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M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus, and spinal cord

(inhibitory neuropeptide/antagonist/release/receptor/nociceptive reflex)

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ABSTRACT **The 20-amino acid peptide M-15 binds with high affinity ($IC_{50} \approx 0.1$ nM) to ^{125}I-labeled galanin (^{125}I-GAL) binding sites in membranes from the ventral hippocampus, midbrain, and rat spinal cord. Receptor autoradiographic studies show that M-15 can displace ^{125}I-GAL from all labeled sites. M-15 acts as a reversible high-affinity antagonist in blocking the inhibitory effects of GAL on the evoked release of acetylcholine *in vivo* in the hippocampus and on the GAL-induced hyperpolarization of locus coeruleus neurons in slices. M-15 also blocks the facilitatory effects of GAL on the spinal flexor reflex. Thus, the chimeric peptide M-15 [GAL-(1–13)–substance P-(5–11) amide] represents the first antagonist to the neuronal actions of GAL.**

Since its discovery in 1983 by Tatemoto, Mutt, and collaborators (1), galanin (GAL) has received much attention as a pluripotent neuropeptide with wide-spread distribution in the endocrine, peripheral, and central nervous system (CNS) (2, 3). Frequently GAL has been shown to coexist with classical transmitters and other peptides (4)—for example, with noradrenaline in the locus coeruleus (LC) (4) and with acetylcholine (AcCho) in some somata in the basal forebrain–septum complex projecting to hippocampus (5). In fact, interactions between classical transmitters and GAL have been shown in several studies. For example, a strong inhibitory action of GAL on the evoked release of AcCho in the ventral hippocampus has been shown in rats *in vitro* and *in vivo* (6), and GAL blocks the slow cholinergic excitatory postsynaptic potential in CA1 pyramidal neurons in the ventral hippocampus (7). GAL also inhibits the amelioration of cognitive deficits in lesioned rats induced by exogenous AcCho (8) and attenuates acquisition in the Morris swim maze model (9). GAL suppresses the firing of LC neurons of rat (10), and in the rat spinal cord, it has biphasic effects on the flexor reflex involving a possible inhibition of substance P's effect (11, 14).

The above studies and many other investigations (see ref. 3) were carried out with exogenously applied synthetic GAL to explore the pharmacological effects of occupancy of GAL receptors, but because of a lack of antagonists to GAL, the specificity of the actions of exogenous GAL could not be established. The present paper describes a GAL receptor antagonist that binds with high affinity to ^{125}I-labeled GAL (^{125}I-GAL) binding sites in the CNS and antagonizes the effects of GAL in all systems examined. The structure of this peptide is based on previous studies (refs. 12, 13, and 15; U.L., T.L., and T.B., unpublished data), which have demonstrated that the N-terminal Gal-(1–16) hexadecapeptide or Gal-(1–15) pentadecapeptide fragment of the 29-amino acid GAL molecule is the part required for high-affinity recognition by receptors in the hippocampus, spinal cord, and pancreas. The antagonist presented here, M-15, is a chimeric, 20-amino acid peptide composed of GAL-(1–13)–substance P-(5–11) amide (U.L., T.L., and T.B., unpublished data). Its ability to bind to GAL receptors is preserved, and it also acts as a high-affinity reversible antagonist at these sites.

MATERIALS AND METHODS

Peptide Synthesis. The peptides were assembled in a stepwise manner on a solid support by using an Applied Biosystems model 431A peptide synthesizer with the standard *N*-methylpyrrolidone /hydroxybenzotriazole solvent-activation strategy on a 0.1-mmol scale (small scale). *tert*-Butoxycarbonyl (*tert*-Boc)-conjugated amino acids were coupled to tert-Boc-amino acid-PAM *p*-acetoxymethyl resin (Nova Biochem, Laufelfingen, Switzerland) or MBHA (4-methylbenzhydrylamine) resin (Bachem) as hydroxybenzotriazole esters. All the solvents and other reagents for automatic peptide synthesis were from Applied Biosystems. The reagents used in deprotection and cleavage steps were of analytical grade and were used without further purification. The peptides were cleaved from resin, deprotected, and purified as described earlier (U.L., T.L., and T.B., unpublished data). Purity of the individual peptides was checked by analytical HPLC and determined to be 99%. Molecular weights of the peptides were determined by using Plasma Desorption Mass Spectrometer model Bioion 20 (Applied Biosystems).

Preparation of Porcine [Mono(^{125}I)iodo-Tyr^{26}]GAL. Synthetic porcine GAL-(1–29) was iodinated by the chloramine-T method to yield porcine [mono(^{125}I)iodo-Tyr^{26}]GAL (specific activity, 1800–2000 Ci/mmol; 1 Ci = 37 GBq) as described (U.L., T.L., and T.B., unpublished data) and was used in equilibrium-binding studies.

Preparation of Membranes. The tissue homogenates of rat ventral hippocampus, midbrain, and spinal cord were prepared as described (15). The resulting pellet (P_2) was resuspended in bacitracin-containing (1 mg/ml) 5 mM Hepes-buffered Ringer solution (137 mM NaCl/2.68 mM KCl/1.8 mM $CaCl_2$/2.05 mM $MgCl_2$/1 g of glucose per liter, pH 7.4) and used immediately in equilibrium binding experiments.

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Abbreviations: GAL, galanin; AcCho, acetylcholine; TTX, tetrodotoxin; LC, locus coeruleus; i.t., intrathecal; CNS, central nervous system; Scop, scopalamine; i.c.v., intracerebroventricularly.

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Displacement of ^{125}I-GAL by GAL and GAL Receptor Ligands. Displacement experiments were carried out in bacitracin-containing (1 mg/ml) Hepes-buffered (5 mM) Ringer solution (pH 7.4) containing 0.05% bovine serum albumin in the presence of 0.1–0.2 nM ^{125}I-GAL, the membrane preparation, and increasing concentrations (1 pM to 1 μM) of unlabeled porcine GAL or of other GAL receptor ligands as described (16). The IC_{50} values of the displacing ligands were calculated, and the experimental data were fitted on a Macintosh SE computer by means of a nonlinear least-squares method with the program KALEIDAGRAPH.

Autoradiography. GAL binding sites were analyzed as described (17, 18). Briefly, fresh tissue of posterior telencephalon including mesencephalon, medulla oblongata, and several levels of spinal cord were mounted on a chuck and cut in a cryostat (Dittes) at a thickness of 14 μm. The sections were incubated with porcine ^{125}I-GAL (2200 Ci/mmol; NEN) in Hepes buffer for 45 min at room temperature, rinsed, and dried in a stream of cold air and then exposed to Hyperfilm-βmax autoradiography film (Amersham) for 1–4 weeks, fixed, and developed. M-15 (1 μM) was added to the incubation medium 10 min before the labeled GAL. Unlabeled GAL (Bachem) at 1 μM was added to the incubation medium to determine unspecific binding.

Dialysis *in Vivo*. In the *in vivo* AcCho-release experiments, female CD-COBS (Charles River Breeding Laboratories) rats (200–280 g) were used. The microdialysis probe (CMA 10, Carnegie Medicine AB) was implanted stereotaxically into the ventral hippocampus of one side at the following coordinates: 5 mm posterior to bregma, 4.8 mm lateral to midline, and 6.8 mm below the surface of the dura mater (19). The day after implantation, the dialysis probe was perfused at a constant rate of 2 μl/min with Ringer solution (147 mM NaCl/2.2 mM $CaCl_2$/4.0 mM KCl) containing 10 μM physostigmine sulfate and was adjusted to pH 7.0 with NaOH. AcCho content was quantified by a specific radioenzymatic method as described (20). The amount of AcCho in each 20-min sample was calculated by linear regression based on the radioactivity of the standards (linear from 10 fmol–25 pmol of AcCho) with the slope equal to 7800 net dpm/pmol. The coefficient of variation of the replicate perfusate samples or AcCho standards was ≈3%. At the end of the release experiment, the placement of the dialysis probe was verified histologically by staining for Nissl substance.

Electrophysiology. Male Sprague–Dawley rats (200–300 g) (Anticimex) were decapitated, the brain was rapidly dissected out, and 300- to 350-μm horizontal Vibratome slices were made in oxygenated ice-cold Ringer solution (124 mM NaCl/2.5 mM KCl/1.2 mM $MgSO_4$/1.24 mM NaH_2PO_4/2.4 mM $CaCl_2$/25 mM $NaHCO_3$/10 mM glucose). A slice containing the LC was transferred to a submersion-type slice chamber maintained at 37°C. A Ringer solution saturated with 95% O_2/5% CO_2 at 37°C was perfused through the chamber at 1.5 ml per min. Slices were allowed to remain in the chamber 2–3 hr prior to recording. The LC could be easily identified in the transilluminated slice as a dark oval area on the lateral edge of the fourth ventricle (21). The slice was stabilized by gently pressing a nickel EM grid held in a micromanipulator down on the slice. The LC was then probed with intracellular microelectrodes filled with 3 M potassium acetate (d.c. resistances between 80 and 120 MΩ). Only LC neurons that had resting potentials more negative than −55 mV, action potentials that overshot zero by >5 mV, and membrane resistances greater than 80 MΩ were studied (range = 90–200 MΩ). Generally stable recordings of cells could be maintained for 4–6 hr. The tip of a calibrated (60 nl per division) micropipette (tip o.d. = 10 μm) containing 10 μM GAL in normal Ringer's solution was positioned ≈100–200 μm above the slice. Nitrogen pulses of constant duration and pressure were applied to the end of the pipette via a Picospritzer (General Valve). M-15 (1 μM) was added immediately prior to use. In some experiments, tetrodotoxin (TTX) (1 μM) was added and/or Ca^{2+} was removed and replaced with equimolar Mg^{2+}.

Reflex Experiments. The magnitude of the hamstring flexor reflex in response to activation of high-threshold afferents was examined in decerebrate, spinalized, unanesthetized Sprague–Dawley (Anticimex) rats. The animals were initially briefly anesthetized with methohexital (Brietal, Lilly, 70 mg/kg of body weight, i.p.), and a tracheal cannula was inserted. The rats were mounted in a stereotaxic frame, decerebrated by aspiration of the forebrain and midbrain, and then ventilated. No further anesthetic was administered after decerebration. The spinal cord was exposed by a laminectomy at mid-thoracic level and sectioned at Th8-9. An intrathecal (i.t.) catheter (PE 10) was implanted caudally to the transection with its tip on the lumbar spinal cord (L4-5). The flexor reflex was elicited by test stimuli of single electric subcutaneous shocks (one per min) to the sural nerve innervation area with sufficient strength (1 ms at 10 mA) to activate C fibers. The flexor reflex was recorded as electromyogram activity via stainless steel needle electrodes inserted in the ipsilateral posterior biceps femoris/semitendinosus muscles. The number of action potentials elicited during the reflex was integrated over 2 s. During the experiments, the heart rate and rectal temperature of the rat were monitored. GAL (Bachem) and M-15 were dissolved in 0.9% saline and injected i.t. in a 10-μl volume followed by 10 μl of saline to

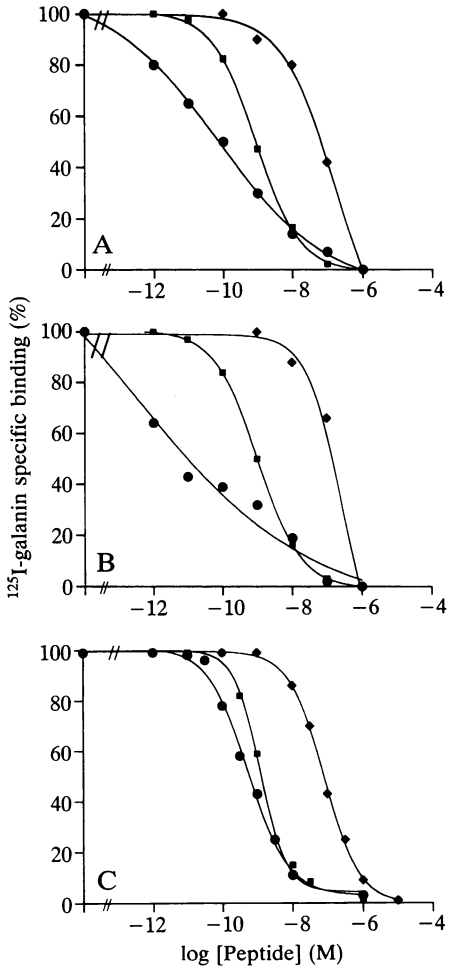

FIG. 1. Displacement of ^{125}I-GAL from membranes of rat ventral hippocampus (*Top*), midbrain (*Middle*), and spinal cord (*Bottom*) by porcine GAL [i.e., GAL-(1–29)] (■), M-15 (●), and the N-terminal fragment [i.e., GAL-(1–13)] (◆).

flush the catheter. Before drug administration, a stable baseline reflex magnitude was established for at least 30 min.

RESULTS

Binding Studies. Displacement of [mono(^{125}I)iodo-Tyr^{26}] GAL from GAL receptors by M-15 was studied in membranes from the rat ventral hippocampus (Fig. 1 *Top*), rat midbrain (Fig. 1 *Middle*), and rat spinal cord (Fig. 1 *Bottom*). M-15 (Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) in all three membrane preparations fully displaced 0.2 nM ^{125}I-GAL in a concentration-dependent manner. The IC_{50} value for M-15 in all three membrane preparations was 0.1 nM while the Hill coefficient of the binding of M-15 for hippocampal and midbrain membrane GAL receptors was significantly less than unity; in spinal cord membranes, the Hill coefficient was not significantly different from unity. Substance P-(5–11) did not bind to GAL receptors in any of these tissues (data not shown). GAL-(1–13) (i.e., the N-terminal portion of M15) is recognized by GAL receptors with a lower affinity by a factor of 1000 than M15. Applying equimolar concentrations of GAL-(1–13) and substance P-(5–11) yielded the same displacement curves as those obtained with GAL-(1–13) alone, showing that GAL-(1–13) and substance P-(5–11) must be covalently joined to yield a high-affinity ($IC_{50} \approx 0.1$ nM) ligand such as M-15. This suggests that there may be intramolecular interactions between the N- and C-terminal portions of the M-15 peptide that account for the high-affinity binding to GAL receptors and also for the antagonist-like properties shown in the functional experiments below.

Autoradiography. A very high density of ^{125}I-GAL binding sites was observed in the dorsal horn of the spinal cord and in the spinal trigeminal nucleus (Fig. 2*A*). A moderately dense binding was seen in the area around the central canal (spinal cord, lower medulla oblongata) and in the ventral hippocampus, extending into subiculum, the posterior amygdaloid complex, and entorhinal cortex in the brain (Fig. 2*a*). Several other brain areas, such as ventral mesencephalon and periaqueductal central grey, exhibited a weak binding (Fig. 2*a*). Addition of 1 μM M-15 peptide blocked binding of ^{125}I-GAL to all of these binding sites (Fig. 2*b*), as did addition of 1 μM GAL peptide (data not shown).

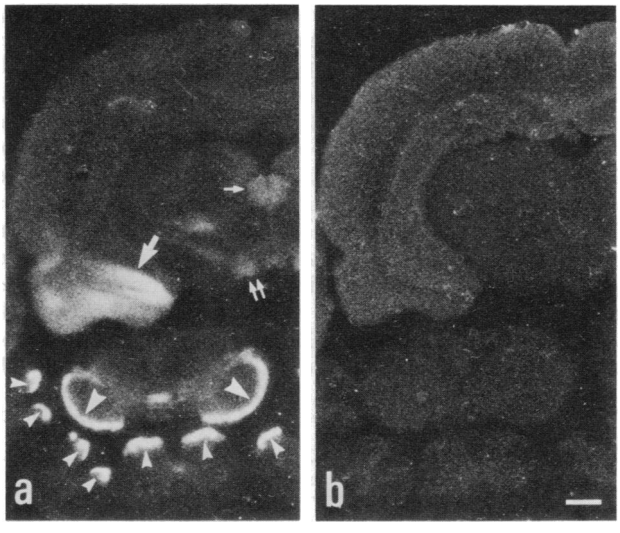

FIG. 2. Film autoradiographs of transverse sections of the brain (mesencephalic level), medulla oblongata, and various levels of the spinal cord after incubation with ^{125}I-GAL (*a*) or ^{125}I-GAL with added 1 μM M-15 (*b*). Semiadjacent sections are shown. Note in *a* strong labeling in the ventral hippocampus/subiculum (arrow), spinal trigeminal nucleus (big arrowheads), and dorsal horns of the spinal cord (small arrowheads) and in *b* its complete blockade by addition of 1 μM M-15. The single small arrow indicates periaqueductal central grey, and the double small arrows indicate the central pigmental area. (Bar = 1 mm.)

***In Vivo* Dialysis.** Release of endogenous AcCho from ventral hippocampus was evoked by the muscarinic antagonist scopolamine (Scop) given s.c. at a dose of 0.3 mg/kg. Scop raised AcCho output by about 460% within 40 min (Fig. 3). The AcCho efflux remained on average 300% above baseline for at least 60 min and then gradually returned to control values. GAL at the dose of 1.56 nmol, i.c.v., reduced by about 50% the Scop-stimulated AcCho release but, by itself, did not affect basal AcCho release from ventral hippocampus (Fig. 3).

The GAL antagonist M-15 (9.36 nmol), injected i.c.v. in combination with GAL, fully prevented the effect of GAL on Scop-evoked AcCho output. At one-third of the above dose (3.12 nmol), it still antagonized GAL's effect by about 15%. M-15 by itself did not affect basal AcCho release from ventral hippocampus either at a 9.36- or 3.12-nmol dose.

Electrophysiology. The initial application of GAL (10 pmol in 1 μl of Ringer solution) induced a hyperpolarization of 20

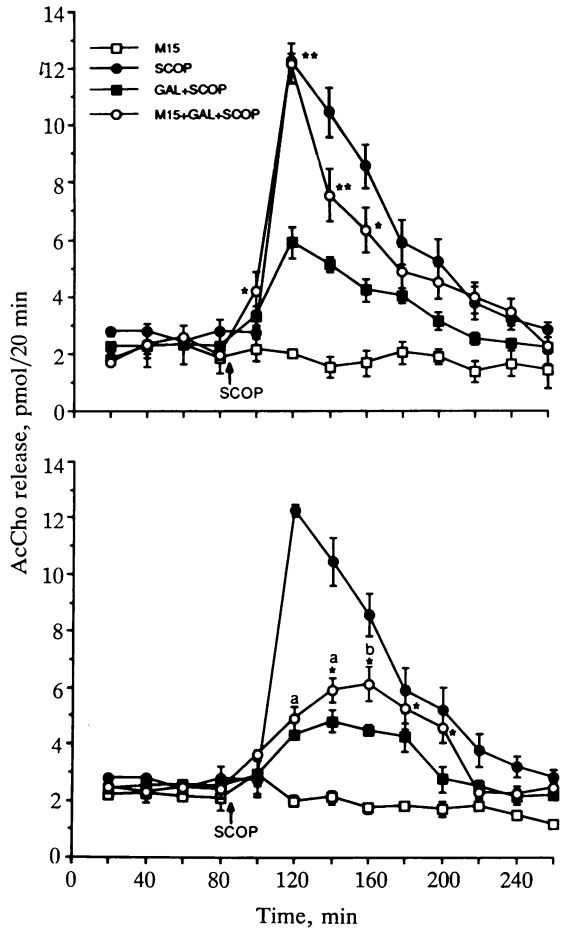

FIG. 3. Dose–response effect of M-15 alone or in combination with GAL on Scop-induced AcCho release as a function of time in rat ventral hippocampus. M-15 [9.36 (*Upper*) or 3.12 (*Lower*) nmol] and GAL (1.56 nmol) were injected intracerebroventricularly (i.c.v.) 2 min before Scop (0.3 mg/kg, s.c.). Perfusate was collected for 80 min (four fractions, 20 min for each fraction) before GAL and/or M-15 and/or Scop injections. AcCho release in GAL + Scop group was significantly ($P < 0.01$) different from that of the Scop group at any time. (*Upper*) Interactions M-15 + GAL + Scop versus GAL + Scop: **, $P < 0.01$; and *, $P < 0.05$. (*Lower*) Interactions M-15 + GAL + Scop versus Scop: a, $P < 0.01$; and b, $P < 0.05$. Split plot and Tukey's test were used.

mV; subsequent applications of GAL resulted in hyperpolarizations of 12 mV (mean; $n = 8$). The effects of M-15 were studied on the latter responses (Fig. 4*A*). This hyperpolarization lasted ≈3 min and had a rapid onset and a slow decay. When dc current injection was used to temporarily restore the membrane potential to resting levels during the GAL-induced hyperpolarization, a decrease in the membrane resistance to hyperpolarizing pulses became apparent. The GAL-induced hyperpolarization was still present, although reduced (mean = 12 mV for 1.3 min; $n = 4$), in neurons that were exposed to media containing 1 μM TTX and low Ca^{2+}/high Mg^{2+}.

M-15 added at 1 μM caused an increase in the spontaneous spike discharge in LC neurons but did not appear to dramatically (<3 mV) alter the resting membrane potential. In LC neurons ($n = 4$) exposed to M-15 for 2–5 min, GAL failed to induce a hyperpolarization, although it reduced spontaneous activity. Partial recovery of the GAL-induced hyperpolarization could be achieved after extended washout of M-15 (3–4 hr). When M-15 was applied to cells ($n = 4$) exposed to Ringer solution with TTX (1 μM), low Ca^{2+}, and high Mg^{2+} to block synaptic activity, it occasionally induced a small depolarization (3–4 mV) and abolished the GAL-induced hyperpolarization (Fig. 4*B*).

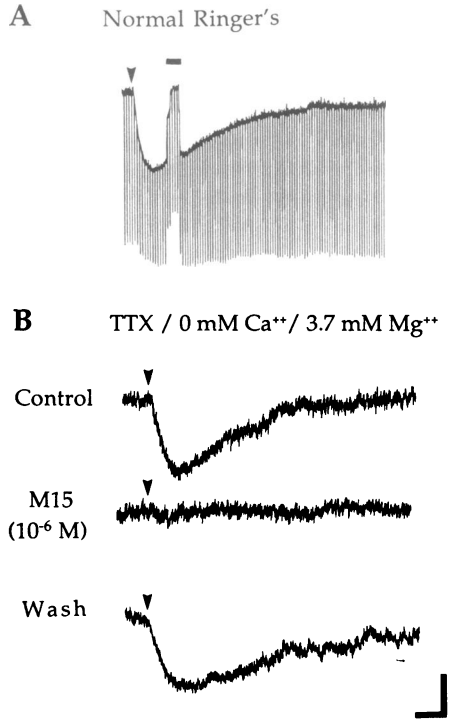

FIG. 4. GAL-induced hyperpolarization of LC neurons *in vitro*. (*A*) Intracellular voltage record of a LC neuron. GAL (10 pmol in 1 μl) was applied at the arrowhead. The membrane resistance was monitored by the application of −200-pA hyperpolarizing pulses. GAL induced a hyperpolarization of the neuron, accompanied by a decrease in membrane resistance, which is revealed when the cell's membrane potential is artificially restored to rest (−55 mV) by the injection of dc current into the cell (at bar). (*B*) The response of a different LC neuron to GAL in a Ringer's solution containing 1 μM TTX, low (0 mM) Ca^{2+}, and high (3.7 mM) Mg^{2+}. All traces are from the same neuron. Identical concentrations of GAL (10 pmol) are applied at the arrowheads. The resting membrane potentials are the same (−54 mV) in all traces. The duration of the GAL-induced hyperpolarization is reduced (note the difference in time scales in *A* and *B*) in this medium, but the amplitude of the response is similar. The GAL-induced hyperpolarization seen in the control medium is abolished in the presence of M-15. The antagonist is then washed out (30 min), and the response to GAL recovers. (Bars = 10mV/40s in *A* and 5 mV/13 s in *B*.).

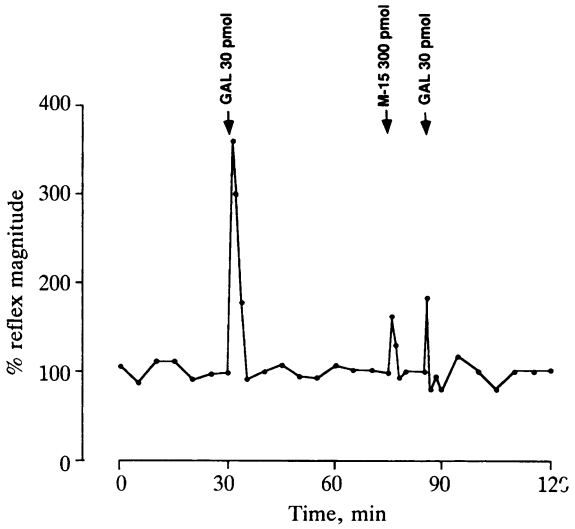

FIG. 5. The antagonism by i.t. M-15 (300 pmol) of the facilitation of the flexor reflex induced by 30 pmol of GAL. Baseline reflex magnitude is expressed as 100%. M-15 was injected 10 min prior to GAL and antagonized the facilitatory effect of GAL by about 70%.

Reflex Experiments. GAL administered i.t. at a dose of 30 pmol briefly facilitated the flexor reflex with a peak increase of 167 ± 42.8% over baseline reflex magnitude for 4.0 ± 0.9 min; i.t. M-15 (30 pmol–3 nmol) also caused facilitation of the flexor reflex. Administered 5–10 min before i.t. GAL, M-15 dose-dependently antagonized the facilitatory effect of GAL (Fig. 5 and Table 1).

DISCUSSION

Structure activity studies on the effects of GAL fragments and analogues in the CNS have shown that it is the N-terminal portion of GAL that is of importance for the recognition by the receptor, and the important amino acids within this fragment have been determined (15). Against this background a series of chimeric, bireceptor-recognizing peptides utilizing the N-terminal Gal-(1–13) fragment and C-terminal portions of some other bioactive peptides have been synthesized. Here we report the effect of one of those chimeric peptides, M-15, also termed galantide (22), in a number of models that previously have been shown to be suitable for analysis of GAL's effects. In all cases, the antagonist was able to completely or markedly reduce the effects of exogenous GAL. Thus, M-15 could displace ^{125}I-GAL when studied in membranes from the rat ventral hippocampus, midbrain, and spinal cord. In autoradiographic experiments on rat brain and spinal cord, ^{125}I-GAL binding was completely blocked in all CNS regions analyzed by 1 μM M-15. In the ventral hippocampus, M-15, when injected i.c.v. in combination with

Table 1. Antagonistic effect of i.t. M-15 on the i.t. GAL-induced facilitation of the flexor reflex

Dose of M-15	*n*	% antagonism
30 pmol	6	26.3 ± 14.4
300 pmol	5	71.4 ± 7.7*
3 nmol	5	87.7 ± 3.0*

The peak facilitatory effect of 30 pmol of GAL was 146.3 ± 32.8% over baseline reflex magnitude. The antagonism of GAL by M-15, injected 5–10 min before GAL, was calculated as the percentage reduction of the peak facilitatory effect of GAL. Data are expressed as means ± SEM. Analysis of variance indicated that there is a significant overall antagonistic effect on GAL-induced reflex facilitation by M-15 ($F_{2,14} = 10.857$; $P < 0.01$). *, $P < 0.005$ compared with zero antagonism with Dunnett's test.

GAL, fully prevented the inhibitory effect of GAL on Scop-evoked AcCho output. M-15 is also shown to be active in the LC, which contains neurons in which noradrenaline and GAL coexist (4). The LC neurons possess GAL binding sites (23, 24), and it has previously been reported in extracellular recording experiments that GAL causes inhibition of the firing of these neurons (10). We here report that GAL produces hyperpolarization of LC neurons, presumably via a direct effect, since it is present also in synaptic blockade media. This inhibition can be completely counteracted by 1 μM M-15. Whether exogenous GAL in this paradigm mimics GAL release from afferent inputs or its effect is related to a local soma-somatic and/or dendro-somatic synapses between LC neurons (25–28) needs clarification (29). The effect of i.t. GAL on the flexor reflex is complex, with facilitation in low doses and inhibition at higher doses (11). Here we show that the antagonist can markedly antagonize the facilitatory effect of low doses of exogenous GAL. Moreover, M-15 alone causes facilitation of the flexor reflex, presumably due to inhibition of the effects of the endogenously released GAL that inhibits the reflex.

The present findings provide evidence for the GAL antagonist M-15 as an additional tool in the analysis of GAL systems in the nervous system. In a parallel study, it has been shown that M-15 in a dose-dependent fashion can antagonize the GAL-mediated inhibition of the glucose-induced insulin secretion from mouse pancreatic islets (22). The antagonist also displaced ^{125}I-GAL from membranes of insulin producing Rin M5F cells (22). Thus, this GAL antagonist is also useful for analysis of endocrine events. Besides M-15, a series of chimeric peptide antagonists have been synthesized, which also show antagonistic properties to GAL. Several of these are, in fact, more potent than M-15 (unpublished results) and should further improve our possibility to establish the functional roles of GAL.

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