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N-terminal galanin-(1–16) fragment is an agonist at the hippocampal galanin receptor

(peptide fragments/acetylcholine/muscarinic receptor/release)

GILBERTO FISONE*, MALIN BERTHOLD*, KATARINA BEDECS*, ANDERS UNDÉN*, TAMAS BARTFAI*, ROSALIA BERTORELLI†, SILVANA CONSOLO†, JACQUELINE CRAWLEY‡, BRIAN MARTIN‡, SIV NILSSON§, AND TOMAS HÖKFELT§

*Department of Biochemistry, University of Stockholm, 106 91 Stockholm, Sweden; †"Mario Negri" Institute for Pharmacological Research, 20157 Milan, Italy; ‡Clinical Neuroscience Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20205; and §Department of Histology and Neurobiology, Karolinska Institute, S-10405 Stockholm, Sweden

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ABSTRACT **The galanin N-terminal fragment [galanin-(1–16)] has been prepared by solid-phase synthesis and by enzymic cleavage of galanin by endoproteinase Asp-N. This peptide fragment displaced ^{125}I-labeled galanin in receptor autoradiography experiments on rat forebrain and spinal cord and in equilibrium binding experiments from high-affinity binding sites in the ventral hippocampus with an IC_{50} of ≈3 nM. In tissue slices of the same brain area, galanin-(1–16), similarly to galanin, inhibited the muscarinic agonist-stimulated breakdown of inositol phospholipids. Upon intracerebroventricular administration, galanin-(1–16) (10 μg/15 μl) also inhibited the scopolamine (0.3 mg/kg, s.c.)-evoked release of acetylcholine, as studied *in vivo* by microdialysis. Substitution of [L-Trp^2] for [D-Trp^2] resulted in a 500-fold loss in affinity as compared with galanin-(1–16). It is concluded that, in the ventral hippocampus, the N-terminal galanin fragment [galanin-(1–16)] is recognized by the galanin receptors controlling acetylcholine release and muscarinic agonist-stimulated inositol phospholipid breakdown as a high-affinity agonist and that amino acid residue [Trp^2] plays an important role in the receptor–ligand interactions.**

The 29-amino-acid-long C-terminal amidated peptide galanin (GAL) (1) has been shown to be widely distributed in the central nervous system of mammals (2–6). GAL-like immunoreactivity has been localized, among other brain regions, in the septal area (4–8) where GAL coexists with acetylcholine in rat (7) and monkey (9) in a subpopulation of cholinergic cell bodies projecting to the hippocampus. Autoradiographic (10–12) and equilibrium binding studies with ^{125}I-labeled GAL (^{125}I-GAL) have shown a high density of putative high-affinity GAL receptors in the ventral part of the hippocampus (13).

In the ventral, but not in the dorsal, hippocampus GAL has been shown to inhibit, in a dose-dependent manner, the release of acetylcholine both *in vitro* and *in vivo* (12). A possible role for GAL as presynaptic modulator of the cholinergic function in the ventral hippocampus has been suggested by a recent electrophysiological study (14) and a behavioral study (15). Interactions between acetylcholine and GAL in the ventral hippocampus are not restricted to presynaptic sites but also involve actions that are considered postsynaptic (16) such as the GAL-mediated inhibition of the muscarinic agonist-stimulated breakdown of inositol phospholipids in the ventral hippocampus (17) and GAL inhibition of acetylcholine on a t-maze memory task in ventral forebrain lesioned rats (33).

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Previous studies carried out on intestinal smooth muscle showed the importance of the N-terminal portion of GAL for biological activity (18, 19). Furthermore, N-terminal GAL fragments and analogs were able to mimic the effects of GAL on the pancreatic β-cell line Rin m 5F, inhibiting forskolin-stimulated cAMP production and insulin release (20).

In this study, we focus our attention on the structure–activity relationship for the N terminus of GAL at receptors in the ventral hippocampus of the rat. Using equilibrium binding and autoradiographic techniques, we compare the synthetic N-terminal fragment GAL-(1–16) with rat GAL-(1–29) for its ability to displace ^{125}I-GAL from its receptors. GAL-(1–16) is also tested for its ability to inhibit the scopolamine-induced acetylcholine release *in vivo* and carbachol-stimulated inositol phospholipid breakdown in a slice preparation from the rat ventral hippocampus. The importance of [Trp^2] in the activity of the N-terminal fragment is examined.

MATERIALS AND METHODS

Materials. Na^{125}I (2500 Ci/mmol; 1 Ci = 37 GBq) and *myo*-[^{3}H]inositol (35 Ci/mmol) were purchased from Amersham. All other reagents were from Sigma. Amino acid derivatives and resins were from Bachem.

Animals. CD–COBS adult male rats (180–250 g) were used in the *in vivo* experiments. For all other studies, Sprague–Dawley adult male rats (200 g) were used. Porcine ^{125}I-GAL (specific activity, 250–300 Ci/mmol), iodinated by the chloramine-T method, was prepared as described (13) and used in both the equilibrium binding experiments and the receptor autoradiographic analysis.

Preparation of GAL Fragments. Peptides were synthesized manually on *p*-methylbenzhydrylamine resin (0.35 mmol/g). Protected *t*-butoxycarbonyl- amino acids were serine (benzyl-), threonine (benzyl-), tyrosine (*p*-bromobenzyloxycarbonyl-), histidine (dinitrophenyl-), and D- and L-tryptophan (formyl-). Asparagine and glutamine were coupled as active hydroxybenzotriazole esters. All other amino acids were activated by *N*,*N*′-dicyclohexylcarbodiimide in a molar ratio of 1:1. The protocol for stepwise solid-phase peptide synthesis using the *t*-butoxycarbonyl–benzyl protective group strategy has been published elsewhere (21).

Removal of protection was carried out by low trifluoromethanesulfonic acid (22), and the resin was washed with trifluoroacetic acid (2 × 1 min), dichloromethane (3 × 1 min), dimethylformamide (3 × 1 min), ethanol (2 × 1 min), and diethyl ether (1 × 1 min). The resin was dried under vacuum followed by final removal of protection by hydrogen fluoride containing 8% *p*-cresol and 2% ethanedithiol at 0°C for 40

Abbreviation: GAL, galanin.

min. Purification of the cleaved peptides was carried out by HPLC on a C_{18} reversed-phase column.

Synthetic porcine GAL-(1–29) (Bachem) was digested with endoproteinase Asp-N (Boehringer Mannheim) at an enzyme/substrate ratio of 1:250 to generate three peptide fragments, GAL-(1–16), -(17–23), and -(24–29). The resulting peptides were separated by HPLC on a reversed-phase C_{18} column.

Ligand Binding Studies. Displacement experiments were performed in a final vol of 400 μl of 5 mM Hepes/Krebs–Ringer solution [137 mM NaCl/2.68 mM KCl/1.68 mM $CaCl_2$/2.05 mM $MgCl_2$/glucose (1 g/liter) 0.05% bovine serum albumin, pH 7.4], containing 1 nM porcine ^{125}I-GAL and 70–100 μg of a ventral hippocampal membrane preparation (P2) obtained as described (13). Samples were incubated for 30 min at 37°C. Incubation was terminated by the addition of 10 ml of Hepes/Krebs–Ringer solution followed by rapid filtration over Whatman GF/C filters, precoated for 5–6 hr in 0.3% polyethylenimine solution, and a subsequent rinse with 10 ml of cold buffer. Specific binding was defined as that displaceable by GAL (1 μM).

Measurement of [^{3}H]Inositol Phosphate Accumulation. Inositol phospholipid breakdown was determined according to the method of Brown *et al.* (23) with *myo*-[^{3}H]inositol-labeled tissue slices from ventral hippocampi from male CD–COBS rats (150–200 g) as described by Palazzi *et al.* (17). GAL-(1–29) and GAL-(1–16) and [D-Trp^2]GAL-(1–16) were added at 1 μM 2 min before the carbachol stimulation, which lasted for 45 min in the presence of LiCl (5 mM). The inositol phosphates were separated by chromatography on Dowex resin as described (17).

Autoradiographic Binding Studies. The autoradiographic analysis of ^{125}I-GAL binding sites was carried out according to the procedure of Young and Kuhar (24) as described (11). Briefly, porcine GAL was iodinated with Na^{125}I by the chloramine-T method. Rats were perfused with ice-cold Tyrode's solution, and the brain and spinal cord were sectioned on a cryostat, followed by incubation with ^{125}I-GAL (≈1.5 nM) for 45 min at room temperature. Sections were rinsed, dried by a stream of cold air, exposed to formalin vapors, and covered by ^{3}H-sensitive film (Hyperfilm, Amersham). Unlabeled GAL-(1–29) and GAL-(1–16) fragment both at 0.1 or 1.0 μM were added to the incubation medium.

***In Vivo* Experiments.** In the *in vivo* release experiments, a thin dialysis fiber was implanted, essentially as described by Ungerstedt (25) and Benveniste *et al.* (26), in the hippocampi of anesthetized rats. The dialysis probe was implanted vertically into the ventral hippocampus of one side. The day after implantation, the dialysis tube was perfused at a constant rate of 2 μl/min with Ringer's solution (147 mM NaCl/3.4 mM $CaCl_2$/4 mM KCl, pH 6.1) containing 10 μM physostigmine. The perfusate was discarded during the first 40 min and then collected at 20-min intervals. Endogenous acetylcholine collected in the samples was assayed by a sensitive and specific radioenzymatic method, as described by Consolo *et al.* (27).

RESULTS

The specific binding of ^{125}I-GAL-(1–29) (1.0 nM) could be fully displaced by the N-terminal fragments GAL-(1–16) and [D-Trp^2]GAL-(1–16) in the concentration range 10^{-11}–10^{-6} M (Fig. 1). The GAL-(1–16) fragment has an ≈5-fold lower affinity than GAL-(1–29). Substitution of [L-Trp^2] for [D-Trp^2] caused a 500-fold decrease in the affinity of this fragment. Autoradiographic analysis showed that GAL-(1–16) could displace the ^{125}I-GAL from receptor sites in forebrain structures and spinal cord labeled by the full-length peptide GAL-(1–29) (Fig. 2). At a concentration of 100 nM, the unlabeled peptide GAL-(1–29) (Fig. 2*b*), but not GAL-(1–16) (Fig. 2*d*), showed a complete block in areas with intense binding (ventral hippocampus, subiculum, dorsal horn of spinal cord) (Fig. 2*d*). At 1 μM concentration, both peptides displaced all ^{125}I-GAL (Fig. 2*c*). The fragment GAL-(1–16), but not [D-Trp^2]GAL-(1–16) at 1 μM, was able to produce an inhibition of the muscarinic agonist-mediated stimulation of the breakdown of [^{3}H]inositol phospholipids in slices of the rat ventral hippocampus (Table 1). The inhibition caused by GAL-(1–16) was identical to that caused by GAL-(1–29).

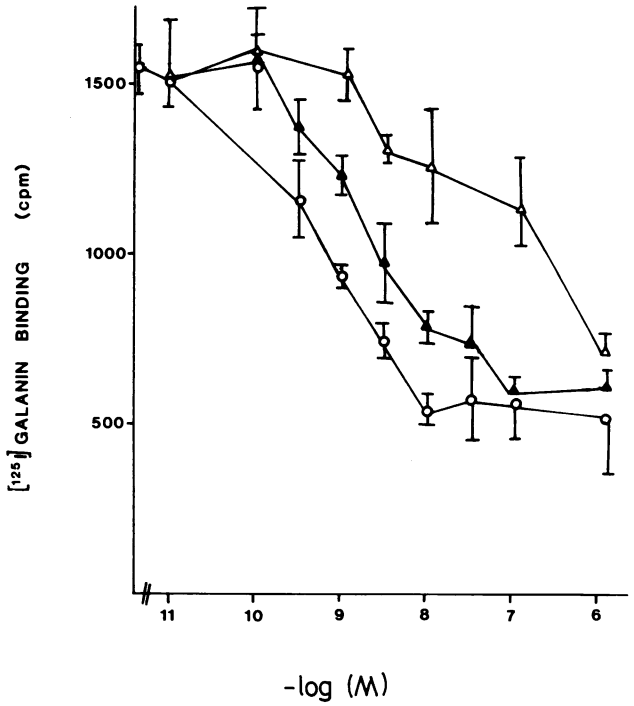

FIG. 1. Displacement of ^{125}I-GAL (1 nM) from binding sites in membranes from the rat ventral hippocampus by GAL (○), GAL-(1–16) (▲), and [D-Trp^2]GAL (△).

The fragment GAL-(1–16), when applied intracerebroventricularly (10 μg/15 μl), caused a long-lasting inhibition of the scopolamine (0.3 mg/kg, s.c.)-induced release of acetylcholine in the rat ventral hippocampus (Fig. 3). This inhibition was dose dependent in the range of 5–20 μg/15 μl, but it was not complete as observed earlier with GAL-(1–29) at 10 μg/15 μl (13). The GAL-(1–16) (5–20 μg/15 μl) did not affect the basal release of acetylcholine.

DISCUSSION

The endogenous ligand GAL is a 29-amino-acid-long C-terminally amidated peptide (1). The amino acid sequence of bovine (28), porcine (1), and rat (29) GAL differs only in four amino acid residues. These changes all occur in the 15–29 C-terminal fragment, whereas the N-terminal fragment is conserved among these species.

We demonstrate here that the N-terminal portion of the GAL molecule [GAL-(1–16)] is sufficient for recognition by high-affinity receptor sites for ^{125}I-GAL in the rat forebrain and in the hippocampus in particular, as well as in the spinal cord. This is evidenced by the ability of GAL-(1–16) to displace the ^{125}I-GAL in both equilibrium binding and autoradiography experiments. The affinity of the GAL-(1–16) is high (K_d ≈ 3 nM).

When this value is compared with the K_d of 0.7 nM for GAL-(1–29) in the same tissue, this suggests that the C-terminal portion [GAL-(17–29)] contributes very little to the free energy of the binding to the receptors in the ventral hippocampus. It is worth noting that [Trp^2] plays such a

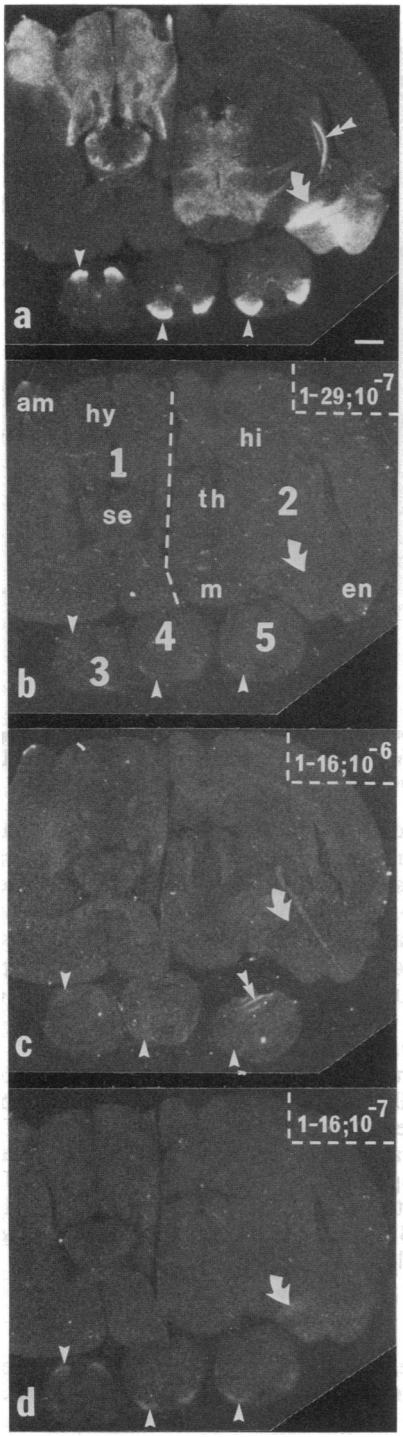

FIG. 2. Autoradiographs of transverse sections of the forebrain (*1* and *2*) at rostral (*1*) and caudal (*2*) hypothalamic levels and of three transverse sections of spinal cord (*3–5*) after incubation with ^{125}I-GAL (*a*), ^{125}I-GAL plus GAL-(1–29) (0.1 μM) (*b*), ^{125}I-GAL plus GAL-(1–16) (1 μM) (*c*), or ^{125}I-GAL plus GAL-(1–16) (0.1 μM) (*d*). Five sections from forebrain and spinal cord are shown as indicated by numbers *1–5* in *b*. Dotted line in *b* shows border between rostral (*1*) and caudal (*2*) brain slice. Note that *1* as well as spinal cord slices *4* and *5* are upside down. (*a*) Very strong binding is seen in the ventral hippocampus/subiculum (arrow in *2*) and the dorsal horns of the spinal cord (arrowheads in *3–5*). Less strong binding is seen in the amygdala (am), entorhinal cortex (en), hypothalamus (hy), and thalamic midline structures (th). (*b–d*) GAL-(1–29) blocks binding completely at 0.1 μM and GAL-(1–16) blocks binding completely at 1 μM (*c*) but not fully at 0.1 μM (*d*). In *d*, arrow and arrowheads point to weak binding. m, Mammillary body; hi, dorsal hippocampus; double arrowheads point to artifactual binding. (Bar = 1 mm.)

Table 1. Effect of GAL and GAL-(1–16) and its analog on muscarinic stimulation of inositol phospholipid breakdown in *myo*-[^{3}H]inositol-labeled tissue slices of rat ventral hippocampus

	[^{3}H]Inositol phosphate accumulated, % of total radioactivity incorporated
Control	6.66 ± 0.82 (n = 8)
Carbachol (0.1 mM)	29.38 ± 2.95* (n = 8)
GAL (1 μM) + carbachol (0.1 mM)	23.09 ± 2.37† (n = 8)
GAL-(1–16) (1 μM) + carbachol (0.1 mM)	24.71 ± 2.07† (n = 8)
[D-Trp^2]GAL-(1–16) (1 μM) + carbachol (0.1 mM)	29.05 ± 1.47‡ (n = 4)

GAL and GAL-(1–16) (1 μM) did not affect the incorporation of *myo*-[^{3}H]inositol or the breakdown of [^{3}H]inositol phospholipids.
*Significantly different from control.
†Significantly different from carbachol (0.1 mM) alone.
‡Not different from carbachol (0.1 mM) alone.

central role in the binding to the receptor that [D-Trp^2]GAL-(1–16) has 500-fold lower affinity than GAL-(1–16). The key role of [Trp^2] in binding to GAL receptors on Rin m 5F pancreatic β cells has also been shown recently (20). It should also be noted that the GAL-(1–16) that is generated both enzymatically and by solid-phase synthesis carries a free C-terminal carboxyl group, while the holopeptide GAL has a C-terminal amide.

Studies on the effect of GAL-(1–16) on the muscarinic agonist-mediated breakdown of [^{3}H]inositol phospholipids demonstrate that the fragment is not only a ligand but a full agonist, as it produces the maximal inhibition caused by GAL-(1–29) at the supramaximal concentration (1 μM) (17). It is also shown that [D-Trp^2]GAL-(1–16) at 1 μM cannot mimic the effects of GAL-(1–29). This is probably not fully explained by its substantially lower affinity, since some degree of receptor occupancy at 1 μM is expected. Thus, this substitution may affect both affinity and efficacy.

In vivo studies on the inhibition of the scopolamine-induced release of acetylcholine show that GAL-(1–16) behaves as an agonist in the presynaptic inhibitory control of acetylcholine release (12). The fragment GAL-(1–16) appears to be less efficient than GAL-(1–29) when applied in the same dose (10 μg/15 μl) (12). This may be a reflection of the somewhat lower affinity in combination with different diffusion and degradation rates for the fragment and for the holopeptide; alternatively, the fragment GAL-(1–16) may be more effective at post- than at presynaptic receptors in the hippocampus.

The above results together with those obtained on smooth muscle preparations with GAL-(1–10) (18) or GAL-(1–20) (19) and with GAL-(1–15) on the pancreatic β-cell line Rin m 5F (20) suggest that, unlike many other neuropeptides, the biological activity of the GAL molecule may reside in the N-terminal portion of the molecule rather than in the C-terminal portion. Studies with the C-terminal fragments [GAL-(10–29)] (20) and -(17–29) (G.F., unpublished data) showed no activity in the pancreas or the hippocampus, respectively.

It is important to study the effects of fragments and analogs of neuropeptides at different receptor sites that differ in their second messenger coupling such as the hippocampal (12, 13) GAL receptor that affects inositol phospholipid breakdown (17) versus pancreatic (30) GAL receptor, which inhibits K^+ channels (31) and inhibits adenylate cyclase activity (32). The GAL fragments may provide tools to define possible receptor subtypes. Another aim of the structure–activity relationship studies is to define the important amino acid residues and to produce ideas concerning the possible structure of neuropep-

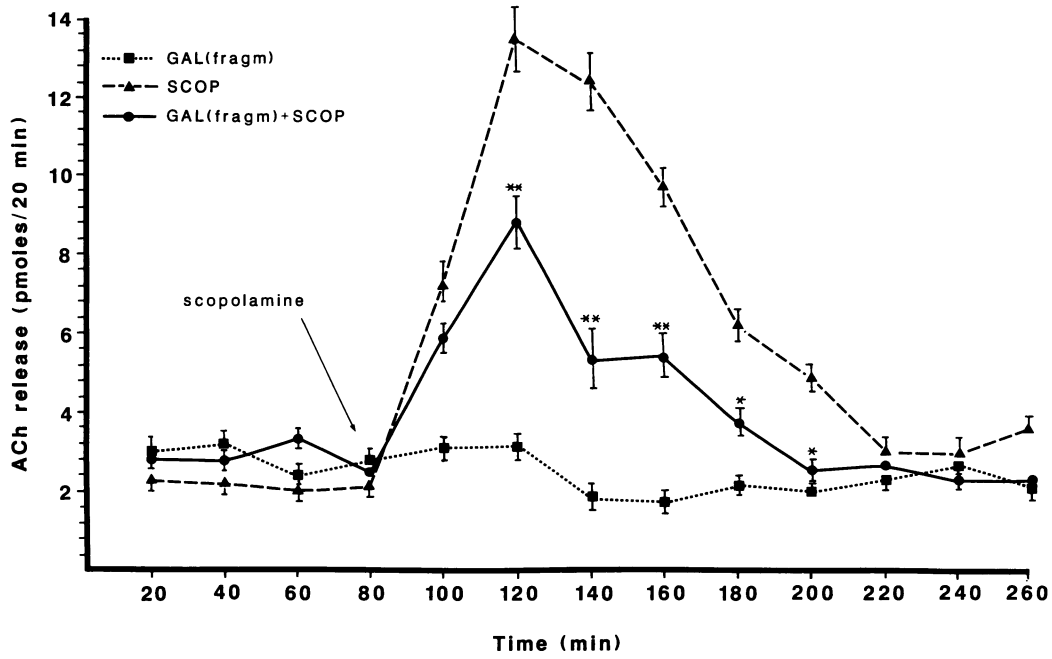

FIG. 3. Effect of GAL-(1–16) (10 μg/15 μl, intracerebroventricularly) on the scopolamine (0.3 mg/kg, s.c.)-induced release of acetylcholine (ACh) as a function of time in ventral hippocampus. GAL-(1–16) was injected 2 min before scopolamine. The Ringer's perfusion solution contained 10 μM physostigmine sulfate; the perfusion rate was 2 μl·min^{-1}. Perfusate was collected for 1 hr (three fractions, 20 min each fraction) before injection of GAL-(1–16) and/or scopolamine. The data are the means ± SEM from three animals. Interaction between GAL fragment and scopolamine: $^{*}P < 0.05$ and $^{**}P < 0.01$, split-plot and Tukey's test.

tide receptor ligands with pharmacological activities. Shortening the peptide by 13 amino acid residues, while retaining full agonist properties, may be a step in this direction.

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