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GALANIN: STRUCTURAL REQUIREMENTS FOR BINDING AND SIGNAL TRANSDUCTION IN RINm5F INSULINOMA CELLS

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Receptors for galanin, a neuropeptide inhibiting insulin release, have been described on RINm5F insulinoma cells. To characterize structural requirements for binding and biological activity of galanin, we studied binding and inhibition of hormone stimulated intracellular cAMP-production of N-terminal galanin fragments and -analogues in RINm5F cells. Half-maximal binding and potency were the same for all peptides used. Active peptides had the following rank of potency: galanin = galanin(1-22^{23}Cys) > galanin(1-29^{4}NLe) > galanin(1-18) > galanin(1-29^{7}DAla) > galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) > galanin(1-29^{2}DTrp). Galanin(3-29) was inactive. Therefore the first two amino acids of the galanin molecule with the indole side chain of the tryptophane residue in the right steric position are crucial for receptor binding.

Galanin is a 29 amino acid containing neuropeptide isolated on the basis of its amidated C-terminus by Tatemoto et al. (1) from porcine intestine. Galanin is widely distributed not only in the gut, but also the central nervous system (2-4). One of the biological actions of galanin is the inhibition of insulin release (5) from the pancreatic B-cell. Binding sites for galanin have been described in RINm5F cells, a cell line derived from a transplantable rat insulinoma (6). Galanin inhibits insulin release by a direct inhibition of adenylate cyclase and a consecutive inhibition of cAMP production that involves an inhibitory GTP regulatory protein (7, 8). Studies with C-terminal galanin fragments, the N-terminal fragment galanin(1-15) and galanin analogues having a substitute for the tryptophane in position 2 showed that the N-terminus of the molecule seems to be important binding and signal transduction of the molecule (9, 10). In order to characterize structure-function relationships of galanin further, we studied inhibition of [^{125}I]-galanin binding and of GLP-1(7-36)amide stimulated cAMP production by galanin fragments and -analogues in RINm5F cells, comparing binding properties and biological activity in the same system. GLP-1(7-36)amide is a hormone cleaved from the glucagon precursor in the small intestine by posttranslational processing (11, 12). It is a strong stimulator of insulin secretion and may

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play an important role in the regulation of insulin secretion in mammals (11, 13, 14). Binding sites for GLP-1(7-36)amide have been characterized in RINm5F cells (15-17). Following binding of GLP-1(7-36)amide to RINm5F cells, adenylate cyclase is stimulated, leading to an increase in cAMP production (15). The aim of this study was to investigate the influence of galanin on the GLP-1(7-36)amide induced stimulation of cAMP production. Using synthetic N-terminal galanin fragments and analogues with multiple amino acid substitutions, we characterized the galanin domain that is crucial for receptor binding and inhibition of hormone-induced cAMP synthesis. By comparing half-maximal binding and half-maximal biological activity of these galanin analogues, possible galanin-antagonistic properties were studied.

MATERIALS AND METHODS

MATERIALS

Synthetic galanin and GLP-1(7-36)amide were obtained from Bissendorf, Hannover, F.R.G.. A radioimmunoassay kit to determine cyclic AMP levels was obtained from New England Nuclear, Dreieich, F.R.G.. All other chemicals were of the highest purity grade available.

CELL PREPARATIONS

RINm5F cells were grown in plastic culture bottles under standard conditions as described by Praz, Halban, Wollheim et al. (18). The cells were detached from the surface of the bottles before experimentation using phosphate-buffered-saline containing 0.7 mM EDTA. Cell concentrations were determined by measurement of cellular DNA (19) or by counting cells in a Neubauer chamber. On average, 22 µg DNA corresponded to 10^6 cells.

IODINATION OF GALANIN

Peptide iodination was carried out by the Chloramin T method (20) and [^{125}I]-labelled galanin was purified by reversed-phase high performance liquid chromatography (HPLC).

BINDING STUDIES

Binding studies on detached RINm5F cells were performed as described in (15, 17). Briefly, RINm5F cells were incubated for 30 min at 25°C with radiolabelled galanin (20000 cpm) and the desired amount of unlabelled peptides. Incubation was stopped by centrifugation of the cell suspension through an oil layer. After centrifugation cell-bound radioactivity in the pellet was measured as described by Flatt, Swanston-Flatt, Hampton et al. (21).

DETERMINATION OF cyclic AMP CONCENTRATIONS

Cells were incubated for 3 min at 37°C in the presence of 0.2 mM isobutylmethylxanthine with different concentrations of peptides as described by Gallwitz et al. (17). Incubation was terminated by the addition of 0.2 ml 12%(w/v) trichloroacetic acid. The cell suspension was sonicated and centrifuged (11500Xg for 5min). After mixing the supernatants with 50 µl 0.1 M HCl and extraction with diethylether (3 x 1 ml), the samples were freeze dried and redissolved in 0.2 ml sodium acetate buffer. Concentrations of cyclic AMP were determined by a cyclic AMP radioimmunoassay according to the manufacturer's instructions (New England Nuclear, Dreieich, F.R.G.).

PEPTIDE SYNTHESIS

Fragments and -analogues of galanin were synthesized by solid-phase methodology on polyamide resins supplied by Pharmacia-LKB, Freiburg, F.R.G. using N-fluorenyl-methoxy-carbonyl protected amino acid pentafluorophenylesters in an automatic peptide synthesizer (LKB Biolynx, Pharmacia-LKB, Freiburg, F.R.G.) (22, 23). Galanin(1-18), galanin(1-22^{23}Cys) and galanin(1-29^{4}NLe) were cleaved off the resin with trifluoroacetic acid/thioanisol (95%/5%; v/v) and precipitated with diethylether. Galanin(1-29^{2}DTrp), galanin(1-29^{7}DAla) and galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) were cleaved off the resin with trifluoroacetic acid and trimethylbromosilan (TMBS). Thioanisol was extracted by consecutive diethylether washes. Purification to homogeneity was achieved by preparative reverse-phase HPLC on a C-18 Nucleosil 300-7 wide pore MN column (30 nm, 1 x 25 cm) (Machery and Nagel, Düren, F.R.G.) using a Waters (Waters Associates, Milford, MA., U.S.A.) HPLC system with a dual-wavelength detector (214/280 nm). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid and 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Peptides were eluted at a flow of 2.5 ml/min with a linear gradient. Following HPLC purification, amino acid composition of the peptides was determined with a Durrum D-500 automatic amino acid analyser (Durrum Corporation, Palo Alto, Ca., U.S.A.) after HCl-gas-phase hydrolysis. If necessary, synthetic peptides were sequenced manually by Edman degradation (24) using 4'-NN-dimethylaminoazobenzene 4'-isothiocyanate and an HPLC α-amidation assay according to Schmidt et al. (25), confirming C-terminal α-amidation of galanin analogues.

STATISTICAL ANALYSIS

Data are expressed as means ± S.E.M.. Effects on cyclic AMP concentrations were analyzed using Student's t-test for paired data. P-values of lesser than 0.05 were considered to be significant.

RESULTS

PREPARATION OF [^{125}I]-GALANIN

[^{125}I]-labelled galanin was eluted as a sharp symmetrical peak of radioactivity from a Nova-Pak C-18 reversed phase HPLC column (Machery and Nagel, Düren, F.R.G.) at an acetonitrile concentration of approximately 35% (v/v). The specific activity of the label was 74 TBq/mM, labelled peptide was sufficiently separated from unlabelled galanin. Binding of the label was tested by incubating approximately 10^6 cells for 30 min at 25°C in the presence or absence of 1 μM unlabelled galanin. Non-specific binding, defined as binding in the presence of 1 μM galanin ranged from 1-3% and total binding from 6-10%. Specific binding was determined as the difference between total and non-specific binding. Tracer was used, if specific binding was higher than 3%. Binding of [^{125}I]-galanin was saturated under the conditions chosen.

BINDING OF GALANIN FRAGMENTS AND ANALOGUES TO RINm5F CELLS

The ability of galanin, galanin fragments and -analogues to inhibit binding of [^{125}I]-galanin to RINm5F cells is shown in figure 1. Except for GAL(3-29) all synthetic peptides inhibited [^{125}I]-galanin binding in a concentration dependent manner, in fol-

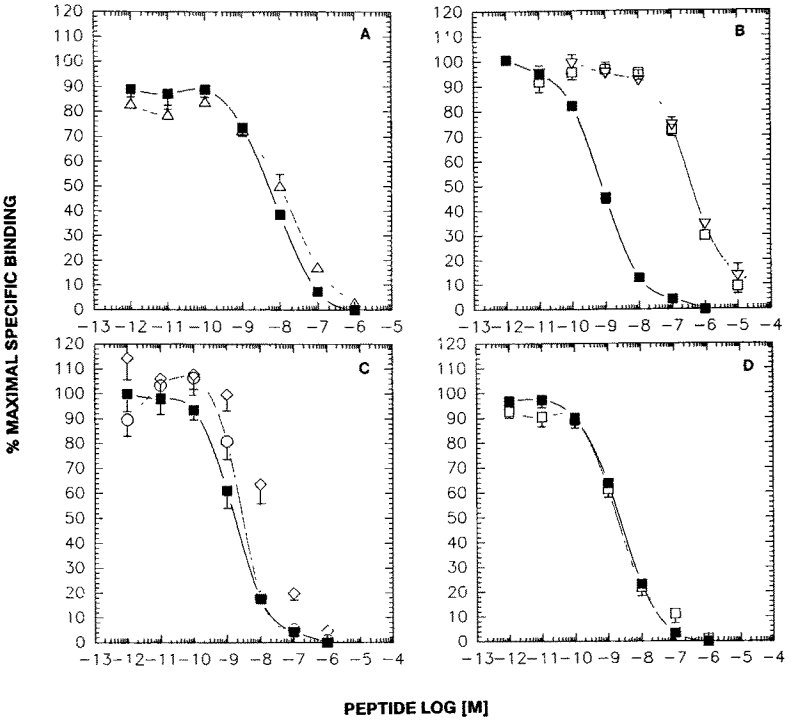

Figure 1. Displacement of binding of [^{125}I]-galanin by unlabelled galanin, its fragments and analogues to RINm5F cells. Cells were incubated for 30 min at 25°C with various concentrations of peptides and [^{125}I]-galanin (20000 cpm). 100% of maximal specific binding corresponded to cell bound radioactivity in the absence of unlabelled peptides; non-specific binding was defined as cell-bound radioactivity in the presence of 1 μM galanin. Galanin (■) and galanin(1-18) (△) (Fig. 1A), galanin(1-29^{2}DTrp) (▽) and galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) (□) (Fig. 1B), galanin(1-29^{4}NLe) (○) and galanin(1-29^{7}DAla) (◇) (Fig. 1C), galanin(1-22^{23}Cys) (□) (Fig. 1D),. Values are means ± S.E.M. for at least 5 separate experiments.

lowing rank order of affinity: galanin(1-29) = galanin(1-22^{23}Cys) > galanin(1-29^{4}NLe) > galanin(1-18) > galanin(1-29^{7}DAla) > galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) > galanin(1-29^{2}DTrp). The IC_{50}-values, defined as the concentrations of unlabelled peptide which inhibits binding of [^{125}I]-galanin by 50% of the galanin fragments and -analogues, are given in table 1.

EFFECT OF GALANIN FRAGMENTS AND -ANALOGUES ON cAMP CONCENTRATIONS IN RINm5F CELLS

Incubation of RINm5F cells with galanin, its fragments and analogues alone did produce a significant change in intracellular cAMP levels. GLP-1(7-36)amide-stimulated cAMP levels, however, were supressed by galanin in a concentration dependent manner. This effect was not due to a change in receptor affinity to galanin or GLP-1(7-36)amide, as either galanin or GLP-1(7-36)amide binding were not changed by simultaneous incubation of cells with both hormones (results not shown).

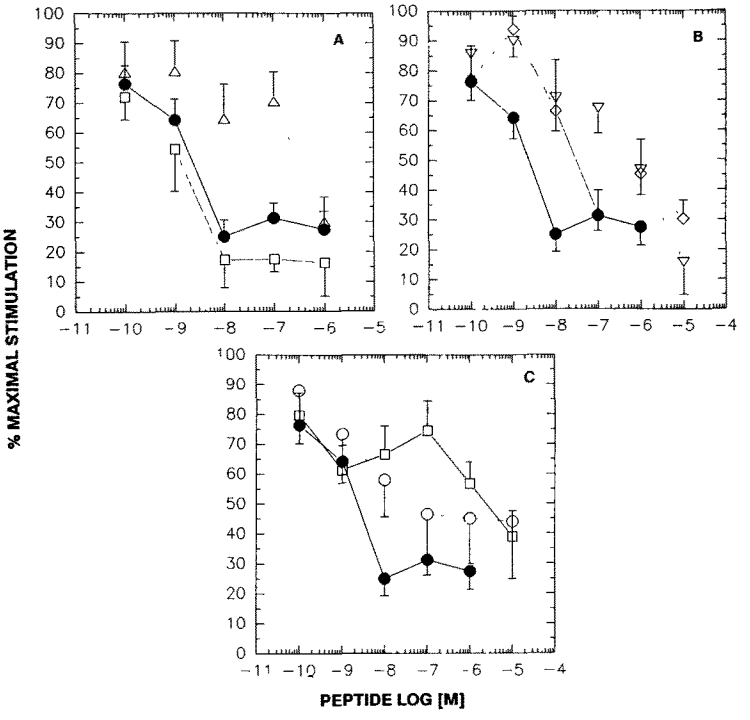

Figure 2. Inhibition of glucagon-like peptide-1 (7-36)amide (GLP-1(7-36)amide)-dependent cAMP production by galanin, its fragments and analogues. Cells were incubated for 3 min at 37°C without peptides (basal controls = 0% maximal stimulation), with 10^{-8} M GLP-1(7-36)amide alone (stimulation controls = 100% maximal stimulation) or simultaneously with 10^{-8} M GLP-1(7-36)amide and various concentrations of galanin (●), galanin(1-18) (△) and galanin(1-22^{23}Cys) (□) (Fig. 2A), galanin(1-29^{7}DAla) (◇) and galanin(1-29^{2}DTrp) (▽) (Fig. 2B), galanin(1-29^{4}NLe) (○) and galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) (□) (Fig. 2C). Values are means ± S.E.M. for at least 5 separate experiments.

Table 1

HALF-MAXIMAL INHIBITION OF [^{125}I]-GALANIN BINDING AND GLP-1(7-36)AMIDE-DEPENDENT cAMP PRODUCTION BY GALANIN, ITS FRAGMENTS AND ANALOGUES IN RINm5F CELLS

PEPTIDE	K_{D50} [nM]	RELATIVE POTENCY	E_{D50} [nM]	RELATIVE POTENCY
GALANIN(1-29)	2.9 ± 0.42	1	2.9 ± 0.9	1
GALANIN(1-22^{23}Cys)	2.4 ± 0.66	1.21	1.7 ± 0.7	1.71
GALANIN(1-18)	12.6 ± 3.50	0.23	59.7 ± 31.4	0.05
GALANIN(1-29^{2}DTrp)	479.8 ± 46.00	0.006	667.0 ± 240.0	0.004
GALANIN(1-29^{4}NLe)	3.6 ± 0.50	0.81	5.1 ± 1.9	0.57
GALANIN(1-29^{7}DAla)	20.5 ± 7.00	0.14	44.6 ± 12.3	0.07
GALANIN(1-29^{2}DTrp^{4}NLe^{7}DAla)	398.5 ± 32.00	0.007	400.0 ± 35.4	0.007

Displacement of [^{125}I]-galanin binding was measured as described in Fig. 1, inhibition of GLP-1(7-36)amide-stimulated cAMP-production was measured as described in Fig. 2. Relative potencies of peptides are referred to native galanin. Values are means ± S.E.M. for at least 5 separate experiments.

The ability of galanin, galanin fragments and -analogues to inhibit GLP-1(7-36)amide induced stimulation of cAMP production is shown in figure 2. The ED_{50}-values, defined as the concentrations that reduce the GLP-1(7-36)amide induced stimulation of cAMP production by 50%, are summarized in table 1. All peptides showed dose-dependent effects on stimulated cAMP levels with potencies that are similar to the binding potencies of these peptides on RINm5F cells.

DISCUSSION

Until recently, little was known about the mechanism of galanin action. By using the insulinoma cell line RINm5F, binding sites for this neuropeptide could be characterized (6). It has been shown that galanin inhibits insulin secretion by decreasing adenylate cyclase activity and intracellular cAMP production via a pertussis-toxin-sensitive pathway, involving an inhibitory GTP-binding protein (7, 8). In addition, galanin was reported to activate ATP-sensitive potassium channels in this cell line (26, 27). Experiments using galanin fragments for binding studies (10) revealed that the N-terminal portion of galanin is very important for interaction with its receptor. Furthermore, the tryptophane residue in position 2 of native galanin has been shown to play a crucial role in the inhibition of forskolin-stimulated cAMP production and insulin release from RINm5F cells (9) and in the inhibition of insulin release from the perfused dog pancreas (28).

In this study we used N-terminal fragments and analogues of galanin with substitutions of the naturally occurring amino acids in the positions 2, 4 and 7. In position 2 and 7, the D-stereoisomers were substituted. In position 4 leucine was changed to norleucine. Binding of galanin(1-22^{23}Cys) and inhibition of hormone-stimulated cAMP production by this fragment confirm that the N-terminal 22 amino acids of galanin are important for binding and biological activity. Galanin(1-22^{23}Cys) shows no significant difference in binding capacity or biological activity compared to native galanin. The 7 C-terminal amino acids do not seem to play an important role in the interaction of galanin with its receptor. In contrast to galanin(1-15) (9, 10), we demonstrated that galanin(1-18) is a significantly more potent receptor agonist. The introduction of nor-leucine for leucine in position 4 also does not change binding or biological effect of the analogue compared to the natural galanin molecule. Using the analogue galanin(1-29^{7}DAla) reveals that the naturally occurring L-stereoisomer of alanine in position 7 is required for full potency. This could be due to conformational changes of the galanin molecule caused by the above mentioned L- to D- stereoisomer exchange. Both analogues galanin(1-29^{2}DTrp) and galanin(1-29^{2}DTrp^{4}NLe^{7}DAla) showed substantially impaired binding and inhibition of stimulated cAMP production. In addition to the findings of Amiranoff et al. (9) and Lagny-Pourmir (10), the study with these two analogues show that in position 2 not only the presence of the indole ring of tryptophane is important for receptor recognition and signal transduction, but also the exact steric position of this part of the molecule

in relation to the surrounding amino acids. It is possible that substitution of the L-tryptophane by a D-tryptophane residue either induces a different tertiary structure of the galanin molecule, or directly interferes with the interaction of the peptide and the recognition site of its receptor. Interestingly, none of the galanin fragments or analogues used have antagonistic properties. They are agonists with different potencies, all showing the same efficacy for binding and biological activity. These data are not only applicable to galanin receptors on RINm5F cells, similar results were obtained in motility studies using ileal muscle strips, (29) or rat fundus strips (30). Therefore galanin receptors in the pancreas, the intestinal smooth muscle as well as the central nervous system (10) require similar structural properties of their ligand for transmitting an intracellular signal.

The ability of galanin to inhibit cAMP production stimulated by the insulinotropic peptide GLP-1(7-36)amide supports the assumption that both peptides interact in modulating postprandial insulin release by acting directly on adenylate cyclase. Similarly, it has been shown that galanin inhibits GIP- and forskolin stimulated insulin release in RINm5F cells (7), involving a pertussis toxin-sensitive GTP regulatory protein. Thus, galanin may counteract or attenuate the action of other insulinotropic peptides, so-called "incretins" in the entero-insular axis (13).

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