[Ala⁶, D-Trp⁸]GALANIN(1–15)ol IS A POTENT GALANIN ANTAGONIST ON INSULIN RELEASE

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ABSTRACT

Among the analogues derived from galanin(1–15)ol, [Ala⁶, D-Trp⁸]galanin(1–15)ol was found to be a potent antagonist against the inhibitory effect of galanin on glucose or forskolin-induced insulin release from the isolated rat pancreas and rat insulinoma RINm5F cells. [Ala⁶, D-Trp⁸]galanin(1–15)ol at 10^{–7} M suppressed the inhibitory effect of rat galanin (10^{–9} M) on glucose-induced insulin release from the isolated rat pancreas. The analogue at 10^{–6} M also counteracted the inhibitory action of rat galanin on forskolin-stimulated insulin release in RINm5F cells. Furthermore, [Ala⁶, D-Trp⁸]galanin(1–15)ol displaced the binding of ¹²⁵I-rat galanin on RINm5F cell membranes with the same binding potency (IC₅₀, about 0.9 nM) as galanin(1–15)ol, suggesting strongly that [Ala⁶, D-Trp⁸]galanin(1–15)ol blocks the galanin-mediated inhibition of insulin release by competitively occupying galanin receptor. The present study has shown that [Ala⁶, D-Trp⁸]galanin(1–15)ol may be a key compound for developing a more potent galanin antagonist.

Galanin is a 29-residues peptide amide originally isolated from porcine upper small intestine using a chemical detection method (32). However, human galanin has been reported to be non-amidated form at the C-terminus with 30 amino acid residues (5, 27). The amino acid sequences of mammalian galanins so far isolated are identical in the amino-terminal 1–15 sequence of the molecules, but differ at several positions in the C-terminal region (16, 29). On the other hand, galanin isolated from yellow fin tuna pituitary in our laboratory showed two substitutions in the N-terminal 1–15 sequence (11). Galanin-like immunoreactivity is widely distributed in the central and peripheral nervous systems in mammals (21, 30). Galanin has been known to inhibit the glucose-induced insulin release probably via sympathetic nerve terminals which innervate pancreatic islets (28). The primary structure of galanin receptor has been recently deduced to be a member of the G protein-coupled seven-trans-

membrane domain receptor superfamily (10, 25). The N-terminal fragments of galanin such as galanin(1–15) or galanin(1–16), have been shown to exhibit high binding affinity to galanin receptors in rat insulinoma cells and brain tissue (6, 18, 19). In addition, autoradiographical study revealed that specific binding sites for the radio-iodinated galanin(1–15) existed in the rat brain (13). Both galanin(1–15) and galanin(1–16) were shown to exhibit a wide range of biological actions which are comparable to those of galanin (2, 6, 12, 17).

Our previous studies on structure and function of galanin have revealed that the N-terminal 1–15 sequence is essential for the inhibitory effect on glucose-induced insulin release from the isolated perfused rat pancreas (23, 33) and on the neurally-evoked circular muscle contraction in guinea-pig ileum (17). Especially, galanin(1–15)ol, in which an alcoholic hydroxyl group was introduced at the C-terminus, was found to be equipotent to galanin on the suppression of the neurally-evoked circular muscle contraction (33). These results strongly indicate the existence of major active site of

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	1	5	10	15	20	25	29
Rat galanin	NH ₂ -G-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-I-D-N-H-R-S-F-S-D-K-H-G-L-T-NH ₂						
	6						
Tuna galanin	NH ₂ - -A- -G- -G- -T-L-G- -P- -A-NH ₂						
Galanin(1-15)ol	NH ₂ - -ol						
	8						
[D-Trp ⁸]galanin(1-15)ol	NH ₂ - -D-W- -ol						
	8						
[D-Ala ⁸]galanin(1-15)ol	NH ₂ - -D-A- -ol						
	6 8						
[Ala ⁶ , D-Trp ⁸]galanin(1-15)ol	NH ₂ - -A- -D-W- -ol						

Male Wistar rats, weighing 250–300 g (Japan SLC, Hamamatsu, Japan), were used. They were fasted for 16–24 h with free access to water. Anesthesia was induced with sodium pentobarbital (50 mg/kg).

Isolated rat pancreas was perfused by a slight modification according to Grodsky and Fanska (8). Briefly, a polyethylene inlet cannula was inserted into the celiac artery, and the outlet inserted into the portal vein. All other vessels were ligated. The pancreas was transferred to a chamber and suspended in saline maintained at 37°C. The perfusate consisted of Krebs-Ringer-bicarbonate buffer (KRBB) (pH 7.4) with supplement of 0.2% (w/v) bovine serum albumin (BSA) (Sigma Chemicals, St. Louis, MO, U.S.A.), 5.0 mM glucose and 4.0% (w/v) dextran T-70 (Meito Sangyo, Nagoya, Japan). The vascular perfusate was saturated constantly with a mixture gas of 95% O₂ and 5.0% CO₂ and run at a flow rate of 1.9 ml/min with a peristaltic pump. After perfusion period of 25 min, the perfusate was collected as a basal secretion for 10 min. Then, peptide was infused for 30 min at the flow rate of 0.1 ml/min through a branch tubing by means of an infusion pump (Harvard Apparatus, South Natic, MA, U.S.A.). Glucose-free KRBB was alternatively infused at the same rate as a control. Ten minutes later after the infusion of peptide, 13.9 mM glucose and 1 nM rat galanin were simultaneously infused for 20 min at a flow rate of 0.1 ml/min, followed by perfusing of KRBB alone for 10 min. Portal effluents were collected into chilled glass tubes containing aprotinin (100 KIE/tube, Novo Research Institute, Copenhagen, Denmark) and stored at -20°C until assayed for insulin.

The response of insulin release when peptides added, was expressed as a percent of the total amount of insulin release induced by the addition of 13.9 mM glucose. Insulin concentration was detected by radioimmunoassay (23). Briefly, synthetic rat insulin (Novo Research Institute, Copenhagen, Denmark) as a standard antigen or unknown samples were incubated with ¹²⁵I-porcine insulin (Dainabot, Tokyo) and guinea-pig anti-porcine insulin serum No. 2 (final concentration 1:160,000) for 48 h at 4°C. After incubation, normal guinea-pig serum (1:300), anti-guinea-pig IgG serum (1:400) and 10% (w/v) polyethylene glycol (60,000) were added. The mixture was incubated for 3 h at 4°C and centrifuged. Free ¹²⁵I-insulin was aspirated and the precipitate was counted in a gamma counter (Aloka, ARC1000, Tokyo). The minimum detection of the assay was about 10 pg/tube.

Iodination of Rat Galanin

Synthetic rat galanin was labeled with ¹²⁵I-Na (7.4 MBq) (Amersham International, U.K.) by chlo-

ramine T method (14). Iodinated peptide was separated by HPLC on a Waters μ Bondasphere C-18-100A column (0.39 × 15 cm) in a linear gradient of CH₃CN/0.01 N HCl solvent system. Fractions containing ¹²⁵I-peptide were collected and used for the receptor binding assay and radioimmunoassay. The specific activity of the ¹²⁵I-labeled peptide was determined as approximately 3.7 MBq/ μ g by the self displacement in immunoassay (31).

Cell Culture

The rat insulinoma cell line RINm5F was a generous gift from by Dr Marco Laburthe (INSERM, France). RINm5F cells were cultured as described elsewhere (1). Briefly, the cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum, (Dainippon Pharmaceutical, Osaka), 100 IU/ml penicillin and 100 μ g/ml streptomycin in 5% CO₂/90% air at 37°C. They were routinely passaged every 6–7 days.

Preparation of Membrane Fraction from RINm5F Cells

Membrane fraction from RINm5F cells was prepared by a slight modification of the method of Lindskog *et al.* (20). The cells were detached by treatment of 0.05% (w/v) trypsin in 1 mM EDTA phosphate buffer and centrifuged at 2,000 × *g* for 5 min at 4°C and then exposed in hypo-osmotic 5 mM HEPES buffer (pH 7.4) for 15 min. The suspension was centrifuged at 20,000 × *g* for 20 min. The resulting pellet was suspended in 5 mM HEPES-buffered Krebs-Ringer solution (137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl₂, 1.8 mM CaCl₂, 0.1% w/v, glucose, pH 7.4) containing bacitracin (1 mg/ml), and used immediately in equilibrium binding experiments.

Radioreceptor Assay in RINm5F Cell Membrane

Radioreceptor assay was performed by the modified method according to Lindskog *et al.* (20). The membrane fraction from RINm5F cells was incubated with peptide and ¹²⁵I-rat galanin for 30 min at 37°C in 5 mM HEPES-buffered Krebs-Ringer solution (pH 7.4) containing 0.05% (w/v) of BSA and 1 mg/ml bacitracin. The incubation was terminated by adding 1 ml of ice-cold incubation buffer. The mixture was filtered on a Whatman glass filter GF/B (Maidstone, U.K.) presoaked with 0.3%

Fig. 1 Inhibitory effect of galanin(1-15)ol analogues on total insulin release from the isolated perfused rat pancreas. The response of insulin release in each experiment was expressed as a percent of total insulin release induced by 13.9 mM glucose. Rat galanin and galanin(1-15)ol analogues were administered at 10^{-8} M and 10^{-7} M, respectively. Values are the mean $\pm$ SEM (n = 6-7). *, $P < 0.05$; **, $P < 0.01$ vs control

(w/v) polyethylenimine (Sigma Chemicals, St. Louis, MO, U.S.A.). The filter was washed three times with 3 ml of ice-cold incubation buffer. The radioactivity retained on the filter was determined using in a gamma-counter. The measurement in one experiment was performed in triplicate. Specific binding was calculated as the difference between 125 I-rat galanin binding in the absence (total binding) and the presence (non-specific binding) of 10^{-6} M rat galanin.

Forskolin-Stimulated Insulin Release in RINm5F Cell

RINm5F cells (0.5×10^5 – 1×10^5 cells per well) in DMEM supplemented with 10% (v/v) fetal calf serum were plated on 24-well micro plates and cultured for three days. Cells were washed twice with DMEM and incubated in 1 ml of incubation medium (DMEM supplemented with 2%, w/v, bovine serum albumin) containing peptides and 0.1 mM forskolin for 30 min at 37°C. Insulin content released in the medium was determined by radioimmunoassay as described (23). The experiments were performed with at least four different cell preparations. Each value was determined by using 7-8 wells.

Statistical Analysis

Results were expressed as mean $\pm$ SEM and ana-

Fig. 2 Antagonistic effect of galanin(1-15)ol analogues against galanin on glucose-induced insulin release from the isolated perfused rat pancreas. The response of insulin release in each experiment was expressed as a percent of total insulin release induced by 13.9 mM glucose. ■, rat galanin at 10^{-9} M plus galanin(1-15)ol analogues at 10^{-7} M. Values are the mean $\pm$ SEM (n = 6-7). *, $P < 0.05$ vs the control

lyzed by one-way analysis of variance (one way ANOVA). Statistically significant difference was assessed with Scheffé test and probability values less than 5% were considered to be statistically significant.

RESULTS

Effect of Insulin Release from the Isolated Perfused Rat Pancreas

As shown in Fig. 1, rat galanin (10^{-8} M) potently inhibited glucose (13.9 mM) induced insulin release from the isolated perfused rat pancreas. Total glucose-induced insulin release was suppressed in the presence of rat galanin to $10.7 \pm 1.1\%$ of the control (glucose alone). On the other hand, galanin(1-15)ol, [D-Trp⁸]galanin(1-15)ol, [D-Ala⁸]galanin(1-15)ol and [Ala⁶, D-Trp⁸]galanin(1-15)ol at 10^{-7} M showed weak inhibitory effects as compared to rat galanin. Among the galanin(1-15)ol analogues examined, [D-Trp⁸]galanin(1-15)ol showed the highest inhibition of insulin release in response to glucose ($54.1 \pm 10.2\%$ total insulin release in response to glucose at 10^{-7} M and $61.4 \pm 9.7\%$ at 10^{-8} M, respectively). Effects of 10^{-7} M galanin(1-15)ol analogues in the presence of 10^{-9} M rat galanin on glucose-induced insulin release from the isolated perfused rat pancreas were examined (Fig. 2). Rat galanin at 10^{-9} M alone reduced to $55.1 \pm 7.4\%$ of the control level. The inhibitory activity of rat galanin was potentiated in the pres-

Fig. 3 Antagonistic effect of $[Ala^6, D-Trp^8]galanin(1-15)ol$ against rat galanin on 13.9 mM glucose-induced insulin release from the isolated perfused rat pancreas. (●), $[Ala^6, D-Trp^8]galanin(1-15)ol$ at $10^{-7} M$ plus rat galanin at $10^{-9} M$; (○), rat galanin at $10^{-9} M$ alone; (Δ), 13.9 mM glucose only (control). Each peptide was administered during the arrow indicated. Values are the means ($n = 6$).

Fig. 4 Dose-response of $[Ala^6, D-Trp^8]galanin(1-15)ol$ in the presence of $10^{-9} M$ rat galanin on glucose-induced insulin release from the isolated perfused rat pancreas. The response of insulin release in each experiment was expressed as a percent of total insulin release induced by 13.9 mM glucose. Values are the mean $\pm$ SEM ($n = 6$). *, $P < 0.05$ vs rat galanin at $10^{-9} M$. (—), expressed the absence of peptide; (+), expressed the presence of peptide

ence of galanin(1-15)ol and $[D-Trp^8]galanin(1-15)ol$ to $35.9 \pm 6.9\%$ and $28.0 \pm 6.0\%$ of the control release, respectively. On the other hand, $[D-Ala^8]galanin(1-15)ol$ showed a weak but no significant antagonistic action on rat galanin, while $[Ala^6, D-Trp^8]galanin(1-15)ol$ strongly antagonized the inhibitory effect of rat galanin ($92.0 \pm 13.7\%$ of

Fig. 5 Binding displacement curves of galanins and galanin(1-15)ol analogues on the membranes from RINm5F cell. Rat galanin (○), tuna galanin (■), galanin(1-15)ol (Δ), $[D-Trp^8]galanin(1-15)ol$ (▲), $[D-Ala^8]galanin(1-15)ol$ (□), $[Ala^6, D-Trp^8]galanin(1-15)ol$ (●). Results are means of at least three separate experiments. Each value was determined in triplicate. Non-specific binding was defined as ^{125}I -rat galanin binding in the presence of $1 \mu M$ rat galanin.

the control). The time course analysis of insulin release revealed that $[Ala^6, D-Trp^8]galanin(1-15)ol$ suppressed the effect of rat galanin mainly in the second phase (Fig. 3). Furthermore, antagonistic effect of $[Ala^6, D-Trp^8]galanin(1-15)ol$ was shown to be dose-dependent (Fig. 4).

Binding Affinity to the Plasma Membrane from RINm5F Cells

Tuna galanin and galanin(1-15)ol analogues such as galanin(1-15)ol, $[D-Trp^8]galanin(1-15)ol$ and $[Ala^6, D-Trp^8]galanin(1-15)ol$ showed less displacement than rat galanin in membranes from RINm5F cells, while $[D-Trp^8]galanin(1-15)ol$ possessed higher binding affinity than rat galanin, although it showed a low inhibitory effect on insulin release as compared with that of galanin (Fig. 5). Table 2 summarizes IC_{50} values for galanin and galanin(1-15)ol analogues examined. $[D-Trp^8]galanin(1-15)ol$ showed 30-fold potent binding activity than rat galanin. The binding affinity of $[Ala^6, D-Trp^8]galanin(1-15)ol$ was found to be nearly equipotent to that of galanin(1-15)ol, although activities of both analogues on insulin release differed each other. $[D-Ala^8]galanin(1-15)ol$ and tuna galanin also displace ^{125}I -rat galanin with

Table 2 Binding Ability of Rat and Tuna Galanins and Galanin(1–15)ol Analogues in RINm5F Cell Membranes

Peptide	IC ₅₀ (pM)	
Rat galanin	79.1 ±	23.7
Tuna galanin	730.2 ±	142.9
Galanin(1–15)ol	1,076.8 ±	426.1
[D-Trp ⁸]galanin(1–15)ol	4.5 ±	1.0
[D-Ala ⁸]galanin(1–15)ol	5,810.2 ±	3,751.7
[Ala ⁶ , D-Trp ⁸]galanin(1–15)ol	938.4 ±	466.7

Data are calculated from the results shown in Fig. 5. Results are means ± SEM from at least three separate experiments.

potency approximately 10-fold less than that of rat galanin.

Antagonistic Effect of [Ala⁶, D-Trp⁸]galanin(1–15)ol on Forskolin-Stimulated Insulin Release from RINm5F Cells

Rat galanin inhibited forskolin-stimulated insulin release from RINm5F cells in a dose-dependent manner at the concentration of 10^{−9} M–10^{−6} M. In fact, total forskolin-stimulated insulin release was decreased approximately 55% of the control by rat galanin at 10^{−6} M (Fig. 6). On the other hand, the inhibitory effect of rat galanin at 10^{−9} M–10^{−6} M on insulin release was antagonized by [Ala⁶, D-Trp⁸]galanin(1–15)ol at 10^{−6} M. In contrast, a single administration of [Ala⁶, D-Trp⁸]galanin(1–15)ol showed no effect on insulin release.

DISCUSSION

Previously, galanin(1–15)ol, which has been prepared in our laboratory, as a stable compound against carboxypeptidase digestion, have been shown to be nearly equipotent to galanin on the inhibitory activity of the neurally evoked circular muscle contraction in guinea-pig ileum (33). In fact, this analogue as well as galanin(1–15) showed high affinity to the receptors on the membranes in hippocampus and pancreas, suggesting that the N-terminal 1–15 sequence is the active site of galanin (15, 18). Based on these observations, attempt has been made to develop galanin(1–15)ol analogues having antagonistic activity against galanin.

In the present study, [D-Trp⁸]galanin(1–15)ol has been found to show relatively higher binding affinity when compared to that of rat galanin on plasma

Fig. 6 Antagonism by [Ala⁶, D-Trp⁸]galanin(1–15)ol (10^{−6} M) against the effect of the rat galanin (10^{−9} M–10^{−6} M)-mediated inhibition of the forskolin (10^{−4} M) stimulated insulin release in RINm5F cell. Response of insulin release in each experiment was expressed as a percent of insulin content induced by the addition of 10^{−4} M forskolin. Results are means of at least four separate experiments (n = 7–8). Vertical bars represent SEM. *, P < 0.05 vs rat galanin

membranes of rat insulinoma cell line RINm5F. Furthermore, [D-Trp⁸]galanin(1–15)ol showed significantly inhibitory effect on glucose-induced insulin release in rat pancreas, though less potent than rat galanin. Thus, D-Trp substitution at position 8 in galanin(1–15)ol was found to cause higher receptor binding affinity but lower inhibitory activity on insulin release, when compared with those of galanin(1–15)ol. These results also suggest that [D-Trp⁸]galanin(1–15)ol may be one of the leading peptide analogue for developing potent galanin antagonist.

In addition, tuna galanin in which two substitutions occur at position 6 and 15 in the active region of mammalian galanin was found to displace [¹²⁵I]-rat galanin binding dose-dependently on rat insulinoma cell as well as rat hippocampus membranes (22), although this peptide did not cause any inhibition of glucose-induced insulin release from the isolated perfused rat pancreas even at the concentration of 10^{−7} M (22). Based on these results, [Ala⁶, D-Trp⁸]galanin(1–15)ol was synthesized and examined for the effect on glucose-induced insulin release from the isolated perfused rat pancreas. The results show that [Ala⁶, D-Trp⁸]galanin(1–15)ol antagonized rat galanin action to inhibit the glucose-induced insulin release, although the analogue alone did not effect on glucose-induced insulin

release even up to the concentration of 10^{-7} M.

It has been reported that galanin inhibited forskolin-stimulated insulin release through inhibition of adenylate cyclase in RINm5F cell (1) and that the inhibition of adenylate cyclase activity by galanin was mediated by the G-protein G_{i3} (26). [Ala⁶, D-Trp⁸]galanin(1–15)ol in the present study was shown to suppress significantly the inhibitory action of rat galanin on forskolin-stimulated insulin release from RINm5F cells in a dose-dependent manner. However, [Ala⁶, D-Trp⁸]galanin(1–15)ol alone did not suppress forskolin-stimulated insulin release even at the concentration of 10^{-6} M. These findings indicate clearly that [Ala⁶, D-Trp⁸]galanin(1–15)ol is a specific and potent galanin antagonist on the insulin release.

Previously, several chimeric galanin analogues have been reported to function as galanin receptor antagonists (3, 7, 20, 34). The chimeric antagonist M15, galanin(1–13)-substance P (5–11), has been shown to antagonize the action of galanin in the central nervous system (3) and also the inhibitory effect of galanin on glucose-induced insulin release from mice pancreatic islets (20). In contrast, it acts as a full agonist in the gastrointestinal motility in rat (9). There is disadvantage using the chimeric peptide, such as M15 as galanin antagonists, since the C-terminal portion consisting of a partial sequence of substance P might interact with receptors of other regulatory peptides. In fact, M15 has been reported to recognize substance P receptor (4). Moreover, galanin is widely distributed and co-localized with other neuropeptides and hormones in various tissues (4, 21). Accordingly, galanin antagonists should be developed to retain specificity to galanin receptor alone. [Ala⁶, D-Trp⁸]galanin(1–15)ol developed in the present study was designed as a small size of peptide consisting of the 1–15 sequence of active site with proteolysis-resistant substitution. In fact, [Ala⁶, D-Trp⁸]galanin(1–15)ol was found to be the most potent galanin antagonist among analogues synthesized in the present study on the glucose-induced insulin release from isolated perfused rat pancreas and rat insulinoma cell line.

Further studies for developing antagonists with higher potency are now in progress. Some of these analogues will be useful for investigating physiological function of galanin.

Received 13 November 1996; and accepted 6 December 1996

REFERENCES

1. AMIRANOFF B., LORINET A. M., LAGNY-POURMIR I. and LABURTHE M. (1988) Mechanism of galanin-inhibited insulin release. Occurrence of a pertussis-toxin-sensitive inhibition of adenylate cyclase. *Eur. J. Biochem.* **177**, 147–152
2. AMIRANOFF B., LORINET A. M., YANAIHARA N. and LABURTHE M. (1989) Structural requirement for galanin action in the pancreatic β cell line Rin m5F. *Eur. J. Pharmacol.* **163**, 205–207
3. BARTFAI T., BEDECS K., LAND T., LANGE Ü., BERTORELLI R., GIROTTI P., CONSOLO S., XU X., WIESENFLD-HALLIN Z., NILSSON S., PIERIBONE V. A. and HÖKFELT T. (1991) M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus, and spinal cord. *Proc. Natl. Acad. Sci. USA* **88**, 10961–10965
4. BARTFAI T., HÖKFELT T. and LANGE Ü. (1993) Galanin-A neuroendocrine peptide. *Crit. Rev. Neurobiol.* **7**, 229–274
5. BERSANI M., JOHNSEN A. H., HØJRUP P., DUNNING B. E., ANDREASEN J. J. and HOLST J. J. (1991) Human galanin: primary structure and identification of two molecular forms. *FEBS Lett.* **283**, 189–194
6. FISONE G., BERTHOLD M., BEDECS K., UNDNÉN A., BARTFAI T., BERTORELLI R., CONSOLO S., CRAWLEY J., MARTIN B., NILSSON S. and HÖKFELT T. (1989) N-terminal galanin-(1–16) fragment is an agonist at the hippocampal galanin receptor. *Proc. Natl. Acad. Sci. USA* **86**, 9588–9591
7. GREGERSEN S., LINDSKOG S., LAND T., LANGE Ü., BARTFAI T. and AHRÉN B. (1993) Blockade of galanin-induced inhibition of insulin secretion from isolated mouse islets by the non-methionine containing antagonist M35. *Eur. J. Pharmacol.* **232**, 35–39
8. GRODSKY G. M. and FANSKA R. E. (1975) The in vitro perfused pancreas. *Meth. Enzymol.* **39**, 364–372
9. GU Z.-F. A., ROSSOWSKI W. J., COY D. H., PRADHAN T. K. and JENSEN R. T. (1993) Chimeric galanin analogs that function as antagonists in the CNS are full agonists in gastrointestinal smooth muscle. *J. Pharmacol. Exp. Ther.* **266**, 912–918
10. HABERT-ORTOLI E., AMIRANOFF B., LOQUET I., LABURTHE M. and MAYAUX J. F. (1994) Molecular cloning of a functional human galanin receptor. *Proc. Natl. Acad. Sci. USA* **91**, 9780–9783
11. HABU A., OHISHI T., MIHARA S., OHKUBO R., HONG Y.-M., MOCHIZUKI T. and YANAIHARA N. (1994) Isolation and sequence determination of galanin from pituitary of yellow fin tuna. *Biomedical Res.* **15**, 357–362
12. HEDLUND P. B., FINNMAN U. B., YANAIHARA N. and FUXE K. (1994) Galanin-(1–15), but not galanin-(1–29), modulates 5-HT_{1A} receptors in the dorsal hippocampus of the rat brain: possible existence of galanin receptor subtypes. *Brain Res.* **634**, 163–167
13. HEDLUND P. B., YANAIHARA N. and FUXE K. (1992) Evidence for specific N-terminal galanin fragment binding sites in the rat brain. *Eur. J. Pharmacol.* **224**, 203–205
14. HUNTER W. M. and GREENWOOD F. C. (1962) Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* **194**, 495–496
15. KAKUYAMA H., SUZUKI M., IIZUKA M., MOCHIZUKI T., IGUCHI K., HOSHINO M. and YANAIHARA N. (1995) Structural requirements of galanin for occupancy of galanin receptor and cell growth in human small cell lung carcinoma cell line SBC-3A. In *Peptide Chemistry*

- (ed. OHNO M.) Protein Research Foundation, Osaka, pp. 61–64
16. KAPLAN L. M., SPINDEL E. R., ISSELBACHER K. J. and CHIN W. W. (1988) Tissue-specific expression of the rat galanin gene. *Proc. Natl. Acad. Sci. USA* **85**, 1065–1069
 17. KUWAHARA A., OZAKI T. and YANAIHARA N. (1990) Structural requirements for galanin action in the guinea pig ileum. *Regul. Pept.* **29**, 23–29
 18. LAGNY-POURMIR I., LORINET A. M., YANAIHARA N. and LABURTHE M. (1989) Structural requirements for galanin interaction with receptors from pancreatic beta cells and from brain tissue of the rat. *Peptides* **10**, 757–761
 19. LAND T., LANGE Ü., LÖW M., BERTHOLD M., UNDÉN A. and BARTFAI T. (1991) Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor. *Int. J. Pept. Protein Res.* **38**, 267–272
 20. LINDSKOG S., AHRÉN B., LAND T., LANGE Ü. and BARTFAI T. (1992) The novel high-affinity antagonist, galantide, blocks the galanin-mediated inhibition of glucose-induced insulin secretion. *Eur. J. Pharmacol.* **210**, 183–188
 21. MELANDER T., HÖKFELT T., RÖKAEUS Å., FAHRENKRUG J., TATEMOTO K. and MUTT V. (1985) Distribution of galanin-like immunoreactivity in the gastro-intestinal tract of several mammalian species. *Cell Tissue. Res.* **239**, 253–270
 22. MOCHIZUKI T., HABU A., KAKUYAMA H., TAKATSUKA N., HOSOE H., MIHARA S., IGUCHI K. and YANAIHARA N. (1994) Synthesis of tuna galanin and differential biological activity. *Biomedical Res.* **15**, 363–368
 23. MOCHIZUKI T., ISHIKAWA J., OSHIMA K., GREELEY G. H. and YANAIHARA N. (1992) Effects of galanin fragments on insulin release from the isolated perfused rat pancreas. *Biomedical Res.* **13**, 203–213
 24. MOCHIZUKI T., SATO T., TAKATSUKA N., KAKUYAMA H., YAMABE K., YANAIHARA C. and YANAIHARA N. (1994) Synthesis of galanin antagonist. In *Peptide Chemistry* (ed. OKADA Y.) Protein Research Foundation, Osaka, pp. 325–328
 25. PARKER E. M., IZZARELLI D. G., NOWAK H. P., MAHLE C. D., IBEN L. G., WANG J. and GOLDSTEIN M. E. (1995) Cloning and characterization of the rat GALR1 galanin receptor from Rin 14B insulinoma cells. *Mol. Brain Res.* **34**, 179–189
 26. PHILIPPE D. M., PAUL K. G. and WEINSTEIN L. S. (1994) Inhibition of adenylate cyclase activity by galanin in rat insulinoma cell is mediated by the G-protein G_{i3} . *Biochem. J.* **303**, 369–375
 27. SCHMIDT W. E., KRATZIN H., ECKART K., DREVS D., MUNDKOWSKI G., CLEMENTS A., KATSOLIS S., SCHÄFER H., GALLWITZ B. and CREUTZFELD W. (1991) Isolation and primary structure of pituitary human galanin, 30-residue nonamidated neuropeptide. *Proc. Natl. Acad. Sci. USA* **88**, 11435–11439
 28. SHIMOSEGAWA T. and TOYOTA T. (1993) Galanin in pancreas. *Biomedical Res.* **14**, Suppl. 3, 97–106
 29. SILLARD R., LANGE Ü. and JÖRNVALL L. H. (1991) Isolation and characterization of galanin from sheep brain. *Peptides* **12**, 855–859
 30. SKOFITSCH G. and JACOBOWITZ D. M. (1986) Quantitative distribution of galanin-like immunoreactivity in the rat central nervous system. *Peptides* **7**, 609–613
 31. STADI F. and REHFELD J. F. (1972) Preparation of 125 I-labelled synthetic human gastrin I for radioimmunoanalysis. *Scand. J. Clin. Lab. Invest. Dec.* **30**, 361–368
 32. TATEMOTO K., RÖKAEUS Å., JÖRNVALL H., McDONALD T. J. and MUTT V. (1983) Galanin: Novel biologically active peptide from porcine intestine. *FEBS Lett.* **164**, 124–128
 33. YANAIHARA N., MOCHIZUKI T., IGUCHI K., HOSHINO M., NAGASHIMA T., TAKATSUKA N., ISHIKAWA J., GREELEY G. H., YANAIHARA C. and KUWAHARA A. (1991) Structure-function relationships of galanin. In: *Galanin* (ed. HÖKFELT T., BARTFAI T., JACOBOWITZ D. and OTTOSON D.) Macmillan Education, London, pp. 185–196
 34. XU X.-J., WIESENFELD-HALLIN Z., LANGE Ü., BEDECS K. and BARTFAI T. (1995) New high affinity peptide antagonists to the spinal galanin receptor. *Brit. J. Pharmacol.* **116**, 2076–2080