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Characterization of a high-affinity galanin receptor in the rat anterior pituitary: Absence of biological effect and reduced membrane binding of the antagonist M15 differentiate it from the brain/gut receptor

(galanin fragment/hemolytic plaque technique/prolactin)

DAVID WYNICK*, DAVID M. SMITH*, MOHAMMAD GHATEI*, KAREN AKINSANYA*, RANJEV BHOGAL*, PAUL PURKISS†, PETER BYFIELD†, NOBORU YANAIHARA‡, AND STEPHEN R. BLOOM*

*Department of Medicine, Hammersmith Hospital, London W12 ONN, United Kingdom; †Haemostasis Research Group, Clinical Research Centre, Harrow, Middlesex, HA1 3UJ, United Kingdom; and ‡Department of Bio-organic Chemistry, University of Shizuoka, Shizuoka, Japan

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ABSTRACT **Structure–activity studies demonstrate that galanin fragments 1–15 and 2–29 are fully active, whereas fragment 3–29 has been reported to be inactive, in a number of different *in vivo* models. M15, a chimeric peptide comprising galanin 1–13 and substance P 5–11, has recently been found to be a potent galanin antagonist. Direct effects of galanin at the level of the pituitary have been defined, yet, paradoxically, a number of studies have been unable to demonstrate galanin binding to an anterior pituitary receptor. Porcine galanin stimulated prolactin release from dispersed rat anterior pituitary cells up to 180% ± 12% (mean ± SEM) of control secretion. The addition of a specific galanin antiserum caused a profound inhibition of basal prolactin release, maximal inhibition being 12% ± 0.5% of control secretion. Addition of M15 produced no effect on basal or galanin-stimulated prolactin release. Galanin fragment 3–29 was fully active when compared to galanin 1–29. Fragments 5–29 and 8–29 stimulated prolactin release to a lesser extent and galanin 1–15, 10–29, and 20–29 had no significant prolactin-releasing activity. Using [mono(^{125}I)iodo-Tyr^{26}]galanin or porcine ^{125}I-labeled Bolton–Hunter [mono(^{125}I)iodo-Lys^{25}]galanin, no anterior pituitary membrane binding was observed. In contrast, ^{125}I-labeled Bolton–Hunter N-terminally labeled galanin allowed characterization of a single high-affinity anterior pituitary galanin receptor with a K_d of 4.4 ± 0.34 nM and a B_{max} of 79 ± 8.3 fmol/mg of protein. The IC_{50} for porcine galanin was 0.51 ± 0.04 nM but for M15 was in excess of 10 μM. Galanin 3–29 fully displaced the label with an IC_{50} of 0.96 ± 0.7 nM. The IC_{50} for galanin 5–29 was 200 nM, whereas 8–29 and 1–15 were >1 μM. Galanin 10–29 and 20–29 failed to displace the label. These data suggest the presence of a high-affinity pituitary galanin receptor, designated GAL-R_2, in which region 3–10 and amino acid 25 are crucial for membrane binding and biological activity, in contrast to the known gut/brain galanin receptor (designated GAL-R_1). A number of tissues known to bind or respond to galanin were screened. GAL-R_2 would appear to be expressed only in the anterior pituitary and hypothalamus.**

Galanin, a 29-amino acid peptide, was originally isolated from porcine intestine (1) and subsequently reported to be widely distributed in gut, pancreas, and the peripheral and central nervous systems (2, 3). Highest levels of galanin synthesis and storage occur within the hypothalamus in the median eminence (4), but it is also abundantly expressed in the anterior pituitary, where it has been shown to be estrogen inducible (5).

Various studies have demonstrated effects of galanin on basal and stimulated release of prolactin (6, 7), growth hormone (8–11), and luteinizing hormone (12, 13) either from dispersed pituitary cells or at the hypothalamic level modulating dopamine, somatostatin (SRIF; somatotropin release-inhibiting factor), and gonadotropin-releasing hormone (GnRH) release into the portal circulation. Recently galanin has been shown to be episodically released into the hypothalamo-pituitary portal circulation at concentrations in the nanomolar range (12), providing further evidence for its putative role as a modulator of pituitary function.

In view of the high concentrations of galanin reaching or secreted by the anterior pituitary it is puzzling that a number of studies have failed to demonstrate anterior pituitary binding of [mono(^{125}I)iodo-Tyr^{26}]galanin (^{125}I-Tyr-galanin) by membrane assay or by autoradiography in the rat or pig (14, 15), although these studies demonstrated good thalamic- and/or hypothalamic-specific galanin binding.

Bartfai *et al.* (16) have recently synthesized a high-affinity galanin antagonist by fusing galanin 1–13 with substance P 5–11, which they termed M-15. This antagonist has been shown to be a potent inhibitor, at doses of 1 nM or less (with an IC_{50} of 0.1 nM), of the actions of galanin on the pancreas, smooth muscle, and hippocampus.

The following studies demonstrate that galanin stimulates the release of prolactin from cultured anterior pituitary cells and that a specific galanin antiserum blocks basal prolactin secretion. The galanin antagonist M15 has no effect on basal or galanin-stimulated prolactin release. Using galanin ^{125}I-labeled with the Bolton–Hunter (^{125}I-BH) reagent at the N terminus, a single high-affinity galanin receptor in the pituitary is characterized. Unlike the brain/gut galanin receptor, the C-terminal part of the peptide is crucial for pituitary membrane binding.

MATERIALS AND METHODS

Pituitary Dispersion. Anterior pituitaries were removed from randomly cycling female Wistar rats (Interfauna, Huntingdon, U.K.) immediately after death by decapitation and dispersed as described (17). Tissue was incubated while shaking at 37°C for 1 hr in Hanks' balanced salt solution (HBSS, GIBCO; containing 0.1% bovine serum albumin, pH 7.4) containing 0.7 mg of collagenase per ml (Boehringer

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Abbreviations: BH, Bolton–Hunter; SRIF, somatostatin (somatotropin release-inhibiting factor); GHRH, growth hormone-releasing hormone; TRH, thyroliberin (thyrotropin-releasing hormone); GnRH, gonadotropin-releasing hormone.

Mannheim), followed by incubation for 45 min in 0.125% (vol/vol) trypsin (Sigma) and 0.1 mg DNase per ml (type IV; Sigma). The resulting cell suspension was rinsed by centrifugation and resuspended in fresh culture medium. The final yield was normally 2.0–2.6 × 10^6 cells per pituitary, with a viability of >98% assessed by trypan blue exclusion. Before further study, cells were cultured for 48 hr at 37°C in 5% CO_2 on Petri dishes coated with poly(lysine) (0.5 mg/ml, Sigma) to prevent cell clumping.

Measurement of Prolactin Release. The reverse hemolytic plaque assay was used to measure prolactin release from single lactotrophs as described (18). Dispersed cells were briefly trypsinized (0.125%, vol/vol) for 5 min on the day of use to remove the cells from the plates. Cunningham chambers were constructed using poly(lysine)-coated microscope slides, double-sided adhesive tape, and coverslips. A mixture of 10^5 pituitary cells and 1 ml 18% (vol/vol) sheep erythrocytes (Tissue Culture Services, Buckingham, Kent) conjugated to protein A (Sigma) by chromium chloride was infused into the chambers and allowed to attach to the slide for 1 hr. Excess cells were then removed by washing with Dulbecco's minimal essential medium (DMEM, GIBCO). Maximal plaque formation (i.e., all lactotrophs had a visible plaque around them) occurred after 3 hr in the basal state, as characterized (19), and this time point was used for all subsequent experiments. Plaque formation was induced by the addition of 1:50 (vol/vol) guinea pig complement (GIBCO) for 45 min. Plaque area was measured using the Cue-2 image analysis system (Olympus, Tokyo). Results are expressed as mean ± SEM (n = 5) in all cases. Statistical analysis was performed using the Student's t test.

Peptide Synthesis. Porcine galanin, its fragments 2–29, 3–29, 5–29, 8–29, 10–29, and 20–29, and the galanin antagonist M15 were synthesized on Rink's amide-type resin (Nova Biochem, Nottingham, U.K.) using fluorenemethyloxycarbonyl-protected amino acids and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflurophosphate activation chemistry in an automated peptide synthesizer (model 431A, Applied Biosystems). Peptide material was purified to homogeneity by HPLC on C_8 columns (Aquapore RP300, Anachem, Luton, U.K.). Fidelity of synthesis was verified by amino acid analysis and mass spectroscopy, confirming the reduced form of the peptide in the case of M15. Galanin 1–15 used in these studies was synthesized as described (20).

Preparation of Porcine and Rat [Mono(^{125}I)iodo-Tyr]Galanin and Porcine Galanin Labeled with ^{125}I-BH Reagent. Synthetic porcine and rat galanin 1–29 were iodinated by the IodoGen method to yield [mono(^{125}I)iodo-Tyr]galanin as described (21).

Synthetic porcine galanin was iodinated by the BH method: 5 nmol in 20 μl of 0.2 M borate buffer (pH 8.2) was incubated at 4°C for 2 hr with 1 nmol of ^{125}I-BH reagent and separated from the free reagent on a C_{18} reverse-phase HPLC column.

Preparation of Membranes. Fifty female rats for each experiment were killed by decapitation, the top of the skull was removed with dissection scissors, and the brain was removed onto ice. The anterior pituitary was carefully dissected free from the posterior and neuro-intermediate lobes. The hypothalamus was removed by block dissection to reveal the thalamus caudal to it, which was then, in turn, dissected free of surrounding tissue and removed. Crude membranes were prepared as described (22). Briefly, tissues were homogenized for 2 min in 50 mM Hepes buffer (pH 7.4) containing 0.25 M sucrose and protease inhibitors, using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 1500 × g at 4°C for 20 min and the supernatant was then centrifuged at 4°C for 1 hr at 100,000 × g. The resulting pellet was resuspended in homogenization buffer without sucrose and centrifuged for a further 1 hr at 4°C at 100,000 × g. The final pellet was then resuspended in buffer (without sucrose) at a concentration of 2.5 mg/ml and frozen at −70°C in aliquots (500 μl) for future use in equilibrium binding experiments.

^{125}I-Galanin Binding to Tissue Membranes. Experiments were performed in 20 mM Hepes buffer (pH 7.4) containing 5 mM $MgCl_2$ (Sigma), 0.1% (wt/vol) bacitracin, 40 kallikrein inhibitor units of aprotinin per ml, 1 mM EDTA (Sigma), and 1% (wt/vol) bovine serum albumin fraction V in the presence of 0.1 nM of ^{125}I-galanin, 50 μg of the membrane preparation, and increasing concentrations of unlabeled porcine galanin or of other galanin receptor ligands. Nonspecific binding was assessed in the presence of 200 nM galanin. All assays were carried out in siliconized polypropylene tubes (final volume, 500 μl) with an incubation period of 45 min at 22°C. Bound and free label were separated by centrifugation at 15,600 × g for 2 min at 4°C. Specific binding was calculated from the nonspecific binding subtracted from the total binding expressed as absolute radioactivity or as a percentage of total binding. Results are expressed as mean ± SEM (n = 3) in all cases.

RESULTS

Effects of Galanin on Prolactin Release. Rat and porcine galanin caused an equipotent dose-dependent stimulation of prolactin release up to a maximum of 180% ± 12% of control secretion at a dose of 100 nM, with an ED_{50} of 6 nM for both peptides. This compares to a 220% ± 19% increase in release by 100 nM thyroliberin (TRH; thyrotropin-releasing hormone), previously shown to be the maximal response obtainable in this experimental paradigm (19) (Fig. 1).

The addition of a specific galanin antiserum, raised in rabbits, caused a profound, dose-dependent inhibition of basal prolactin release to a minimum of 12% ± 0.5% of control secretion using an addition of 1:100 dilution of the antiserum (Fig. 1). Controls included substitution of the galanin antiserum with (*i*) 1:100 nonimmune rabbit serum and (*ii*) eight other antisera at the same dilution raised in rabbits against glucagon or insulin and (*iii*) galanin antisera preincubated for 24 hr at 4°C with 25 μg of porcine galanin. All controls failed to demonstrate any inhibitory effect on prolactin release. The galanin antisera, when used at a 1:100 dilution, had no effect on basal or growth hormone-releasing hormone (GHRH) stimulation of growth hormone secretion.

Addition of M15 at concentrations up to 1 μM had no effect on basal prolactin release. Similarly, when the antagonist was used at a concentration of 1 μM, concurrently with 100 nM galanin, no attenuation of stimulation was observed (Fig. 1).

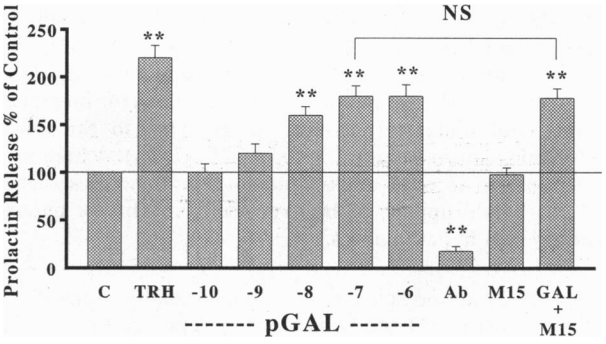

FIG. 1. Prolactin secretion from dispersed anterior pituitary cells was measured by reverse hemolytic plaque assay and expressed as a percentage of basal release in response to thyrotropin-releasing hormone (TRH, 100 nM), increasing doses of porcine galanin (pGAL), galanin antisera (Ab, 1:100 dilution), galanin antagonist M15 (1 μM), and a combination of 100 nM galanin and 1 μM M15. Results are expressed as mean ± SEM; n = 5 separate experiments. **, $P <$ 0.01; NS, not significantly different.

Various N- and C-terminal galanin fragments were also tested in the reverse hemolytic plaque assay. Galanin 2–29 and 3–29 were fully active at concentrations of 1 μM when compared to galanin 1–29. Equimolar concentrations of galanin 5–29 and 8–29 were less active than 3–29 (Fig. 2). Galanin 1–15, 10–29, and 20–29 had no significant prolactin-releasing activity when tested at a concentration of 1 μM.

Binding Studies. Specific binding to the thalamic membrane preparation (at a single ligand concentration) was 94.5 ± 6.1 fmol/mg of protein for porcine and rat ^{125}I-Tyr-galanin, whereas pituitary membrane specific binding was insignificant at 2.3 ± 0.14 fmol/mg of protein.

Iodination of porcine galanin using ^{125}I-labeled BH reagent produced two peaks when purified on HPLC (designated fractions 46 and 48). With thalamic membranes, both fractions gave identical specific binding to that obtained with either the porcine or rat ^{125}I-Tyr-galanin. Fraction 46 also bound the pituitary membranes with a specific binding of 50.2 ± 2.2 fmol/mg of protein, whereas fraction 48 specific binding was insignificant. Since the BH reagent could label only the free N terminus or the side chain of the Lys^{25} of porcine galanin, further studies were undertaken to determine which of these fraction 46 represented. Both fractions were run on a Sephadex G-25 column [using a 0.01 M phosphate buffer containing 0.5% bovine serum albumin (wt/vol)] after incubation for 6 hr at 37°C with either a 50 mM Hepes buffer (pH 7.4) containing 1 mM cobalt chloride (Sigma) or the above buffer containing 0.5 units of aminopeptidase M (Boehringer Mannheim). Aminopeptidase M sequentially cleaves amino acids from the N terminus if the peptide is not blocked. Thus, if the BH reagent had labeled the N terminus, and blocked the free amino group, the peptide would not be cleaved. In contrast, if the BH reagent labeled at the Lys^{25}, then the peptide could be cleaved up to the Pro^{13} (aminopeptidase M is unable to cleave prolylpeptide bonds), leaving a residual labeled 16-amino acid peptide. The results (Fig. 3) suggest that fraction 46 is N-terminally BH labeled, whereas fraction 48 is labeled at the Lys^{25}. All further studies were performed using fraction 46, N-terminal labeled ^{125}I-BH galanin [specific activity = 1481 Ci/mmol (1 Ci = 37 GBq)].

Scatchard analysis of saturation binding data demonstrated a K_d of 1.3 ± 0.12 nM for the thalamic membrane and 4.4 ± 0.34 nM for the anterior pituitary membrane, whereas the B_{max} for the thalamus and pituitary were 222 ± 17 and 79 ± 8.3 fmol/mg of protein, respectively, and were thus in good agreement with existing published brain and hippocampal values (23, 24) (Fig. 4). The IC_{50} values for unlabeled porcine galanin in the anterior pituitary and thalamus were 0.51 ± 0.04 and 0.58 ± 0.06 nM, respectively. The IC_{50} for M15, however, differed greatly, the thalamic membranes giving a value of 0.5 ± 0.05 nM, identical to the published value (16), whereas the pituitary yielded a value of >10 μM (Fig. 5).

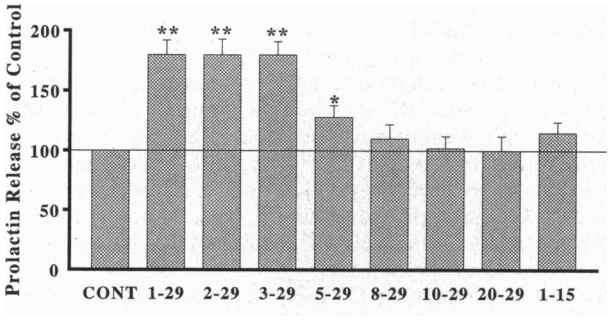

FIG. 2. Prolactin secretion from dispersed anterior pituitary cells was measured by reverse hemolytic plaque assay and expressed as a percentage of basal release in response to the addition of various galanin fragments. All fragments were used at 1 μM. CONT, control. Results are expressed as mean ± SEM; n = 5 separate experiments. **, $P < 0.01$; *, $P < 0.05$.

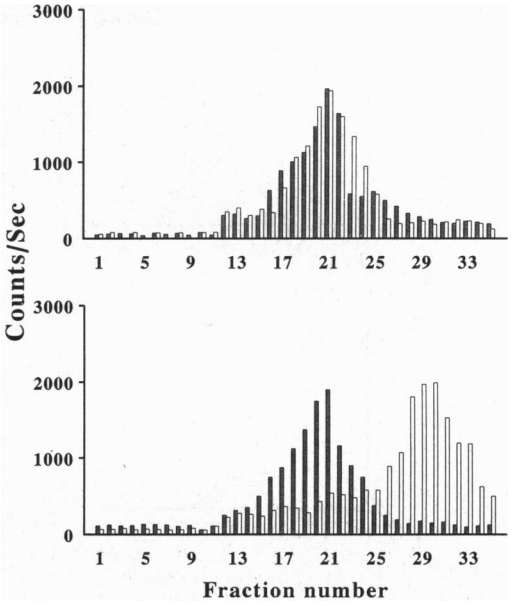

FIG. 3. Iodination of porcine galanin using ^{125}I-BH reagent produced two peaks when purified on HPLC, designated fractions 46 and 48. Fractions 46 (*Upper*) and 48 (*Lower*) were incubated with the enzyme aminopeptidase M and then separated on a Sephadex G-25 column. Crosshatched bars represent untreated fractions and open bars represent fractions following enzymic treatment.

Displacement of the labeled galanin with the various galanin fragments was tested and the results (Fig. 6) paralleled those obtained in the reverse hemolytic plaque assay. Galanin 2–29 and 3–29 fully displaced the label, having IC_{50} values of 0.6 ± 0.04 and 0.96 ± 0.7 nM, respectively. The IC_{50} for galanin 5–29 was 200 ± 13 nM, whereas IC_{50} values for 8–29 and 1–15 were >10 μM. Galanin 10–29 and 20–29 demonstrated no displacement of receptor binding.

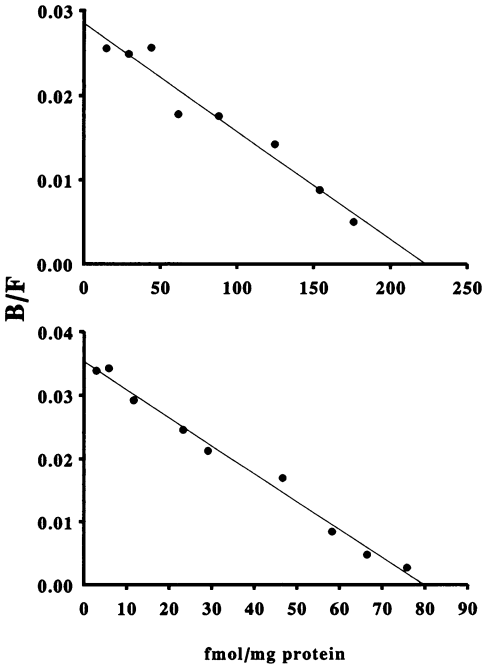

FIG. 4. Scatchard analysis of saturation binding data using the N-terminal labeled ^{125}I-BH porcine galanin from thalamic (*Upper*) and pituitary (*Lower*) membranes. B/F, bound/free.

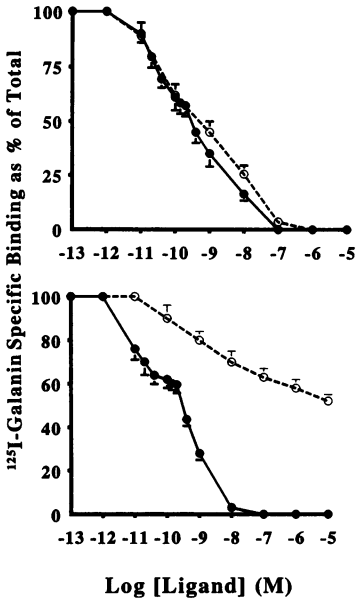

FIG. 5. Displacement of the N-terminal labeled ^{125}I-BH porcine galanin from thalamic (*Upper*) and pituitary (*Lower*) membranes with either unlabeled porcine galanin (●) or the galanin antagonist M15 (○).

Binding of the N-terminal labeled ^{125}I-BH porcine galanin was assessed in a number of tissues, known to express galanin receptors, in the absence or presence of 200 nM unlabeled porcine galanin, galanin fragment 3–29, or M15. Hippocampus, thalamus, cortex, stomach, duodenum, ileum, and pancreas demonstrated equal displacement of the label with porcine galanin and M15 and no displacement with galanin fragment 3–29. In contrast, M15 and galanin 3–29 displacement of the label using the hypothalamic membranes was 83% ± 4.7% and 32% ± 1.7%, respectively, of that observed with unlabeled galanin.

No displacement of the labeled porcine galanin from pituitary membranes was observed with the following peptides, all at 1 μM: TRH, GHRH, corticotropin-releasing factor, GnRH, SRIF, dopamine, vasoactive intestinal polypeptide, neuromedins U and B, calcitonin gene-related peptide, substance P, neuropeptide Y, neurotensin, and pituitary adenylate cyclase-activating peptide.

DISCUSSION

Modulation of the release of the anterior pituitary hormones prolactin, growth hormone, and luteinizing hormone by galanin is now well documented (6–13) and may well represent a summation of a number of different interactions. These include (*i*) changes in the release of hypothalamic stimulating and inhibiting factors (e.g., TRH, GHRH, GnRH, dopamine, and SRIF), (*ii*) effects of hypothalamic galanin secreted into the portal circulation and acting directly on basal and/or stimulated hormone release from the lactotroph, somatotroph, or gonadotroph and (*iii*) locally synthesized and secreted galanin acting in an autocrine manner.

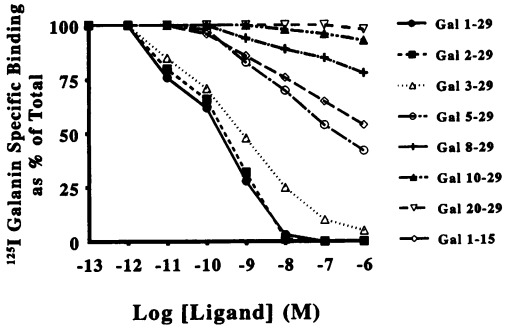

FIG. 6. Displacement of the N-terminal labeled ^{125}I-BH porcine galanin from pituitary membranes with unlabeled galanin fragments. Standard error bars are not shown for the sake of clarity.

We report in this study that galanin stimulates prolactin release from the dispersed lactotroph and that basal prolactin release would appear to be largely dependent on endogenous galanin for the tonic release of prolactin since the galanin antiserum used in our study profoundly inhibits basal prolactin release. The possibility that locally synthesized pituitary galanin is vital for lactotroph function implies that it is acting, at least in part, as an autocrine or paracrine agent. It was expected, therefore, that recent characterization (16) of the highly potent, galanin antagonist M15 would inhibit prolactin release to an extent similar to that obtained with the antiserum and thus allow further study of the role galanin plays in mediating pituitary function.

Failure to demonstrate inhibition of basal and galanin-stimulated prolactin release with M15 and the surprising absence of ^{125}I-labeled galanin specific binding in the anterior pituitary by either autoradiography or membrane displacement, as demonstrated by a number of groups (14, 15), was inexplicable and cast doubt as to the role that galanin may play in pituitary physiology.

To explain these inconsistencies we postulated a separate pituitary galanin receptor. The studies reported here suggest that a single high-affinity galanin receptor in the anterior pituitary does exist (designated GAL-R_2) and that it is different from the previously characterized receptor (designated GAL-R_1) found in the brain, gut, pancreas, and smooth muscle.

The many published structure–activity studies on the effects of galanin fragments and analogues on the GAL-R_1 (25–31) have demonstrated that the active receptor binding part of the peptide resides at the N-terminal portion of the peptide. The presence of the unmodified, first two N-terminal amino acid residues is particularly important for retention of high biological potency, since galanin 2–29 and 1–15 are nearly as potent biologically and at receptor displacement as the full 1–29, whereas 3–29, 10–29, and 20–29 are inactive.

In contrast to the GAL-R_1, the first two N-terminal amino acids are not crucial for binding to the pituitary GAL-R_2 since fragment 3–29 retains full biological activity. Receptor binding and release of prolactin decrease with increasingly shorter C-terminal fragments such that fragments 10–29 and 20–29 have no significant activity. These data indicate that the 3–10 region of galanin is crucial for binding to the GAL-R_2. However, the addition of the ^{125}I-BH reagent to the Lys^{25} of galanin modifies the peptide in some way, thus inhibiting binding to the GAL-R_2. Thus the amino acid in position 25 may also be required for pituitary receptor binding in accord with the inactivity of fragment 1–15.

Given the above data, it is not surprising that M15 (galanin 1–13 and substance P 5–11) is not a pituitary galanin antagonist and only weakly displaces labeled galanin, to a degree similar to that of fragment 1–15. Our data would suggest that since two regions of the peptide appear to be required for GAL-R_2 binding, the preparation of a specific pituitary galanin antagonist is likely to require the 3–10 region and amino acid 25 of the peptide.

To assess whether the GAL-R_2 was unique to the anterior pituitary we screened a number of regions of the brain and gut known to bind or respond to galanin (32–37). In all but one of the tissues tested, M15 and unlabeled galanin equally displaced the label, whereas fragment 3–29 caused no displacement of the label at all, implying that they only possess GAL-R_1 receptors. In the hypothalamus, however, 17%

lower displacement was observed with M15 than with unlabeled galanin, whereas galanin fragment 3–29 was able to displace 32% of the label. Since we have demonstrated that fragment 3–29 is able to fully displace the label from the GAL-R_2 receptor, these data would support the hypothesis that the hypothalamus expresses GAL-R_1 and GAL-R_2, whereas the other tissues examined exclusively express GAL-R_1.

In summary, these data demonstrate a high-affinity pituitary galanin receptor, designated GAL-R_2, in which region 3–10 and amino acid 25 are crucial for biological and membrane binding activity, in contrast to the known gut/brain galanin receptor (designated GAL-R_1), in which activity is dependent only on the first 15 N-terminal amino acids. The previously characterized GAL-R_1 antagonist, M15, does not bind to the GAL-R_2 and has no direct effect on the pituitary.

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