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Isolation and primary structure of pituitary human galanin, a 30-residue nonamidated neuropeptide

(regulatory peptide/gastrointestinal hormone/neuromodulator/amidation)

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ABSTRACT **Galanin (Gal), a 29-amino acid C-terminally amidated neuropeptide, is widely distributed throughout the central and peripheral nervous system. The primary structures of rat and bovine Gals were derived from the cDNA sequences of their precursors. To elucidate the structure of human Gal (hGal), we extracted 280 postmortem pituitaries in trifluoroacetic acid and purified hGal binding activity, by three successive HPLC steps, to homogeneity based on a radioreceptor assay. The primary structure of hGal was determined by automatic Edman degradation to be Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Val-Gly-Asn-His-Arg-Ser-Phe-Ser-Asp-Lys-Asn-Gly-Leu-Thr-Ser-COOH. The structure was confirmed by plasma desorption time-of-flight mass spectrometry, revealing a mass of 3156.1. Compared to the 29-residue porcine, rat, and bovine Gals, hGal uniquely comprises 30 amino acids possessing an additional nonamidated serine residue as C terminus. The nonamidated carboxylic group at the C terminus was proven by synthesis of amidated and nonamidated hGal and by mass spectrometry after selective methylation of all free carboxylic groups. Synthetic hGal possesses full biological activity on isolated rat fundus muscle strips.**

Galanin (Gal) is a C-terminally amidated 29-residue peptide deriving its name from the N-terminal glycine and C-terminal alanine residue. It was isolated from porcine intestinal extracts by Tatemoto *et al.* (1) using its C-terminal alanine amide as isolation criterion. Porcine Gal (pGal) shows no significant sequence homology to any regulatory peptide family. Gal-like immunoreactivity is expressed in the central and peripheral nervous system (2–5), gastrointestinal tract (6–8), adrenal medulla (9, 10), pituitary (2, 4), and pancreas (11) of several species (2). Biological actions of Gal include contraction or relaxation of gut smooth muscles, inhibition of insulin and somatostatin release, and modulation of hormone secretion from the pituitary and adrenal gland (2, 12–14). Gal may play an important role as neuromodulator of endocrine secretion and synaptic transmission (2, 12).

The primary structures of porcine, bovine, and rat prepro-galanin have been deduced from their respective cDNA precursors (9, 10, 15, 16). Although human Gal (hGal)-like immunoreactivity has been detected in various endocrine, neuronal, and gastrointestinal tissues (3, 4, 6), the primary structure of the hGal peptide is not known. However, tissue levels of hGal-like immunoreactivity, as measured with antisera raised against pGal, are considerably lower than tissue levels in pigs and rats, indicating sequence heterogeneity (4, 6). Moreover, substantial controversy exists on the effect of Gal on insulin secretion being inhibitory (17–19) or stimulatory (20) in different species.

To define the physiological role of this regulatory peptide in humans, it is essential to elucidate the structure of hGal. To avoid difficulties with poorly reactive pGal-specific antisera, we used a radioreceptor assay based on rat insulinoma cells for the isolation of the human peptide. Here, we report the sequence and biological activity of pituitary hGal, a 30-residue nonamidated peptide.

MATERIALS AND METHODS

Materials. Rat Gal (rGal) was obtained from Bissendorf Biochemicals (Hannover, F.R.G.). Chemicals for peptide synthesis were from MilliGen (Eschborn, F.R.G.).

Radioreceptor Assay for Gal Binding Activity (GBA). The GBA was determined using the rat insulinoma-derived cell line RIN56A (generously provided by S. J. Brand, Massachusetts General Hospital, Boston) expressing high numbers of Gal receptors. Cells were grown, passaged, and harvested, as described (21, 22). For binding assays, 1×10^6 cells were incubated in 0.2 ml of Krebs–Ringer/bacitracin (1 g/liter) for 30 min at 25°C (final volume, 0.2 ml) with ^{125}I-labeled rGal (20,000 cpm) alone or in the presence of unlabeled rGal as standard (1 pM to 1 μM). ^{125}I-labeled rGal was prepared by the Chloramine-T method and purified by HPLC. Cell-bound radioactivity was determined after centrifugation (5 min, 1000 $\times$ *g*). Results were expressed as specific binding (total binding minus unspecific binding, as percent of total binding).

Extraction of Human Pituitaries. Human pituitaries were collected less than 24 h postmortem, immediately frozen in liquid nitrogen, and kept at −80°C. Approximately 280 human pituitaries (132 g) were boiled for 10 min in water (1.8 liters). After cooling to 4°C, trifluoroacetic acid (TFA) was added to a final concentration of 2% (vol/vol), and the tissue was homogenized (Ultraturrax, two 30-sec high-speed homogenizations) and stirred 2 h at 4°C (extract A1, 1.8 liters). The extract was centrifuged (1 h, 16,000 $\times$ *g*), filtered through nylon gauze, and passed through 30 Adsorbex C_{18} cartridges (10 ml per cartridge) (400 mg, Merck) using a vacuum extractor. Peptide material was eluted by 2 ml of 80% (vol/vol) acetonitrile in 0.1% TFA and concentrated by partial evaporation. Combined eluates (extract A2, 155 ml) were centrifuged (1 h, 16,000 $\times$ *g*) and filtered through gauze.

Isolation and Purification of hGal from Human Pituitary Extract. Eluate A2 was separated by HPLC on a preparative

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Abbreviations: Gal, galanin; hGal, rGal, pGal, and bGal, human, rat, porcine, and bovine Gal; GBA, Gal binding activity; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid.

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reverse-phase C_{18} column (Nucleosil 300-7 wide pore, 1 × 25 cm, Macherey & Nagel) at a flow of 2.5 ml/min using a Waters gradient HPLC system (Waters–MilliGen), as described (23). UV-absorption was recorded at 214 and 280 nm. The solvent system consisted of 0.1% TFA (solvent A) and 70% acetonitrile in 0.1% TFA (solvent B). Eluate A2 (11–12 ml per HPLC run) was applied and material was eluted employing a linear gradient (0–80% solvent B in 80 min). Fractions (2.5 ml) were screened for GBA by radioreceptor assay. GBA-containing fractions were partially evaporated and rechromatographed on a Vydac RP 218 TP 54 column (wide pore, 4.6 × 250 mm, The Separations Group). The solvent system was 0.12% heptafluorobutyric acid (HFBA) (solvent A) and 70% acetonitrile in 0.1% HFBA (solvent B). hGal was purified to homogeneity by rechromatography on the Vydac RP 218 TP 54 column in the TFA/acetonitrile solvent system. To prove the C-terminal structure, native hGal was rechromatographed on the same column in 5 mM potassium phosphate at pH 6.0 (solvent A) and 70% acetonitrile in 5 mM potassium phosphate at pH 6.0 (solvent B).

Sequence Determination. The sequence of hGal (0.5 nmol) was determined by Edman degradation on an Applied Biosystems sequencer (model 470A/120A). Amino acid composition was quantified after gas-phase hydrolysis (1 h, 150°C). C-terminal amide assay was performed after thermolysin cleavage, as described (24, 25).

Peptide Synthesis. hGal-COOH (nonamidated) and hGal-$CONH_2$ (amidated) were synthesized by the fluoren-9-ylmethoxycarbonyl (Fmoc) solid-phase strategy, using Pepsyn KA and Ultrosyn C resins, in an automatic synthesizer (Novasyn crystal, Nova Biochem) (26). Synthetic peptides were characterized by mass spectrometry, radioreceptor assay, and HPLC rechromatography in the potassium phosphate/acetonitrile system.

Mass Spectrometry. Molecular masses were determined by plasma desorption time-of-flight mass spectrometry (27) on a Bio Ion 20 instrument (Applied Biosystems). Peptide (50–200 pmol) was applied in 10 μl of 15% acetonitrile in 0.1% TFA to nitrocellulose-coated sample foils. Mass spectra were obtained by bombardment with fission fragments from a 10-μCi ^{252}Cf source (1 Ci = 37 GBq) at an acceleration voltage of 18 kV. Spectra were recorded for 1–20 × 10^6 primary ions. The molecular weight was calculated as average of the single-charged ($[M + H]^+$) and double-charged ($[M + H]^{2+}$) ions. The accuracy of the method is less than ±0.1%.

Methylation of Native hGal. Dried native hGal (0.1 nmol) was esterified in 0.1 ml of 2 M HCl in methanol (0.17 ml of acetylchloride added to 1 ml of ice-cold methanol) for 2 h at 22°C (28). Methylated hGal was purified by HPLC and subjected to mass spectrometry.

Effect of Synthetic hGal on Isolated Rat Fundus Strips. Isolated longitudinal muscle strips from rat fundus were prepared, as described (29). Strips were kept at a resting tension of 2 g in oxygenated (95% O_2/5% CO_2) Krebs solution at 37°C. Responses were measured isometrically with Grass FT03 force displacement transducers connected to a Grass multichannel polygraph (model 79D). Results are expressed as percentage of the maximal response induced by acetylcholine. Potencies (ED_{50}) were calculated by interpolation from the dose–response curve.

Statistical Analysis. Data are expressed as the mean ± SEM. Student's *t* test for paired data was used for statistical evaluation of differences; $P < 0.05$ was considered to indicate statistically significant differences.

RESULTS

Extraction of Human Pituitaries. The initial extract (A1) of 280 human pituitaries (132 g) contained 410 pmol of GBA per g of frozen tissue (total amount, 54 nmol), as determined by the Gal radioreceptor assay. Serial dilutions of human GBA displaced ^{125}I-labeled rGal in an approximately parallel manner (Fig. 1); the K_d was 0.5 nM. The extract was passed through C_{18} cartridges to concentrate peptides and small hydrophobic substances. In the partially evaporated eluate (A2, 155 ml), 29 nmol of GBA was recovered (53.7%).

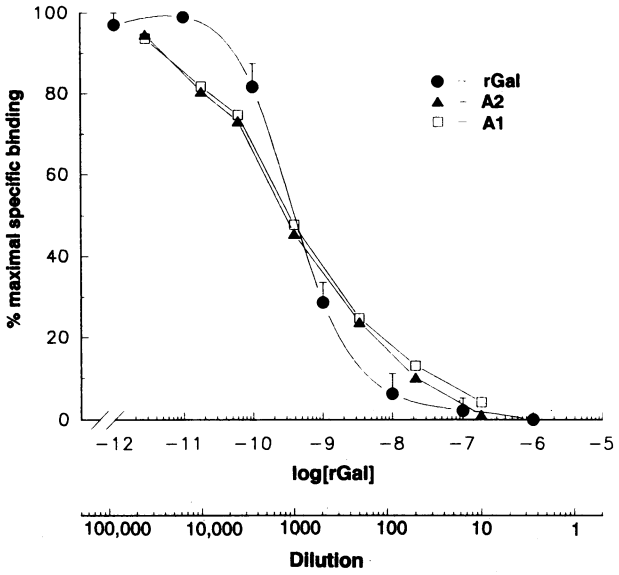

FIG. 1. Displacement of ^{125}I-labeled rGal binding to RIN56A cells by unlabeled rGal and human pituitary extracts A1 and A2. Cells were incubated for 30 min at 25°C with ^{125}I-labeled rGal (20,000 cpm) and rGal standards or various dilutions of lyophilized pituitary extracts (A1 and A2). Maximal specific binding is defined as cell-bound radioactivity in the absence (100%) minus the presence (0%) of unlabeled rGal.

Isolation and Purification of hGal from Human Pituitary Extract. Eluate A2 was chromatographed in successive runs (11–12 ml per injections) by HPLC on a preparative reverse-phase C_{18} column using the TFA/acetonitrile gradient system. Fig. 2*A* shows a representative elution profile. Human GBA, determined by radioreceptor assay, was eluted in two adjacent fractions at 51 and 52 min, respectively. Synthetic rGal used for column calibration was detected at 54 min (data not shown). Total recovery of human GBA from 13 preparative HPLC separations was 13.9 nmol (25.7% of initial). Further purification was achieved by HPLC rechromatography of the main fraction (51 min) on an analytical C_{18} column using the HFBA/acetonitrile solvent system. Fig. 2*B* shows that hGal was eluted as an almost homogeneous peak at 38 min, detected by UV-absorption at 214 nm. All human GBA was present in this single fraction and ≈10 nmol (18.5% of initial) was recovered. HPLC rechromatography of this fraction on the same column in the TFA/acetonitrile solvent system revealed apparent homogeneity of hGal (Fig. 2*C*), characterized by UV-absorption at 214 nm and 280 nm (the latter not shown).

Composition and Primary Structure of hGal and Proof of its C Terminus. Gas-phase sequencing of the homogeneous material (0.5 nmol of GBA) established the amino acid sequence of hGal was as follows.

$$\overset{1}{\text{Gly}}\text{-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-}\overset{10}{\text{Leu}}\text{-}$$

$$\text{Leu-Gly-Pro-His-}\overset{15}{\text{Ala}}\text{-Val-Gly-Asn-His-}\overset{20}{\text{Arg}}\text{-}$$

$$\text{Ser-Phe-Ser-Asp-}\overset{25}{\text{Lys}}\text{-Asn-Gly-Leu-Thr-}\overset{30}{\text{Ser}}$$

All residues could be unequivocally identified. The average repetitive yield was 94.4% (range, 91–98%). The phenyl-

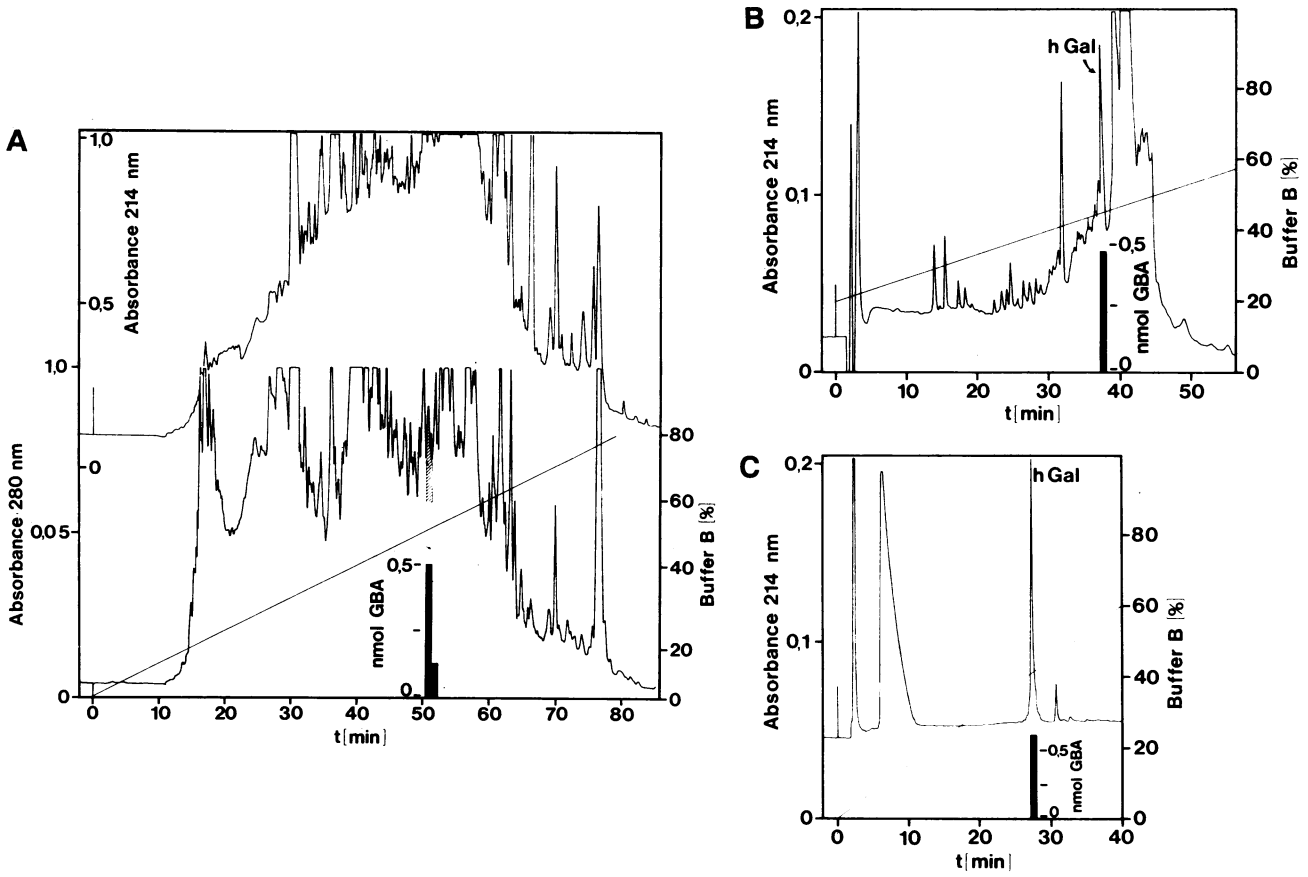

FIG. 2. Isolation of hGal from human pituitary extracts. UV-absorbance was monitored at 214 nm and/or 280 nm. Human GBA was measured by radioreceptor assay and expressed as nmol per fraction (inner histogram); the elution gradient is shown as percent of solvent B. (*A*) HPLC elution profile of the human pituitary extract (12 ml) on a preparative C_{18} column in the TFA/acetonitrile system after concentration on C_{18} cartridges (linear gradient, 0–80% acetonitrile in 0.1% TFA in 80 min). (*B*) HPLC rechromatography of fraction 51 on an analytical C_{18} column in the HFBA/acetonitrile system (linear gradient, 20–60% acetonitrile in 0.1% HFBA in 60 min). Fractions were collected manually. (*C*) HPLC rechromatography of the hGal-containing fraction on a C_{18} column in the TFA/acetonitrile system (linear gradient, 0–60% acetonitrile in 0.1% TFA in 40 min). hGal was eluted as homogeneous peak, characterized by UV-absorbance at 214 nm and GBA.

thiohydantoin derivatives of residues 29 and 30 were detected on the basis of 2.5 and 1.7 pmol, respectively. This primary structure was confirmed by plasma desorption time-of-flight mass spectrometry of 100 pmol of native hGal. A mass of 3156.1 was measured, compared to 3157.4, as calculated from the sequence shown above (Fig. 3*A*). Amino acid analysis of purified hGal (0.3 nmol) revealed the following composition: Asx 4.2 (4), Thr 1.8 (2), Ser 4.4 (4), Pro 1.1 (1), Gly 5.4 (5), Ala 2.4 (2), Val 0.8 (1), Leu 3.5 (4), Tyr 0.8 (1), Phe 1.0 (1), His 1.7 (2), Lys 1.1 (1), Arg 0.8 (1), and Trp not determined, where the numbers in parentheses are theoretical values.

To elucidate whether hGal is C-terminally amidated, the peptide was cleaved by thermolysin and screened for a C-terminal amide residue by a modified amide assay. No amino acid amide was identified. To confirm this result, native hGal (250 pmol) was methylated in acetylchloride/methanol, under reaction conditions that allow selective methylation of free α- and γ-carboxylic groups. An increase in mass of 14.02 indicates methylation of one carboxylic group. The mass of methylated hGal was measured as 3187.1 (calculated, 3157.4 + 28 = 3185.4), shown in Fig. 3*B*. Since hGal contains one γ-carboxylic group (on Asp-24), this result provides evidence that the C-terminal serine residue of hGal is nonamidated.

Synthesis of hGal. To prove the structure of hGal, C-terminally nonamidated hGal (hGal-COOH) and its amidated derivative (hGal-$CONH_2$) were synthesized by fluoren-9-ylmethoxycarbonyl solid-phase chemistry and characterized by mass spectrometry, HPLC, and binding assay. Average masses were determined to be 3158.3 for hGal-COOH (calculated, 3157.4) and 3155.6 for hGal-$CONH_2$ (calculated, 3156.4). By using the TFA/acetonitrile solvent system (pH 2.1), the two forms could not be separated: both eluted with native hGal (retention time, 29 min). HPLC separation in potassium phosphate/acetonitrile at pH 6 revealed unambiguous differences: native hGal eluted with synthetic hGal-COOH at 32.8 min, whereas synthetic hGal-$CONH_2$ eluted more than 2 min later (35.1 min), thereby confirming the nonamidated C terminus of hGal. Both peptides bound equipotently to the RIN cell Gal receptor (results not shown).

Effect of Synthetic hGal on Isolated Rat Fundus Muscle Strips. Synthetic hGal and rGal elicited contractions of isolated longitudinal rat fundus strips. Fig. 4 displays parallel dose–response curves for both peptides from six experiments, expressed as percentage of the maximal contraction induced by acetylcholine. Reproducible effects were observed with both peptides at 6 nM (rGal, 10.4 ± 2%; hGal, 11 ± 2%). Maximal contraction occurred at 180 nM (rGal, 62 ± 6%; hGal, 58 ± 4%), larger concentrations failed to produce a greater response. No significant difference was detected at any concentration. Potencies calculated as ED_{50} values were 13.8 ± 1.6 nM for hGal and 14.5 ± 0.5 nM for rGal. Synthetic hGal-$CONH_2$ was found to be equipotent to hGal-COOH (results not shown).

DISCUSSION

This investigation describes the isolation and amino acid sequence of pituitary hGal, a 30-residue nonamidated neuropeptide. hGal differs in 6 of 30 residues from pGal, orig-

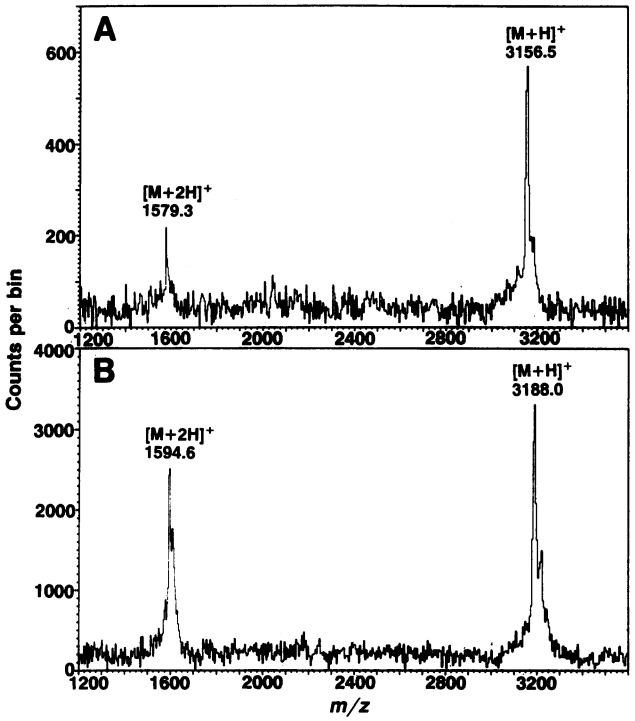

FIG. 3. Plasma desorption time-of-flight mass spectra of native hGal (M_r, 3156.1) (*A*) and methylated hGal (M_r, 3187.1) (*B*). Approximately 100 pmol of native hGal or 50–80 pmol of methylated hGal was applied to nitrocellulose-covered sample foils. Molecular weights were calculated as the average of the single-charged ($[M+H]^+$) and double-charged ($[M+2H]^{2+}$) ions.

inally isolated by Tatemoto *et al.* (1). In comparison to bovine Gal (bGal) and rGal, 7 and 4 residues are different, respectively. All amino acid substitutions are restricted to the C-terminal part of the molecule (positions 16–30). Table 1 compares the sequences of hGal, rGal, pGal, and bGal. The most striking structural feature of hGal is the C terminus: hGal comprises 30 instead of 29 amino acids with an additional nonamidated C-terminal residue. The evidence for this unusual structure of hGal was as follows. (*i*) The amide assay relying on proteolytic cleavage of hGal and identification of the C-terminal amino acid amide residue as phenylthiocarbamoyl-derivative by HPLC (24), a modification of the strategy originally described by Tatemoto and Mutt (25), did not reveal any amidated residue, indicating a free carboxylic group as C terminus. (*ii*) Selective methylation of hGal produced an increase in mass by ≈30, indicating methylation of two free carboxylic groups. These are obviously present in Asp-24 and in the C-terminal Ser-30, thus verifying the nonamidated state of the C terminus. Mass spectrometry of the native peptide cannot be used, since a mass difference of 1 Da (carboxylic group vs. α-amide) is beyond the resolution limit of the technique. (*iii*) hGal coeluted with the synthetic C-terminally nonamidated derivative, whereas synthetic amidated hGal eluted at a different position on HPLC. Thus, hGal is the only Gal peptide described that is extended by one residue and is not amidated C-terminally. The molecular basis for this mutation must await the structural characterization of the hGal gene. In the bGal, pGal, and rGal precursors (9, 10, 15, 16), the Gal sequence is flanked by Gly-Lys-Arg at the C terminus, serving as amide donor (glycine) and dibasic proteolytic processing site (Lys-Arg). Since GGC codes for the glycine residue in these three species, one can speculate that the Ser-Gly substitution in hGal is due to a single-base mutation (from GGC to AGC), which does not affect processing of the hGal precursor.

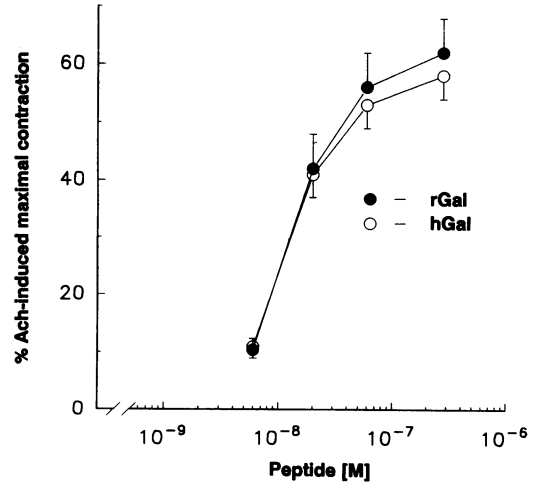

FIG. 4. Dose–response curve for the contractile effect of synthetic rGal and hGal on isolated rat fundus strips. Results are expressed as percentage of the maximal contraction induced by acetylcholine (Ach). Each point represents the mean, calculated from six experiments; vertical bars are SEM.

Table 1. Comparison of the amino acid sequences of hGal, rGal, pGal, and bGal

Peptide	Sequence
	1 5 10 15 20 25 30
hGal	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS.COOH
rGal	——————————ID————H—T.$CONH_2$
pGal	——————————ID———H—Y—A.$CONH_2$
bGal	——————————LDS—^—Q—H—A.$CONH_2$

Residues identical to hGal are indicated by dashes.

C-terminal amidation is a common feature of regulatory gut or brain peptides. Most peptide families like gastrin/cholecystokinin, pancreatic polypeptide/peptide YY/neuropeptide Y, the tachykinins, neurokinins, bombesin/gastrin-releasing peptide, and several opioid peptides require the C-terminal amide group for biological activity, whereas only a few amidated peptides [i.e., glucagon-like peptide 1 (30, 31), growth-hormone-releasing factor (32, 33), and Gal] are equipotent compared with their nonamidated derivatives. The 43-residue nonamidated rat growth-hormone-releasing factor represents another example for a species variation at the C terminus (32).

Synthetic nonamidated hGal was shown to exert full biological activity on isolated rat fundus muscle strips, being equipotent to rGal. Similar results were obtained from binding studies of synthetic hGal with RIN56A cells, which served as radioreceptor assay for the detection of hGal in pituitary extracts. The rGal receptor present on pancreatic B cells and gastric smooth muscle preparations obviously has a similar affinity for rGal and hGal and is fully activated by both, thus confirming the close structural homology between hGal and rGal, as suggested by others (4). Interestingly, nonamidated and amidated hGals showed no differences in biological activity or high-affinity binding (results not shown). These findings are in agreement with several structure–activity studies for the characterization of Gal and the interaction with its receptor: it has been demonstrated that the N-terminal portion of Gal, which is completely conserved in all species, is important for receptor interaction in rat pancreatic B cells (21, 34), central nervous system neurons (35, 36), and isolated smooth muscle strips (14, 29). In contrast, a number of Gal-specific antisera reacting with the C-terminal part of the peptide show poor cross-reactivity among species and divergent tissue levels of Gal (4, 6).

The high expression of Gal in the human pituitary (410 pmol/g) compares well with the distribution of Gal in the porcine central nervous system, showing highest levels of Gal-like immunoreactivity in the pituitary and hypothalamus (4). In rats, Gal has been shown to inhibit dopamine secretion from the median eminence and to release prolactin, growth hormone, and luteinizing hormone (37–39). Gal-immunoreactive cells are abundant in the human hypothalamus (3). Moreover, i.v. infusion of pGal stimulates plasma growth hormone levels in humans (40). These reports seem to indicate a neuromodulatory role for Gal in the regulation of the hypothalamopituitary axis. The structural characterization of pituitary hGal should allow the investigation of the role of Gal in human physiology and endocrine disorders.

Note Added in Proof. Bersani *et al.* (41) reported an identical primary structure for human colonic galanin. In addition, a 19-residue N-terminal fragment was isolated.

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