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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF GALANIN RECEPTOR SITES IN RAT BRAIN

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ABSTRACT - Receptors for galanin are identified and characterized in rat brain membranes. Interaction of [^{125}I]-galanin with its receptors is saturable, time, pH, and ionic strength-dependent. It is reversible and highly peptide specific. Scatchard analysis of binding data reveals the existence of one single class of high affinity binding sites with a K_D of 0.9 nM and a capacity of 101 fmoles/mg membranes protein. Chemical cross-linking of [^{125}I]-galanin to its brain receptor followed by SDS-PAGE analysis leads to the identification of one major protein of 56 kD corresponding to the galanin-receptor complex. Our findings provide the first biochemical characterization of galanin receptors in the central nervous system supporting a role for galanin in the control of brain functions.

Galanin, a 29 amino-acid peptide recently isolated from porcine intestine by a chemical approach that detects the C-terminal amide structure of the peptide (1), is widely distributed in neurons of central and peripheral nervous system (2-10). In consonance with its widespread localization in nerves, galanin exerts numerous biological effects including contraction of rat intestinal smooth muscle (1,11) and rat vas deferens (12), inhibition of insulin and somatostatin secretion (7,13,14) and enhancement of growth hormone (15) and glucagon plasma levels (7).

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Owing to its localization in various areas of brain and in peripheral nervous structures, galanin is a good candidate for being considered as a neurotransmitter. To assess this status the existence of receptors for galanin in central nervous system remains to be demonstrated. In the present work, we describe the first characterization of specific galanin receptors in rat brain membranes.

EXPERIMENTAL PROCEDURES

Materials - Galanin is purified from porcine intestine as described (1). Synthetic porcine secretin and peptide histidine isoleucine amide (PHI) are from Prof. Moroder (Max Planck Institute, RFA), porcine gastric inhibitory polypeptide (GIP), vasoactive intestinal peptide (VIP) from Prof. V. Mutt (Karolinska Institute, Sweden), rat GRF from Prof. R. Guillemin (Salk Institute, San Diego, USA). Peptide (P) having NH_2-terminal tyrosine (Y) and COOH-terminal tyrosine (Y) (PYY), rat pancreatic polypeptide, synthetic glucagon-37, neurotensin, substance P are from Peninsula Laboratories. Bovine insulin, leupeptin, bacitracin, tosyl-L-lysine-chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), EDTA, EGTA, GTP and ATP are from Sigma, bovine serumalbumin (BSA) from Miles Laboratories, chemicals and unstained protein markers for SDS-gel electrophoresis from Bethesda Research Laboratories and ethylene glycol bis-(succinimidyl succinate) (EGS) from Pierce Chemical Company. Carrier free Na-^{125}I (IMS 300, 14.7 mCi/µg of iodine) is obtained from Amersham (Buckinghamshire, England).

Preparation of the radioligand, [^{125}I]galanin.
Purified galanin is radiolabeled with ^{125}I by the chloramine T method (16). In a typical experiment, the following are added sequentially at room temperature to a glass tube: 20 µl of a galanin solution at 160 µg/ml in 0.3M sodium phosphate buffer pH 7.5, 2 µl (1mCi) ^{125}I-solution, and 5 µl of chloramine T (1mg/ml). After 30 seconds, the reaction is stopped by adding 5 µl of sodium metabisulfite (2mg/ml) and 200 µl of 0.3M phosphate buffer containing 1.5% (wt/vol) BSA and 0.04% (wt/vol) bacitracin. Radiolabeled peptide is purified on a Sephadex G-50 fine column (0.9 x 30cm), eluted with 0.2M acetic acid containing 0.5% (wt/vol) BSA and 0.03% (wt/vol) bacitracin (1ml/min). [^{125}I]-galanin is eluted as a single peak with a K_{av}=0.9. Specific activity of [^{125}I]galanin is 900-1000 Ci/mmol in six iodinations. Fractions corresponding to the peak of [^{125}I]galanin on the Sephadex column are pooled and stored frozen at -20°C until use. [^{125}I]galanin retains binding activity for at least 1 month.

Preparation of membranes from rat brain.
Male Wistar rats (180-200g) are killed by carotid section. Brains are rapidly removed and placed in an ice-cold Tris-HCl (50mM) buffer (pH 7.5) containing NaCl 100 mM, $MgCl_2$ 5mM, EDTA 1 mM, bacitracin 100 µg/ml, TLCK 10 µg/ml, PMSF 100 µM, leupeptin 10 µg/ml. All operations are performed at 4°C. Pooled brains are homogenized using a Waring blendor in a 10 fold higher volume of Tris-HCl buffer. After centrifugation at 20,000g, the resulting pellet is washed twice and the final material containing the crude membranes is frozen at -80°C until use. For the study of galanin receptor distribution, rat brains are dissected on ice according to Glowinski and Iversen (17).
Proteins are determined by the method of Bradford (18) using BSA as a standard.

Binding assay.
In standard conditions, binding assays are performed in a final volume of 0.25ml containing membranes (100 µg protein/ml), [^{125}I]galanin 0.2 nM, binding buffer (HEPES 20mM, pH 7.5, BSA 2% [wt/vol], bacitracin 0.1% [wt/vol]) and other compounds when specified. Membranes are incubated for 60 minutes at 15°C. Membrane-bound and free radioactivity are separated by centrifugation as previously described (19). Specific binding (saturable binding) is calculated as the difference between the amount of [^{125}I]galanin bound in the absence (total binding) and presence of 0.1µM unlabeled galanin (non-saturable binding). Non-saturable [^{125}I]galanin binding represents 30-40% of total binding and about 2% of total radioactivity. It is verified that specific [^{125}I]galanin binding is proportional to membranes concentration up to at least 200 µg protein/ml. Each binding measurement within one experiment is performed in duplicate.

Affinity covalent cross-linking of galanin receptor.
The cross-linking reaction is performed as described for other regulatory peptides (20,21), except that ethylene glycol bis-(succinimidyl succinate) (EGS) is used as cross-linker. Membranes (0.2 mg/ml) are incubated until equilibrium in the presence of 10 nM [^{125}I]galanin, and 1 µM of unlabeled galanin when specified. After washing at 20,000 g for 10 min. , membranes containing bound [^{125}I]galanin are resuspended in 1ml of 20 mM HEPES buffer, and incubated with EGS (1mM) for 15 min. at 4°C. Reaction is stopped by adding 20 ul of ice-cold HEPES buffer containing 60 mM lysine as a reagent quench. After centrifugation (20 min. at 20,000 g), the cross-linked material is resuspended in 60 mM Tris-HCl buffer, 3% sodium dodecyl sulfate (w/v), 10% glycerol (w/v), and 0.001% (w/v) Bromophenol Blue, pH 6.8, and incubated for 30 min. at 60°C. Aliquots of solubilized material are then applied to 5-15% polyacrylamide slab-gel, according to the Laemmli method (22), with 3% staking gel. After electrophoresis, the gels are dried and exposed at -80°C to a Trimax type XM film (3M Co) with a Trimax intensifying screen. The molecular weight standards used are alpha-chymotrypsinogen (25.6 kD), ovalbumin (43 kD), bovine serumalbumin (68 kD), phosphorylase b (92.2 kD) and myosin (200kD).

RESULTS

Specific binding of [^{125}I]-galanin to rat brain membranes is time-dependent. Binding is rapid, reaches a steady-state after 60 minutes which is maintained during the following hour (Figure 1). The association rate constant k_1, calculated from the pseudo-first order rate constant k_{obs}, is 2.8 x 10^8 M^{-1} min^{-1} (Figure 1, inset left). [^{125}I]-Galanin receptor complexes rapidly dissociate as a function of time (Figure 1, inset right) when an excess of native galanin (100 nM) is added to prevent the reassociation of the tracer. The first order dissociation constant, k_{-1} is 0.039 min^{-1}. The dissociation constant calculated from these values ($K_D = k_{-1}/k_1$) is 0.14 nM.

As shown in Figure 2, binding of galanin to rat brain membranes depends on pH and is maximal at pH 7. Varying the pH around this value

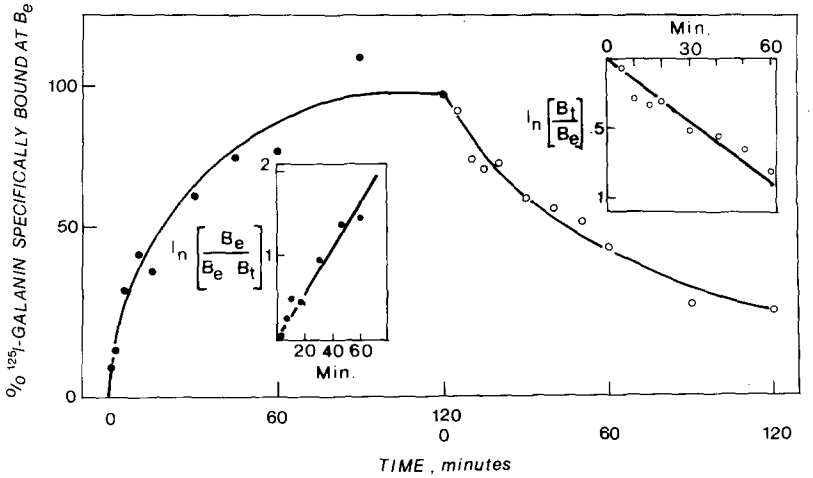

Figure 1. **Association and dissociation time course of specific binding of [^{125}I]-galanin with rat brain membranes.** [^{125}I]-galanin (0.2 nM) is incubated for different lengths of time with rat brain membranes. For dissociation experiment unlabeled galanin (100 nM) is added after association at equilibrium. The inset left shows linearization of the association data according to a pseudo-first order reaction, using the equation previously described (30). The inset right shows linearization of the dissociation data according to a pseudo-first order reaction.

dramatically decreases [^{125}I]-galanin binding to membranes. The interaction of galanin with brain membranes is also dependent on the ionic strength of the medium. Indeed, addition of NaCl or $MgCl_2$ to incubation medium strongly decreases the binding of [^{125}I]-galanin, maximal inhibition being observed at a ionic strength of 0.6 (Figure 2).

The concentration dependence of galanin binding to rat brain membranes is determined at equilibrium by adding increasing concentrations of unlabeled galanin. As shown in Figure 3 inset , native galanin in the concentration range between 10^{-11} and 10^{-8} M competitively inhibits the binding of [^{125}]-galanin to brain membranes, half maximal inhibition being elicited by 0.9 nM peptide. Computerized scatchard analysis of the data gives a straight line, indicating that galanin binds to a single population of binding sites (Figure 3). From six experiments, the dissociation constant K_D is estimated at 0.9 $\pm$ 0.2 nM and the concentration of binding sites at 101 $\pm$ 22 fmoles/mg protein.

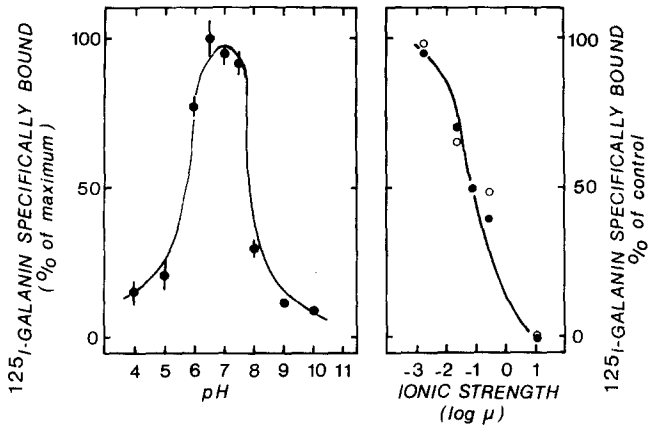

Figure 2. **Dependence of [^{125}I]-galanin binding to rat brain membranes on pH (left) and ionic strength (right).**
Rat brain membranes are incubated with [^{125}I]-galanin (0.2 nM) in 60 mM HEPES buffered at the indicated pH (left) or in 60 mM HEPES, pH 7.5, containing increasing concentrations of (●) NaCl, or (○) $MgCl_2$ (right). Each point is the mean ± SEM of three separate experiments.

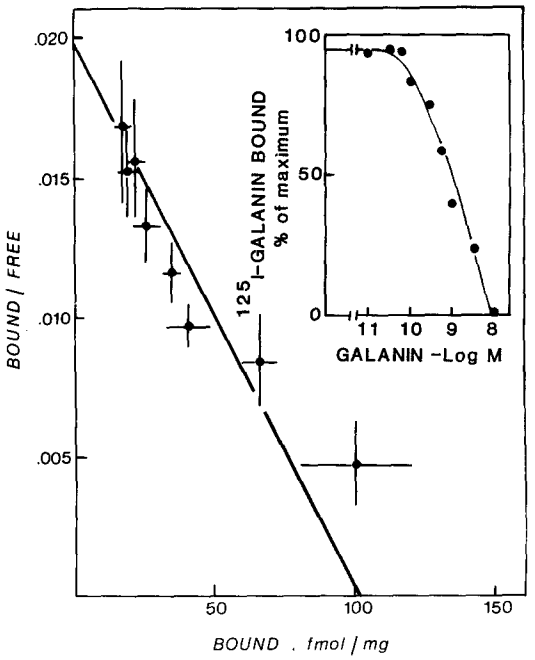

Figure 3. **Scatchard plot of [^{125}I]-galanin binding to rat brain membranes.**
Brain membranes are incubated with a fixed concentration of [^{125}I]-galanin (0.2 nM) and increasing concentrations of unlabeled galanin. The competitive inhibition of [^{125}I]-galanin binding by unlabeled galanin is described in the inset. Each point is the mean ± SEM of six separate experiments.

Table 1. **Regional distribution of specific [^{125}I]-galanin binding activity in rat brain**

Region	[^{125}I]-galanin specifically bound (fmol/mg protein)
Brainstem	2.67 ± 0.80
Cerebral cortex	0.96 ± 0.28
Cerebellum	2.20 ± 0.51
Hippocampus	4.04 ± 0.30
Hypothalamus	7.46 ± 0.30
Midbrain	1.71 ± 0.24
Striatum	1.66 ± 0.17

Results are means ± SEM of three separate experiments.

The specificity of brain galanin binding sites is tested by the ability of a variety of polypeptides to inhibit [^{125}I]-galanin binding to brain membranes. Among these peptides i.e. VIP, Secretin, GIP, Glucagon-37, Insulin, Neurotensin, Substance P, none of them appears to interact with the galanin receptor (not shown), firmly demonstrating the high specificity of the galanin receptor for its ligand.

A preliminary survey of the regional brain distribution of [^{125}I]-galanin binding sites is performed. A high specific binding of [^{125}I]-galanin is much higher in hypothalamus and hippocampus, than in other brain regions tested (Table 1). Values represent determinations at a single ligand concentration (0.2 nM) rather than B_{max}; they are proportional to the total receptor number only if the K_D values are very similar in all regions.

To further analyze the galanin receptors in brain, [^{125}I]-galanin has been covalently cross-linked to the receptor by EGS treatment. When the solubilized membrane proteins are submitted to SDS-PAGE analysis, the resulting autoradiograph (Figure 4, lane A) reveals that [^{125}I]-galanin is covalently cross-linked to a high molecular weight component. One major band is observed, corresponding to a [^{125}I]-galanin-protein complex of molecular weight 56,000. Two other bands (25 and 80 kD) were also detected but were very faint. The specificity of the labeling of these

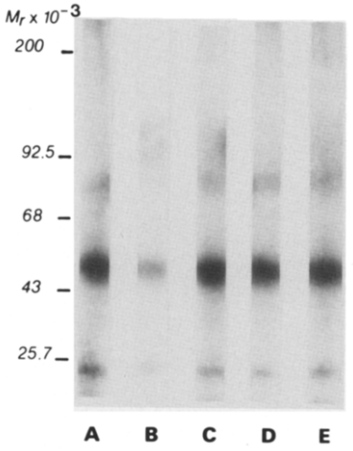

Figure 4. **Covalent labeling of [^{125}I]-galanin to rat brain membrane proteins as shown by SDS-PAGE analysis.**
Rat brain membranes are incubated with [^{125}I]-galanin alone (Lane A) or in the presence of native galanin (1 µM, Lane B), Neurotensin (1 µM, Lane C), Substance P (1 µM, Lane D), VIP (1 µM, Lane E).

components is tested by adding 1 µM unlabeled galanin or other peptide hormones together with the tracer in the incubation medium. As shown in Figure 4 (lane B), the covalent labeling of the major band is completely inhibited by unlabeled galanin while unaffected by 1 µM Neurotensin (lane C), Substance P (lane D) and VIP (lane E). When solubilized membrane proteins are treated with dithiothreitol (100 mM) the labeling of the band is not altered, suggesting that the galanin binding protein in brain does not contain subunits linked by S-S bonds (not shown).

DISCUSSION

The present study deals with the first characterization of galanin receptor in brain. Indeed interaction of galanin with brain membranes fulfills the criteria of specific peptide receptor i.e. saturability, reversibility, high affinity and specificity. Scatchard analysis indicates that interaction of galanin with its receptors fits with a bimolecular association of the peptide and a population of a non interacting receptor site. This is consistent with dissociation experiments (see Figure 1) which indicate simple first-order kinetics.

This study also documents some molecular properties of the galanin receptor protein. Indeed, by use of the cross-linking homobifunctional reagent EGS and SDS-PAGE analysis, a single protein of Mw 56,000 appears to be involved in the specific binding of the peptide. If we assume that one molecule of galanin (Mw 3,200) is linked per receptor, the intrinsic Mw of the receptor is about 53,000. This is in the range of the molecular weights of many other peptide hormone brain receptors, such as VIP (23), Neurotensin (24) and octaCCK (25).

The identification of specific receptors in rat brain membranes agrees well with the preliminary visualization of galanin binding sites detected by autoradiography (26). Moreover, it is highly consistent with the widespread localization of this neuropeptide in cell bodies and neurones of the central nervous systems (2-5), particularly in hypothalamus (3) and hippocampus (27). Thus galanin originating from hypothalamic neurons may control pituitary function through a neuroendocrine pathway, since infusion of galanin in vivo in rats, stimulates the release of growth hormone (28). In addition, galanin appears to stimulate the feeding behaviour of rats (29). The identification of specific galanin receptors in brain provides the first decisive argument assessing the physiological involvement of galanin in brain control, and argues for a role for galanin as a neurotransmitter in the central nervous system.

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