Peptide YY (PYY), somatostatin and neurotensin were measured as previously described (Chayvialle *et al.* 1978, Cuber *et al.* 1990, Dumoulin *et al.* 1995).

Histological analysis

Freshly removed, full-thickness, ileal wall samples were immersed in an ice-cold PBS solution containing 0.4% *p*-benzoquinone for 2 h at 4 °C. They were repeatedly rinsed in PBS containing 10% sucrose at 4 °C and frozen in liquid nitrogen. Adjacent sections were sliced with a Bright cryostat. Galanin and GLP-1 immunoreactivities were detected with the indirect immunofluorescence technique of Coons *et al.* (1955). Antiserum 51162C raised in rabbit against synthetic porcine galanin (Philippe *et al.* 1990) and antiserum 199D were diluted (1:100) in PBS containing 3% Triton X-100 and 5% BSA (pH 7.4) for 1 h in a humid chamber at room temperature. After three washing steps with PBS, sections were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated antirabbit immunoglobulin G diluted 1:100 in PBS. After washing, sections were mounted in a non-aqueous medium (Fluorep, Biomérieux, Marcy l'Etoile, France) and examined with a Leitz fluorescence microscope. To test the specificity of the immunostaining, sections were incubated with antisera 51162C or 199D that had been preadsorbed with galanin and GLP-1 (1 µmol/ml undiluted antiserum) respectively.

Microscopic slides of cell suspensions (1 × 10⁵ cells) obtained after elutriation were prepared using a cyto-centrifuge (Shandon Southern Instruments, Sewickley,

PA, USA). Slides were fixed in acetone for 10 min at 4 °C and washed three times with PBS, pH 7.4. GLP-1 immunoreactivity-containing cells were visualized by indirect immunofluorescence using the antiserum 199D. For control purposes, cells were incubated with GLP-1 antiserum preabsorbed with GLP-1 (1 µmol/ml undiluted antiserum). Cells were also visualized by alkaline phosphatase stain with the fast red stain Cadenzatags kit (Shandon Southern Instruments).

Data analysis

Peptide release (mean ± S.E.M.) was expressed for control and treated cells as fmol/10⁶ cells. Statistical analysis was performed by analysis of variance (ANOVA), followed by Student's *t*-test for comparisons of means. Differences were considered significant at *P* < 0.05.

Results

Immunofluorescence of galanin nerve fibres in ileal mucosa

Galanin immunoreactive fibres could be identified using the antiserum 51162C in the rat ileal mucosa (Fig. 1*a*). Similarly after incubation with the antiserum 199D, several GLP-1 immunoreactive cells were revealed in an adjacent ileal villous (Fig. 1*b*). No labelling was detected after incubation of antisera with homologous antigens. The distribution of galanin immunoreactive nerves in the ileum, in close apposition with the L-cells, thus suggests a possible regulatory function of the neuropeptide on the secretion of GLP-1.

Isolation of GLP-1-enriched cells

After combination of mechanical and enzymatic dispersion, the resulting cell number was 314.8 ± 27.4 10⁶ cells (mean of 31 preparations, each preparation including two rats). GLP-1 concentration was 138 ± 14 fmol/10⁶ cells (*n* = 20). Cell viability, determined by trypan blue exclusion, always exceeded 90%. Figure 2 shows the concentration of GLP-1 and the cell number in the different fractions obtained after counterflow elutriation. Fractions 3 and 4 contained the maximum amount of GLP-1 and were pooled for release studies. GLP-1 concentration in the enriched cell suspension was 929 ± 81 fmol/10⁶ cells, with a 6- to 7-fold enrichment over the crude suspension. Cell content in PYY was also significantly enriched (944 ± 99 vs 155 ± 21 fmol/10⁶ cells, *P* < 0.05). The mean apparent diameter of the GLP-1-enriched cells, as calculated from the rates of centrifugation and elutriation according to Stoke's law, was 13–19 µm. Upon counting the cells labelled at immunofluorescence (Fig. 3) or alkaline phosphatase staining, the elutriated preparation contained 4–5% of L-cells, when they represented

Figure 1 Immunofluorescence micrographs of two adjacent sections of the ileum villous in the rat after incubation with galanin antiserum 51162C (a) and GLP-1 antiserum 199D (b). (a) A galanin-like immunoreactive fibre can be observed in the villous. (b) The adjacent section shows two GLP-1 immunoreactive cells. Scale bars=25 µm.

Figure 2 Concentration of GLP-1 (in fmol/10⁶ cells, bars, left scale) and cell number (points, right scale) in the serial fractions obtained from the elutriator after cell dispersion. Fractions 3 and 4 were kept and pooled for studies on release (means $\pm$ S.E.M. of four separate experiments).

less than 1% in the initial cell suspension. Crude cell preparation also contained immunoreactive neurotensin (343 ± 48 fmol/10⁶ cells) and somatostatin (20 ± 3 fmol/10⁶ cells), that were significantly increased after elutriation (3017 ± 253 and 78 ± 26 fmol/10⁶ cells respectively, $P < 0.05$). Vasoactive intestinal polypeptide, substance P and galanin were undetectable as judged by RIA and by immunofluorescence.

Agonist-dependent release of GLP-1

The responsiveness of the enriched L-cell preparation was assessed with bombesin, which is a potent stimulant of GLP-1 secretion (Fig. 4). Bombesin (10^{-6} M) produced a time-dependent release of GLP-1 (Fig. 4, left panel), significant from 30 min on (65.6 ± 4.3 vs 40.2 ± 5.1 fmol/

10⁶ cells, $P < 0.05$). The secretion increased up to 4 h, representing 210.2 ± 16.2 fmol/10⁶ cells. Bombesin induced over a 1-h incubation period a dose-dependent (10^{-10} – 10^{-6} M) release of GLP-1 (Fig. 4, right panel), which was first significant at 10^{-8} M and maximal at 10^{-6} M (73.7 ± 4.9 fmol/10⁶ cells, $P < 0.05$). Similarly, the insulinotropic peptide GIP, at the pharmacological concentration of 10^{-7} M, and forskolin (10^{-5} M), that directly stimulates adenylate cyclase, significantly ($P < 0.05$) increased the release of GLP-1 (Table 1). A similar rise of GLP-1 secretion was also evidenced in the presence of 10^{-6} M TPA (Table 1). The inactive phorbol ester 4- α phorbol (1 µM) and the carrier solvent DMSO did not alter basal GLP-1 release (data not shown). Interestingly, none of the tested agonists affected the cellular integrity after incubation at 37 °C for 1 h, as judged from trypan blue exclusion and release of lactate dehydrogenase (data not shown), suggesting that the increase in GLP-1 found in the medium was due to an enhanced secretion rather than to leakage from damaged cells.

Galanin inhibition of GLP-1 release

The effects of galanin were investigated on basal and stimulated GLP-1 secretion (Table 1). Galanin alone had no significant effect on the amount of GLP-1 present in the cells nor on GLP-1 release over the concentrations (10^{-11} – 10^{-7} M) tested (Table 1 and data not shown). In contrast, it dose-dependently inhibited bombesin-induced GLP-1 secretion. The inhibition of GLP-1 release stimulated by 10^{-6} M bombesin was significant at 10^{-9} M galanin and maximal at 10^{-7} M, decreasing

Figure 3 Immunocytochemical localization of GLP-1 in the cells after elutriation (magnification: $\times 280$; scale bar=25 μm).

Figure 4 Time- and dose-dependent effect of bombesin (BBS) on GLP-1 release. *Left*, cells were incubated at 37 °C for serial times without (closed squares) or with (open squares) 10^{-6} M BBS. *Right*, cells were incubated at 37 °C for 1 h with or without the indicated concentrations of BBS. Results are expressed as fmol/ 10^6 cells. Data are the means of three separate experiments each performed in triplicate. *Significantly different from control values ($P<0.05$).

GLP-1 secretion to a value close to basal ($P<0.05$). Galanin also inhibited in a dose-dependent fashion (10^{-11} – 10^{-7} M) GIP-stimulated GLP-1 release. Inhibition of 10^{-7} M GIP-stimulated GLP-1 secretion was

significant ($P<0.05$) at 10^{-9} M galanin, and was maximal at 10^{-7} M. The effects on the secretion of GLP-1 induced by direct activators of cAMP production or protein kinase C are shown in Table 1. Addition of galanin

Table 1 Effect of galanin on GLP-1 secretion (in fmol/10⁶ cells) when stimulated by various agonists. Cells were incubated at 37 °C for 1 h without or with 10⁻⁶ M bombesin, 10⁻⁷ M GIP, 10⁻⁵ M forskolin or 10⁻⁶ M TPA, in the absence or presence of increasing concentrations of galanin. Results are expressed as fmol/10⁶ cells. Data are means ± S.E.M. of three separate experiments each performed in triplicate

Treatment	Galanin (M)			
	0	10 ⁻¹¹	10 ⁻⁹	10 ⁻⁷
No agonist	31.3 ± 2.1	38.7 ± 1.3	37.4 ± 2.4	36.8 ± 2.6
Bombesin (10 ⁻⁶ M)	61.1 ± 4.9*	51.4 ± 2.4	43.1 ± 2.1†	31.7 ± 2.5†
GIP (10 ⁻⁷ M)	87.4 ± 4.8*	84.5 ± 5.9	67.3 ± 5.2†	48.2 ± 9.9†
Forskolin (10 ⁻⁵ M)	84.4 ± 11.3*	65.2 ± 9.9	61.4 ± 4.3†	46.1 ± 0.3†
TPA (10 ⁻⁶ M)	76.1 ± 5.2*	74.1 ± 5.5	73.6 ± 13.7	73.9 ± 9.7

Symbols (*,†) denote significant difference from basal and agonist-stimulated GLP-1 release respectively (*P*<0.05).

(10⁻¹¹–10⁻⁷ M) inhibited the forskolin-stimulated GLP-1 secretion in a concentration-dependent manner. At the maximally active dose (10⁻⁷ M), galanin decreased the forskolin-induced GLP-1 release by 74% (*P*<0.05). On the other hand, galanin at the different concentrations tested (10⁻¹¹–10⁻⁷ M) failed to inhibit GLP-1 secretion induced by TPA.

Effect of pertussis toxin on galanin inhibition of GLP-1 release

To study the mechanisms underlying the effects of galanin on GLP-1 release, cells were pretreated for 3 h in the absence or the presence of 200 ng/ml pertussis toxin before a 1-h incubation with combinations of bombesin or GIP, and galanin (Fig. 5). In the absence of pertussis toxin, galanin (10⁻⁷ M) significantly inhibited GLP-1 release stimulated by 10⁻⁶ M bombesin (42.9 ± 2.7 vs 91.6 ± 3.4 fmol/10⁶ cells, *P*<0.05) or by 10⁻⁷ M GIP (36.5 ± 1.2 vs 89.4 ± 1.6 fmol/10⁶ cells, *P*<0.05). Pertussis toxin treatment did not significantly modify cell viability (78 vs 83% without toxin), nor basal, bombesin- or GIP-stimulated release of GLP-1. However under these conditions, galanin was ineffective in inhibiting the stimulatory action of bombesin (70.2 ± 2.5 vs 72.3 ± 1.4 fmol/10⁶ cells) and of GIP (72.2 ± 4.3 vs 81.9 ± 3.2 fmol/10⁶ cells without galanin).

Involvement of ATP-dependent K⁺ channels in the inhibitory effect of galanin

The selective blocker disopyramide was used to assess the role of ATP-sensitive K⁺ channels in the inhibitory effect of galanin on GLP-1 secretion (Fig. 6). Treatment of cells with 200 µM disopyramide preserved the bombesin- and GIP-stimulated GLP-1 secretion (80.2 ± 5.1 and 88.1 ± 4.1 fmol/10⁶ cells respectively, as compared with 37.3 ± 2.1 fmol/10⁶ cells for control, *P*<0.05). Under

these conditions, galanin only reduced by 46 and 27% the bombesin- and GIP-induced GLP-1 release respectively.

Discussion

In the present study, we developed in the adult rat a model of dispersed ileal cells, that were fractionated according to their density by counterflow elutriation, to determine the role of the enteric neuropeptide galanin on GLP-1 secretion. Our results indicate that viable mucosal cells, significantly enriched in L-cells, could be prepared from rat ileum for characterization of the cellular mechanisms of GLP-1 secretion. The release of GLP-1 was stimulated by forskolin and the phorbol ester TPA, that increase cAMP-dependent protein kinase and protein kinase C activities respectively. Moreover, exposure of cells to bombesin and GIP induced a significant increase in GLP-1 secretion. These findings are consistent with previous studies performed either *in vivo* (Roberge & Brubaker 1993, Roberge *et al.* 1996) or in the isolated perfused rat ileum and colon models (Plaisancié *et al.* 1994, Dumoulin *et al.* 1995, Herrmann-Rinke *et al.* 1995). They are also in accordance with results obtained *in vitro* using rat intestinal dispersed cells on the release of GLP-1 (Saïfia *et al.* 1994, Huang & Brubaker 1995) or GLI peptides (Brubaker 1991). In addition, the present levels of GLP-1 secretion are similar to those reported using foetal rat intestinal cells, and effects reached statistical significance at similar concentrations of agonists (Brubaker 1988, 1991, Huang & Brubaker 1995). The present system thus provides a suitable model for studies on the control of GLP-1 secretion.

Our study indicates that galanin inhibits forskolin-, bombesin- and GIP-stimulated GLP-1 secretion from isolated rat ileal cells. Considering the presence of galanin immunoreactive nerves throughout the gastrointestinal tract, with galanin fibres projecting into the mucosa (Rattan 1991), our data suggest that the neuropeptide

Figure 5 Effect of pertussis toxin on galanin inhibition of bombesin (BBS)- and GIP-stimulated GLP-1 release. Cells were preincubated at 37 °C for 3 h without or with 200 ng/ml pertussis toxin (PTX), then incubated for 1 h without or with 10^{-6} M BBS (panel A) or 10^{-7} M GIP (panel B) in the absence or presence of 10^{-7} M galanin (Gal). Results are expressed as fmol/10⁶ cells. Data are the means $\pm$ S.E.M. of three separate experiments each performed in triplicate. * and + significantly different from basal and stimulated GLP-1 release respectively ($P < 0.05$).

could be a candidate to negatively regulate the postprandial release of GLP-1. This hypothesis is consistent with previous reports that demonstrated an inhibitory effect of galanin on proglucagon-derived peptide secretion (Bauer *et al.* 1989, Herrmann-Rinke *et al.* 1996). Interestingly, galanin also exerts inhibitory effects on pancreatic insulin, glucagon and somatostatin secretions (Hramiak *et al.* 1988,

Figure 6 Effect of galanin (Gal) upon K⁺ channel blockade. Cells were preincubated at 37 °C for 5 min with 200 μ M disopyramide, then incubated for 1 h without or with 10^{-6} M bombesin (BBS, hatched bars) or 10^{-7} M GIP (dotted bars) in the absence or presence of 10^{-7} M Gal. Results are expressed as fmol/10⁶ cells. Data are the means $\pm$ S.E.M. of three separate experiments each performed in triplicate. *Significantly different from basal GLP-1 release ($P < 0.05$).

Bauer *et al.* 1989, Amiranoff *et al.* 1990). The present study also showed that galanin could counteract the stimulatory effect of adenylate cyclase activation by forskolin, which is consistent with the inhibitory action of the neuropeptide on forskolin- and GIP-stimulated cellular cAMP levels or adenylate cyclase activity in insulin- and somatostatin-secreting cell lines (Amiranoff *et al.* 1988, 1991). Furthermore, the failure of galanin to inhibit GLP-1 secretion induced by TPA suggests that the inhibitory effects of galanin do not involve protein kinase C, or that galanin is ineffective because TPA has completely desensitized the protein kinase C-dependent pathway.

Because of the heterogeneity of the present preparation of ileal cells, we cannot exclude local/paracrine influences mediating the galanin effects on GLP-1 secretion. Somatostatin is located in nerves and endocrine cells which are in the vicinity of the L-cells. As somatostatin exerts inhibitory effects on endocrine secretions (Sagor *et al.* 1985, Brubaker 1991), it could be speculated that somatostatin released from D-cells restrained GLP-1 release in our preparation. Somatostatin-like immunoreactivity was indeed detected both in crude and enriched cell preparations. However, galanin did not modify somatostatin release in basal conditions nor upon stimulation with the various agonists, thus excluding an indirect effect of galanin on GLP-1 secretion through somatostatin release. On the other hand, we

cannot completely rule out the possibility of an indirect effect of galanin via other cell types, e.g. absorptive cells since they express galanin receptors (Homaïdan *et al.* 1994).

A 3-h treatment of cells with pertussis toxin abolished the inhibitory effect of galanin on bombesin- and GIP-stimulated GLP-1 secretion, indicating that the galanin receptor is coupled to a pertussis toxin-sensitive G-protein. Similar results were reported on the secretion of insulin from the tumoral cell line RINm5F (Amiranoff *et al.* 1988, Lang *et al.* 1994) and from rat pancreatic B-cells (Nilsson *et al.* 1989). Our data are also in concordance with those obtained in the rat islet cell Rin-m and in the entero-endocrine STC-1 cell lines. In these tumour-like cells, a pertussis toxin treatment reversed the inhibitory effects of galanin on somatostatin and cholecystokinin release respectively (Amiranoff *et al.* 1990, Chang *et al.* 1995). The nature of this pertussis toxin-sensitive G-protein, i.e. G_i and/or G_o , coupled to the galanin receptor in L-cells is unknown. Recently, the galanin receptor was reported to interact with G_{ai2} , G_{ai3} and G_{ao1} , leading to inhibition of adenylate cyclase activity and of voltage-gated Ca^{2+} channels in insulin-secreting cells (Kalkbrenner *et al.* 1995, McDermott & Sharp 1995). Further investigations, using native L-cells purified to homogeneity, are needed to identify the α -subunit that mediates the inhibitory effect of galanin on GLP-1 secretion.

Previous studies indicated that galanin can act via an opening of K^+ channels (Ahren *et al.* 1989, Chang *et al.* 1995), which would hyperpolarize the membrane and thereby restrict Ca^{2+} entry via the voltage-dependent Ca^{2+} channels. A similar involvement of K^+ channels was here observed using the ATP-dependent K^+ channel blocker disopyramide, that largely counteracted the inhibitory effect of galanin on the GLP-1 secretion. K^+ channels also play a critical regulatory role in the action of somatostatin on insulin-secreting cells (De Weille *et al.* 1989, Ribalet & Eddlestone 1995). The question arises whether galanin can affect ATP-dependent K^+ channel activity directly, through interaction between the transducing α -subunit of G_i or G_o and the ionic channel with consequent increase of activity, or indirectly via diffusible messengers contributing to alterations in the phosphorylation level of the K^+ channel.

In conclusion, the results of the present study provide evidence of a role for galanin in decreasing GLP-1 release from L-cells as induced by forskolin, GIP and bombesin. Its action involves a pertussis toxin-sensitive G-protein and the activation of ATP-dependent K^+ channels.

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