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Galanin-receptor ligand M40 peptide distinguishes between putative galanin-receptor subtypes

(peptide antagonist/acetylcholine release/nociceptive reflex/feeding/insulin)

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ABSTRACT **The galanin-receptor ligand M40 [galanin-(1–12)-Pro$_3$-(Ala-Leu)$_2$-Ala amide] binds with high affinity to [mono[^{125}I]iodo-Tyr^{26}]galanin-binding sites in hippocampal, hypothalamic, and spinal cord membranes and in membranes from Rin m5F rat insulinoma cells (IC_{50} = 3–15 nM). Receptor autoradiographic studies show that M40 (1 μM) displaces [mono[^{125}I]iodo-Tyr^{26}]galanin from binding sites in the hippocampus, hypothalamus, and spinal cord. In the brain, M40 acts as a potent galanin-receptor antagonist: M40, in doses comparable to that of galanin, antagonizes the stimulatory effects of galanin on feeding, and it blocks the galaninergic inhibition of the scopolamine-induced acetylcholine release in the ventral hippocampus *in vivo*. In contrast, M40 completely fails to antagonize both the galanin-mediated inhibition of the glucose-induced insulin release in isolated mouse pancreatic islets and the inhibitory effects of galanin on the forskolin-stimulated accumulation of 3′,5′-cAMP in Rin m5F cells; instead M40 is a weak agonist at the galanin receptors in these two systems. M40 acts as a weak antagonist of galanin in the spinal flexor reflex model. These results suggest that at least two subtypes of the galanin receptor may exist. Hypothalamic and hippocampal galanin receptors represent a putative central galanin-receptor subtype (GL-1-receptor) that is blocked by M40. The pancreatic galanin receptor may represent another subtype (GL-2-receptor) that recognizes M40, but as a weak agonist. The galanin receptors in the spinal cord occupy an intermediate position between these two putative subtypes.**

Galanin is an important neuroendocrine peptide with multiple biological and pharmacological actions (1). It is a potent inhibitor of glucose-induced insulin release (2), it inhibits hippocampal acetylcholine release (3) induced by systemic administration of scopolamine (4), it impairs cognitive performance (5, 6), it stimulates feeding behavior upon hypothalamic or intracerebroventricular injection (7, 8), it stimulates growth hormone secretion (9), and it has a biphasic effect on the spinal flexor reflex (10). Galanin hyperpolarizes noradrenergic cell bodies in the locus coeruleus (11) and inhibits the release of glutamate, but not of γ-aminobutyric acid, in the hippocampus (12). This fragmentary list of the effects of exogenously applied galanin explains much of the interest in this neuropeptide in psychopharmacology, in endocrinology, in prevention of anoxic damage (13), and in management of chronic pain and explains the interest in galanin antagonists as possible therapeutic agents in Alzheimer disease (14, 15).

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Galanin receptors have been characterized by means of ^{125}I-labeled galanin in binding experiments on cells (16) and on membranes from different tissues (17) and by receptor autoradiography (18, 19) in the central nervous system (CNS) and in the periphery. It was shown that actions mediated by galanin receptors involve pertussis toxin-ADP-ribosylable G proteins of inhibitory (G_i) or regulatory (G_o) type (20–22). Furthermore, crosslinking studies using ^{125}I-labeled galanin have shown that the molecular size of the receptor in SDS gels was 54 kDa (23), close to the value found for most G protein-coupled receptors with seven membrane-spanning domains (24). The cDNA coding for the galanin receptor or its subtypes has not yet been cloned; thus subtyping of galanin receptors must be based on comparing rank order of potency of different galanin-receptor agonists and antagonists.

Accordingly, a galanin-receptor subtype has been suggested, which is composed of nervous tissue and pancreatic galanin receptors that recognize the N-terminal 1- to 15-aa or 1- to 16-aa fragment of galanin as high-affinity agonists, whereas another putative galanin-receptor subtype in smooth muscle requires both the N and C terminus of galanin for binding and biological action (25). On the basis of the differential affinity of galanin (3–29) and of a galanin-receptor antagonist M15 (17), existence of an additional galanin-receptor subtype has been suggested in the rat anterior pituitary (26), which differs from other CNS galanin receptors. Finally, galanin-(1–15)-binding sites have been demonstrated in the dorsal hippocampus, neocortex, and neostriatum, areas that seem to lack galanin-(1–29)-binding sites (27).

The synthesis of chimeric galanin-receptor ligands such as galantide [M15, galanin-(1–12)-Pro-substance P-(5–11) amide] (17), M35 [galanin-(1–12)-Pro-bradykinin-(2–9) amide] (28), M40 [galanin-(1–12)-Pro$_3$-(Ala-Leu)$_2$-Ala amide] (29), and C7 [galanin-(1–12)-Pro-spantide] (29) provided a tool for characterization of certain actions of endogenous galanin, as these compounds and a dozen of their analogs showed high affinity (K_d, 10^{-10} M) for galanin receptors in several tissues. Interest in these compounds has been considerable, and in the past year their use has been reported in numerous experimental systems (cf. refs. 1 and 30).

The synthetic galanin-receptor ligand M40 not only acts as a high-affinity galanin-receptor ligand but also appears to differentiate between different galanin-receptor subtypes in terms of agonist–antagonist efficacy. The results with this ligand may form the pharmacological basis for definition of galanin-receptor subtypes, while awaiting the molecular cloning of galanin receptors.

Abbreviation: CNS, central nervous system.

MATERIALS AND METHODS

Peptide Synthesis. The peptides M40, M35, and galanin were synthesized in a stepwise manner on a solid support by using Applied Biosystems model 431A peptide synthesizer. The synthesis, cleavage from resin, purification, and characterization of the peptides have been described in detail (31). The sequence of the M40 peptide is as follows: galanin-(1–12)-Pro-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide (or GWTLNSAGYLLGPPPALALA amide).

The peptides were analyzed by means of plasma desorption mass spectrometry on a BioIon20 (Applied Biosystems) spectrometer, and the calculated molecular masses were found.

Binding Experiments. Preparation of [mono[^{125}I]iodo-Tyr^{26}]galanin (porcine) and of the membranes from different rat tissues (32) and the subsequent displacement of ^{125}I-labeled galanin by galanin-receptor ligands have been described (17, 32).

Receptor Autoradiography. Galanin-binding sites were analyzed as described (19, 33). Briefly, fresh tissue of rat telencephalon, diencephalon, spinal cord, and pituitary gland was mounted on a chuck and cut in a cryostat (Microm, Heidelberg) at a 4-μm thickness. The sections were incubated with porcine ^{125}I-labeled galanin (2200 Ci/mmol; NEN; 1 Ci = 37 GBq) in Hepes buffer for 45 min at room temperature, rinsed, dried in a stream of cold air, and then exposed to Hyperfilm-βmax autoradiography film (Amersham) for 1–4 weeks, fixed, and developed. Galanin-(1–29) (Bachem) or M40, both at 10^{-6} M, were added to the incubation medium 10 min before the labeled galanin.

Behavioral Testing for Feeding. Galanin-induced feeding behavior of the rats was tested as described (29, 34). Male Sprague–Dawley rats, maintained on a normal rat chow diet, were treated with Ringer vehicle, Ringer plus galanin (0.5 nmol), or M40 (0.25 or 0.5 nmol) plus galanin, microinjected into the paraventricular nucleus of the hypothalamus, 1.8 mm posterior to bregma, 0.4 mm bilateral to bregma, and 8 mm ventral to the surface of the skull, immediately before a 30-min exposure to a palatable wet cookie mash. Grams consumed during the 30-min test session were compared between treatment groups by one way analysis of variance, followed by Student–Newman–Keuls post hoc test.

Microdialysis *in vivo* and Reflex Experiments. The *in vivo* acetylcholine-release experiments and the magnitude of the hamstring flexor reflex in response to activation of high-threshold afferents have been described (17).

Determination of Insulin Secretion. Determination of glucose-induced insulin secretion from incubated isolated mouse pancreatic islets has been reported (35). The collagenase-isolated islets were cultured overnight and, after a 45-min preincubation, incubated for 45 min in Krebs/Ringer/bicarbonate medium supplemented with 3.3 or 11.1 mM glucose with or without addition of synthetic rat galanin or M40 at various dose levels.

Galanin-Mediated Inhibition of Forskolin-Stimulated 3′,5′-cAMP Accumulation. This inhibition was studied by using Rin m5F rat insulinoma cells preincubated at 37°C for 30 min with the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (0.2 mM), aprotinin (10 μg/ml), bacitracin (0.5 mg/ml), and bovine serum albumin (2%, wt/vol). Forskolin (5 μM), galanin (10 nM), and M40 (1, 10, and 100 nM) were added to the cell suspension of 10^6 cells in a total volume of 360 μl. Incubation was then continued for 30 min and stopped by adding 300 μl of ice-cold medium, followed by centrifugation in an Eppendorf microcentrifuge. The supernatant was aspirated off, and 300 μl of boiling EDTA (50 mM) was added to the cells. The 3′,5′-cAMP content was determined by using a 3′,5′-[^{3}H]cAMP-binding assay after freezing and thawing the samples.

RESULTS

The peptide M40 displaced [mono[^{125}I]iodo-Tyr^{26}]galanin from galanin-binding sites in membranes from the hippocampus, hypothalamus, spinal cord, and from Rin m5F rat insulinoma cells (Fig. 1). Peptide M40 had a lower affinity than galanin for all membranes. The IC_{50} values were 6 nM

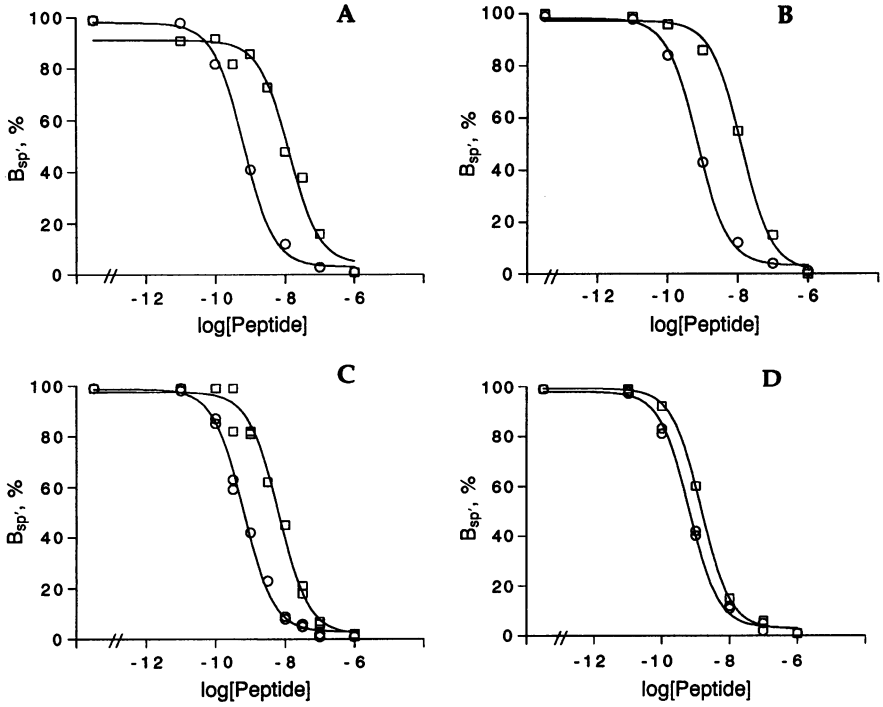

FIG. 1. Displacement of 0.2 nM [mono[^{125}I]iodo-Tyr^{26}]galanin by galanin (○) and M40 (□) from the membranes of rat ventral hippocampus (*A*), rat hypothalamus (*B*), rat lumbar dorsal spinal cord (*C*) and from rat insulinoma Rin m5F cells (*D*). B_{sp}' is defined as binding (cpm)/total binding (cpm).

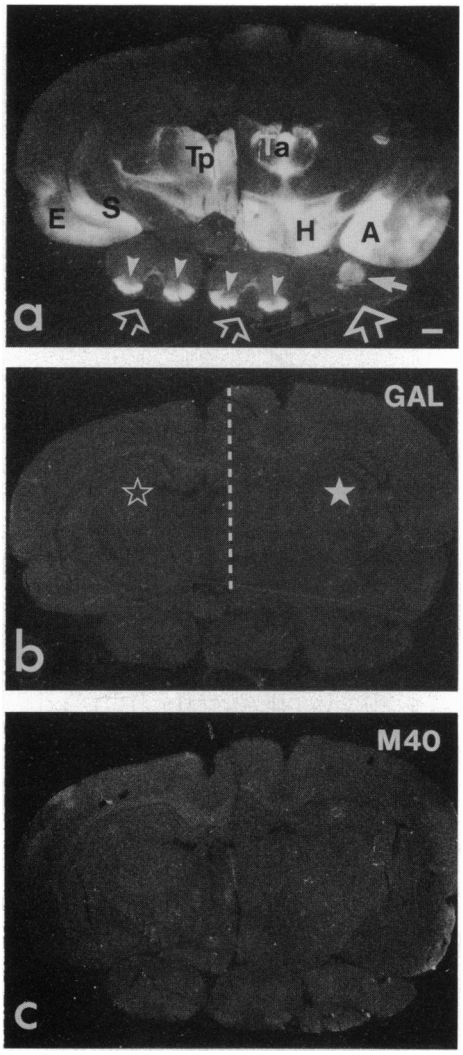

FIG. 2. Film autoradiograms of semi-adjacent, frontal sections of a "cocktail" of brain slices at midhypothalamic (star in *b*) and mesencephalic (open star in *b*) levels and of spinal cords (small open arrows) and pituitary gland (big open arrows) of rat after incubation with ^{125}I-labeled galanin (*a*), ^{125}I-labeled galanin plus 1 μM galanin-(1–29), or ^{125}I-labeled galanin plus 1 μM M40. Dotted line in *b* shows border between the two brain slices. (*a*) Strong labeling is seen in anterior (Ta) and posterior (Tp) thalamic areas, in amygdaloid (A) and entorhinal (E) cortices, in subiculum (S), the dorsal horns (arrowheads) of the spinal cord and the posterior lobe (arrow). H, hippocampus. (*b* and *c*) No binding is seen after addition of galanin-(1–29) (GAL) (*b*) or M40 peptide (*c*) to the incubation mixture. (Bar = 1 mm.)

in the hippocampus, 15 nM in the hypothalamus, 12 nM in the spinal cord, and 3 nM in the insulinoma cells.

In the receptor autoradiographic experiments a strong binding was observed for ^{125}I-labeled galanin in many brain regions, including anterior and posterior thalamic regions, hypothalamus, the amygdaloid and entorhinal cortices, subiculum/ventral hippocampus, the dorsal horns, and the posterior lobe of the pituitary gland (Fig. 2*a*). Addition of galanin-(1–29) (Fig. 2*b*) or M40 (Fig. 2*c*), both at 10^{-6} M, completely blocked all these ^{125}I-labeled galanin-binding sites.

Upon intracerebroventricular injection, M40 did not affect by itself the basal acetylcholine release from the ventral hippocampus of awake rats, but given in combination with galanin, it reversed the galanin inhibitory effect on scopolamine-induced acetylcholine release (Fig. 3).

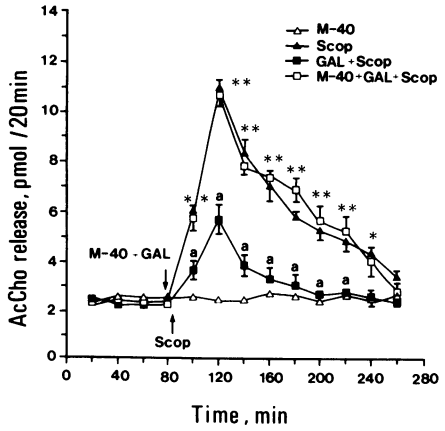

FIG. 3. Reversal of galaninergic inhibition of scopolamine (0.3 mg/kg s.c.)-evoked acetylcholine (AcCho) release in the ventral hippocampus by M40 peptide. M40 at the dose of 9.36 nmol and galanin (GAL) at 1.56 nmol were injected intracerebroventricularly 2 min before scopolamine (Scop). Perfusate was collected for 80 min (four fractions, 20 min for each fraction) before drug injections. Data are mean ± 0.5 SEM (bars) values (n = 6) and are not corrected for recovery *in vitro*, which was 17.6 ± 0.5% for a probe 3 mm long. Acetylcholine release in galanin plus scopolamine group differed significantly ($P < 0.01$) from that of the scopolamine group at each time. Interactions were as follows: M40 plus galanin plus scopolamine vs. galanin plus scopolamine; **, $P < 0.01$ and *, $P < 0.05$; galanin plus scopolamine vs. scopolamine; a, $P < 0.01$ (split-plot ANOVA followed by Tukey's test for unconfounded means).

M40 peptide inhibited the galanin-mediated facilitation of the spinal flexor reflex in a dose-dependent manner, but partial antagonism (20–40%) of the facilitation by galanin was achieved only when M40 was applied at a 1000-fold excess over galanin (Fig. 4). Fig. 4 also shows that the earlier introduced nonsubtype-specific antagonist M35 in equimolar doses antagonized the effects of galanin on the spinal flexor reflex (28).

Feeding induced by injection of galanin (0.5 nmol) into the paraventricular nucleus was antagonized by paraventricular nucleus injection of M40 in a dose-dependent manner. Equimolar doses of M40 fully blocked the stimulatory effect of galanin on food intake (Fig. 5).

The galanin-mediated inhibition of glucose-induced insulin release from isolated mouse pancreatic islets was not blocked by M40 peptide, even when added in a 1000-fold excess over galanin (Fig. 6*A*); instead, M40 appeared as a weak galanin

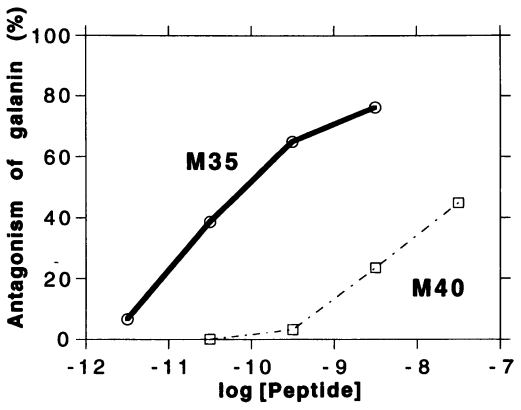

FIG. 4. Inhibition of the galanin (30 pmol, intrathecally) -mediated facilitation of the spinal flexor reflex by M40 (3×10^{-11}–3×10^{-8} M) injected 10 min before galanin into decerebrate, spinalized unanesthetized Sprague–Dawley rats. For comparison, the antagonist effects exhibited by the nonsubtype-specific antagonist M35 are shown.

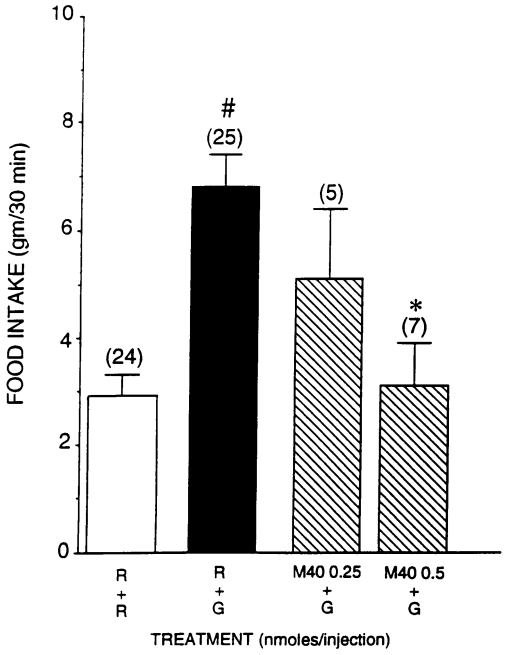

FIG. 5. M40 dose-dependently blocked feeding induced by galanin (0.5 nmol per 0.3 μl), microinjected into the paraventricular nucleus of the hypothalamus of satiated rats. Data are expressed as mean ± SEM. R, Ringer's vehicle; G, rat galanin-(1–29). Number of animals per treatment group is indicated in parentheses. #, $P < 0.05$ for Ringer plus galanin (0.5 nmol) as compared with Ringer plus Ringer; *, $P < 0.05$ for M40 (0.5 nmol) plus galanin (0.5 nmol) as compared with Ringer plus galanin (0.5 nmol), by Student–Newman–Keuls post hoc analysis after a significant one-way ANOVA (modified method from ref. 34).

agonist when applied at concentrations >100 nM. The nonsubtype-specific galanin-receptor antagonist M35 antagonized the inhibitory action of galanin on insulin release when applied in equimolar concentration with galanin, as shown earlier (36).

The galanin-mediated inhibition of the forskolin-stimulated 3′,5′-cAMP accumulation in Rin m5F cells was not reversed by M40 peptide (1–100 nM); instead M40 (10 and 100 nM) acted as a weak agonist at galanin receptors (Fig. 6*B*). Again M35 peptide (10 nM) antagonized the effects of galanin (Fig. 6*B*).

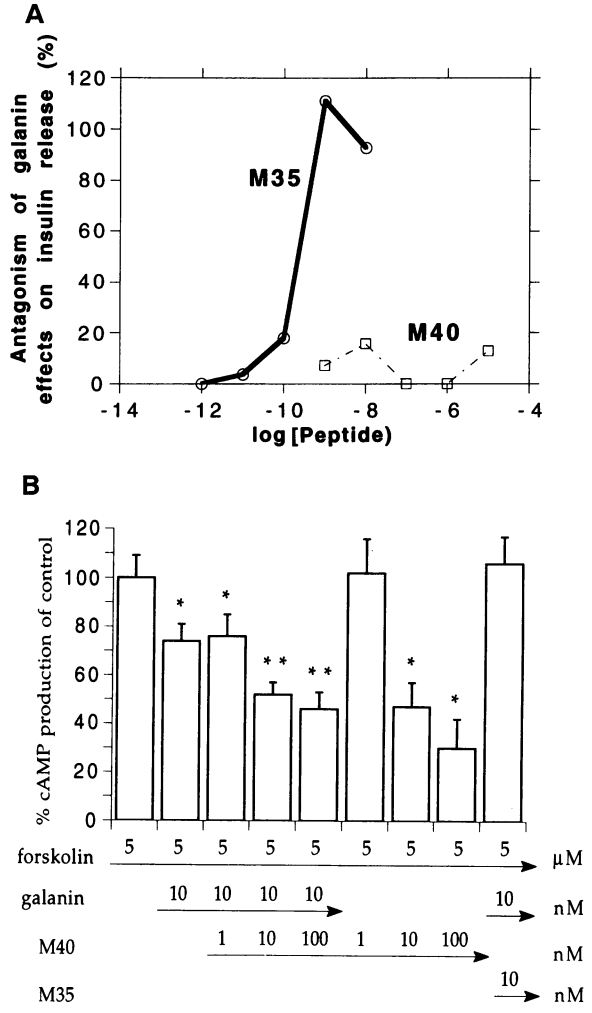

FIG. 6. (*A*) Lack of antagonism by M40 (10^{-9}–10^{-5} M) of the galanin (1 nM)-mediated inhibition of the glucose (11.1 mM)-induced insulin release from incubated isolated mouse pancreatic islets. For comparison, the antagonist effects of the nonsubtype-specific antagonist M35 are shown. (*B*) Lack of antagonism by M40 (1–100 nM) of the galanin (10 nM)-mediated inhibition of forskolin (5 μM) stimulated adenylate cyclase activity (n = 8–12 for all samples). *, $P < 0.05$, significantly different from forskolin (5 μM) alone; **, $P > 0.05$, significantly different from forskolin (5 μM) plus galanin (10 nM).

DISCUSSION

The results suggest that M40 is a high-affinity galanin-receptor ligand in all four tissues examined because it can fully displace [mono[^{125}I]iodo-Tyr^{26}]galanin from receptors in hippocampal, hypothalamic, spinal cord, and pancreatic membranes. The affinity of M40 peptide is somewhat lower (K_d = 3–15 nM) than that of the endogenous ligand galanin (K_d = 0.8 nM), but when used in a 100-fold excess over galanin, M40 peptide fully displaces galanin in all four tissues. However, in two of the systems examined, in pancreatic islets and rat spinal cord, a 100-fold excess of M40 did not antagonize the effects of galanin. For the spinal cord flexor reflex a 1000-fold excess of M40 was required for a significant, but still not full, blockade of the facilitatory effects of galanin. In the mouse pancreatic islets M40 did not antagonize the effects of galanin on glucose-induced insulin release, even when present in a 1000-fold excess. Furthermore, M40 peptide did not antagonize the inhibitory effects of galanin on the forskolin-stimulated accumulation of 3′,5′-cAMP in the Rin m5F insulinoma cells. In fact, in these models of pancreatic actions of galanin, M40 peptide acted as a weak galanin-receptor agonist.

In contrast, the earlier introduced galanin-receptor antagonists such as M15 (17) and M35 (28), which possess ≈100-fold higher affinity than M40, act in a nonsubtype-specific manner and block the CNS, spinal cord, as well as the pancreatic actions of galanin (17, 28).

It is possible that the failure of M40 peptide to act as antagonist at the pancreatic galanin receptors is related to its lower affinity and to the presence of a large excess of spare receptors for galanin on the pancreatic islets and Rin m5F cells. If so, M40 peptide with its lower affinity can never occupy all galanin receptors in the presence of competing galanin. In addition, we have to account for the galanin-agonist-type effect of M40 peptide at the pancreatic galanin receptors when M40 is applied alone because M40 shows no galanin agonist activity in the areas of CNS studied here. These different effects of M40 peptide suggest that chemical differences may exist between galanin receptors in the CNS and the pancreas.

On the basis of the above data we suggest a classification of galanin-receptor subtypes using the differential efficacy of

M40 peptide. At the putative CNS galanin receptors of GL-1 subclass, such as the receptors in the hippocampus and hypothalamus, M40 is a highly potent antagonist. The pancreatic galanin receptors (GL-2 receptors) are receptors that recognize M40 peptide, but M40 acts as a weak agonist there. M40 peptide acts as a weak antagonist in the lumbar spinal cord, where galanin receptors, thus, may represent an intermediate between one class of CNS (GL-1) and the pancreatic galanin-receptor (GL-2) subtypes.

In addition to these subtypes of galanin receptors, it is likely that galanin receptors in smooth muscle (25), in the anterior pituitary (26), and in cortex, dorsal hippocampus, and the caudate putamen (27) may constitute additional subclasses of galanin receptors, as differentiated by their ligand-binding properties.

It is, nevertheless, clear that this tentative subclassification will be followed by characterization of the receptor subtypes with fully subtype-specific ligands of peptide and/or peptidomimetic character. The molecular cloning of the receptor subtypes showing differences at the level of the primary sequence of receptor subtypes is needed to finally settle the number and also the chemical basis of the differences in ligand-binding properties of the different galanin-receptor subtypes. Meanwhile, M40 peptide provides an interesting tool to study, for example, central galaninergic mechanisms in the control of food intake while not affecting pancreatic function.

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