Inhibition of bombesin-stimulated gastrin release from isolated canine G cells by bombesin antagonists

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This study investigated the effects of two putative bombesin antagonists, [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]substance P and [Leu[13]-Ψ-CH_2NH-Leu[14]]bombesin, on bombesin-stimulated gastrin release from isolated canine G cells following short-term culture. Canine antral tissue was dispersed by sequential collagenase and EDTA treatment, and counterflow elutriation was used to enrich for G cells. Plates were seeded with 2×10^6 cells/mL in each well and cultured for 2 days prior to testing. Gastrin-containing and somatostatin-containing cells were identified by immunocytochemistry using the biotin–avidin–peroxidase method and accounted for 8.5 and 1%, respectively, of adhered cells. Basal gastrin secretion was 1.91 ± 0.48% of total cell content. After a 2-h incubation period, bombesin (0.01–100 pM) stimulated gastrin release in a concentration-dependent fashion. The substance P analog, at a concentration of 1 μM, modestly inhibited bombesin-stimulated gastrin release from canine G cells. This analog also produced weak stimulation of basal gastrin release. In contrast, the bombesin analog, at a concentration of 1 μM, did not affect basal gastrin secretion. The bombesin analog completely blocked bombesin-stimulated gastrin release from 0.01 to 1 pM and produced greater than 50% inhibition at higher doses. The ability of the bombesin analog to directly inhibit bombesin-stimulated gastrin release from cultured canine G cells underscores its usefulness in studies involving the role of bombesin and its mammalian counterpart, gastrin-releasing peptide, in the control of gastrin cell function.

Key words: gastrin, bombesin, antagonists, cultured cells.

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Cette étude a examiné les effets de deux antagonistes putatifs de la bombésine, substance P [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]] et bombésine [Leu[12]-Ψ-CH_2NH-Leu[14]], sur la libération de gastrine stimulée par la bombésine des cellules G isolées du chien après une culture de courte durée. Le tissu antral canin a été dispersé par traitement en série à l'EDTA et à la collagénase, et les cellules G ont été enrichies par élutriation. Les plaques ont été ensemencées avec 2×10^6 cellules/mL dans chaque puits et cultivées pendant 2 jours avant le test. Les cellules contenant de la gastrine et celles contenant de la somatostatine ont été identifiées par immunocytochimie en utilisant la méthode biotine–avidine–peroxydase et ont totalisé 8,5 et 1%, respectivement, des cellules adhérentes. La sécrétion basale de gastrine a été de 1,91 ± 0,48% de la concentration cellulaire totale. Après 2 h d'incubation, la bombésine (0,01–100 pM) a stimulé la libération de gastrine en fonction de la concentration utilisée. L'analogue de la substance P, à une concentration de 1 μM, a inhibé légèrement la libération de gastrine induite par la bombésine des cellules G canines. Cet analogue a aussi provoqué une légère stimulation de la libération basale de gastrine. À l'opposé, l'analogue de la bombésine, à une concentration de 1 μM, n'a pass affecté la sécrétion basale de gastrine. L'analogue de la bombésine a complètement bloqué la libération de gastrine stimulée par la bombésine de 0,01 à 1 pM et a provoqué une inhibition de plus de 50% à des doses plus élevées. La capacité de l'analogue de la bombésine d'inhiber directement la libération de gastrine stimulée par la bombésine des cellules G canines cultivées souligne son utilité dans les études impliquant le rôle de la bombésine et de son équivalent chez les mammifères, le peptide de libération de la gastrine, dans le contrôle de la fonction celluaire de la gastrine.

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Introduction

Bombesin-like immunoreactivity has been localized in neurons in the mammalian stomach (Dockray et al. 1979). The ability of bombesin (BN) and its mammalian counterpart, gastrin-releasing peptide (GRP), to stimulate gastrin secretion in vivo and from the isolated perfused stomach preparation is well known (Bertaccini et al. 1974; DuVal et al. 1981; Martindale et al. 1982; McDonald and Fox 1984; Bunnett et al. 1985). However, a direct stimulatory role could not be attributed to BN–GRP from such studies owing to the presence of multiple paracrine, endocrine, neural, and luminal influences on the G cell. Recently, Giraud et al. (1987) and Sugano et al. (1987) have provided convincing evidence that the effect of BN–GRP on G-cell function was a direct one. These investigators used isolated canine antral G cells in primary culture to demonstrate potent receptor-mediated release of gastrin by BN–GRP.

To date, three classes of BN–GRP receptor antagonists have been described. One class behaved as substance P(SP) analogs and were also SP antagonists (Jensen et al. 1984; Jensen et al. 1988). The second class was [D-Phe[12]]BN analogs (Heinz-Erian et al. 1987; Merali et al. 1988), and the third class included BN analogs with reduced peptide bonds (Coy et al. 1988). The latter group of compounds featured the replacement of the peptide bond between amino acids with a methylamide (CH_2NH) moiety. These three classes of BN–GRP receptor antagonists have been shown to be potent inhibitors of BN-stimulated amylase

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secretion from dispersed pancreatic acini, but their effectiveness in reducing BN-evoked gastrin release remained equivocal. The antagonist [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP attenuated the gastrin response to exogenous GRP and blocked vagally mediated gastrin release in pigs (Holst et al. 1987*a*, 1987*b*). However, another SP analog, [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP (spantide) failed to alter BN-stimulated gastrin secretion from isolated canine G cells in short-term culture (Giraud et al. 1987).

In the present study, a method for the isolation, enrichment, and culture of canine antral G cells has been utilized to investigate the ability of two putative BN–GRP antagonists, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP and [Leu^{13}-Ψ-CH$_2$NH-Leu^{14}]-BN, to inhibit BN stimulation of gastrin secretion.

Materials and methods

Cell separation, enrichment, and culture

The antrum was dissected from the canine stomach and washed in Hanks' balanced salt solution (Gibco Laboratories, New York, NY) containing 0.1% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Missouri) (HBSS–BSA). The mucosa was carefully dissected from the submucosa, weighed, and minced into small fragments (about 4 mm^3) using a pair of scalpel blades. Antral tissue (8–10 g/10 mL) was exposed to an initial collagenase (type I; Sigma) digestion of 1 mg/mL in basal medium Eagle (Gibco) containing 0.1% bovine serum albumin for 15 min at 37°C. The resulting cell suspension largely comprised mucous cells and was discarded. The remaining tissue was further dispersed by sequential collagenase treatment, 1 mg/mL for 1 h then 2 mg/mL for 1 h, with each step followed by the addition of 300 μL of 0.5 M EDTA (final concentration about 3 mM; BDH Inc., Toronto, Ont.) for 15 min. The cell suspensions resulting from the second and third collagenase digests were filtered through Nitex mesh (240 μm; B. & S. H. Thompson, Scarborough, Ont.) and washed with HBSS–BSA, then with HBSS–BSA containing 0.01% dithiothreitol (Sigma) (HBSS–BSA–DTT), and resuspended in 20 mL of HBSS–BSA–DTT. Prior to elutriation, the cell suspensions were pooled and filtered through finer Nitex mesh (62 μm; B. & S. H. Thompson).

Further enrichment for G-cell content was achieved using the Beckman counterflow elutriation system, which separated cells on the basis of their sedimentation rates. The elutriation tubing and rotor were sterilized with H_2O_2 followed by 70% ethanol and washed with sterile water. Cells were collected under sterile conditions using HBSS–BSA as eluant at a loading density of 2×10^8 cells/run. Cells were loaded at a rotor speed of 2500 rpm and a flow rate of 25 mL/min and washed for 3 min under the same conditions. Maintaining the same flow rate, the rotor speed was reduced to 2100 rpm and a 100-mL fraction was collected (fraction 1). The rotor speed and flow rate were then adjusted to 2000 rpm and 55 mL/min, respectively, and a 100-mL fraction was collected (fraction 2). Immunocytochemical and radioimmunoassay results later showed that the majority of immunoreactive G cells eluted in fraction 2, whereas the greater proportion of immunoreactive D cells eluted in fraction 1. Consequently, 12-well collagen-coated culture plates (Costar, Cambridge, MA) were seeded at a density of 2×10^6 cells/mL in each well with cells from fraction 2. The culture medium consisted of Dulbecco's modified Eagle medium (Gibco) (DMEM) containing 5.5 mM glucose (Abbott Laboratories, Montréal, Que.) supplemented with 5% dog serum, 2 ng/mL nerve growth factor (Collaborative Research Inc., Bedford, MA), and 8 μg/mL insulin, 1 μg/mL hydrocortisone, and 50 μg/mL gentamycin sulfate (all three from Sigma). The cultures were maintained at 37°C in a humidified atmosphere of 95% O_2 – 5% CO_2 for 2 days.

Gastrin-release studies

After a 40- to 48-h incubation period, the culture medium was aspirated and the adherent cells were washed twice with 1 mL of release medium consisting of DMEM (Gibco) containing 2.7 mM glucose supplemented with 0.1% BSA (RIA grade; Sigma) and 1% aprotinin (500 Kallikrein inhibitor units/mL; Trasylol, Miles Pharmaceuticals, Rexdale, Ont.). The test compounds were dissolved in 100 μL of 10 mM acetic acid (BDH) and made up to a 40-fold concentration with release medium immediately prior to the studies. Stock solutions of reagents were added to duplicate wells in 25-μL aliquots to achieve the desired final concentration in a total volume of 1 mL. After a 2-h incubation period, the release medium was collected and centrifuged for 2 min in a Beckman microfuge (Microfuge B, Beckman Instruments). The supernatant was collected and stored at −70°C for future radioimmunoassay. Adherent cells from control wells were extracted in 1 mL of 2 M acetic acid, boiled for 10 min, centrifuged, and the supernatant collected for determination of total cell content of immunoreactive gastrin (IR-G) and somatostatin-like immunoreactivity (SLI).

Secretagogues

Bombesin and [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P were purchased from Institut Armand-Frappier (Montréal, Qué.). Synthesis of the [Leu^{13}-Ψ-CH$_2$NH-Leu^{14}]bombesin analog was undertaken as previously described (Coy et al. 1988).

Radioimmunoassay

IR-G levels in the supernatants and cell extracts were measured by specific radioimmunoassay employing synthetic human gastrin I (Research Plus Inc., Bayonne, NJ) as standard. Gastrin antibody 1611 (a gift from Dr. J. Walsh, Center for Ulcer Research and Education (CURE)) was used in the assay at a final dilution of 1 : 250 000. This antibody binds to C-terminal residues and recognizes gastrin 34 and gastrin 17 equally well (Walsh et al. 1982). The radioimmunoassay of gastrin has been previously described (Rosenquist and Walsh 1980). SLI was also determined by radioimmunoassay using the monoclonal antibody S3 as previously described (McIntosh et al. 1987). This antibody recognizes somatostatin 14 and somatostatin 28.

Immunocytochemistry

Immediately after the release studies, the cells in the culture wells were fixed in Bouin's solution for 10–15 min at room temperature and washed twice in 70% ethanol and twice in phosphate-buffered saline. Gastrin-containing cells and somatostatin-containing cells were localized with the monoclonal antibodies 109–121 (a gift from Dr. J. Walsh, CURE) at a dilution of 1:100 and S8 at a dilution of 1:1000 (Buchan et al. 1985), respectively. Immunoreactive cells were then identified using the biotin–avidin–peroxidase method as previously described (Buchan et al. 1982).

Expression of results

IR-G and SLI were initially determined in picograms per millilitre. IR-G release was further expressed as percent of total cell content (%TCC) owing to considerable interanimal variation. Statistical significance was determined using the Wilcoxon sign rank test.

Results

Culture of canine antral gastrin cells

Radioimmunoassay of cell extracts from postelutriation fractions 1 and 2 revealed that IR-G levels from the latter were approximately two- to three-fold greater than the former. Consequently, cells from fraction 2 were cultured for 48 h at 37°C. Immunocytochemical examination of the culture plates demonstrated that gastrin-containing G cells and somatostatin-containing D cells accounted for 8.5 ($n = 2$) and 1% ($n = 2$), respectively, of the total viable adherent cells. Mucin cells comprised the remainder of the adherent population (Fig. 1).

Pattern of immunoreactive gastrin release during basal conditions

Basal gastrin release was found to be 1.91 ± 0.48% TCC (Fig. 2). BN, from 0.01 to 100 pM, potently stimulated gastrin release from cultured G cells in a concentration-dependent fashion ($p < 0.05$, Fig. 2). Maximal gastrin response was