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Identification and characterization of glucagon-like peptide-1 7–36 amide-binding sites in the rat brain and lung

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High-affinity binding sites for glucagon-like peptide-1 7–36 amide (GLP-1 7–36 NH_2) were identified in rat brain and lung membranes. Binding of [^{125}I]GLP-1 7–36 NH_2 was rapid, reversible, specific, saturable and pH dependent. Specific binding in the central nervous system was particularly high in the hypothalamus and the brain stem. Oxyntomodulin, glucagon-like peptide-1, glucagon-like peptide-2 and glucagon were 100–1000-fold less potent than GLP-1 7–36 NH_2 in competition for this binding site.

Glucagon-like peptide 7–36 amide; Receptor; (Brain, Lung)

1. INTRODUCTION

The proglucagon gene also encodes two peptides which share approx. 50% sequence homology with glucagon and are named glucagon-like peptide-1 and -2 (GLP-1 and GLP-2) [1]. The GLP-1 sequence is identical in hamster, man, ox and rat [2] and this high degree of sequence conservation suggests that GLP-1 has a significant biological role which has yet to be determined. Apart from its weak insulinotropic effect [3], the only known actions of GLP-1 are its binding in the hypothalamus and pituitary and the subsequent adenylate cyclase activation [4,5]. While GLP-1 is the major processed form found in the pancreas a smaller fragment GLP-1 7–36 NH_2 predominates in the intestine [6]. GLP-1 7–36 NH_2 occurs naturally, and is a potent stimulator of insulin secretion in the isolated rat pancreas [7], a rat β-cell line [3], and the most potent insulinotropic hormone known in man [6]. The existence of GLP-1 7–36 NH_2 receptors in rat insulinoma cell lines has been reported [8,9]. Recently we have reported the presence of GLP-1 7–36 NH_2 in the brain [10]. This peptide has also been shown to be a potent inhibitor of gastric acid secretion [11]. We have studied the binding of [^{125}I]GLP-1 7–36 NH_2 in various tissues to obtain more information about the possible biological activities of GLP-1 7–36 NH_2.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Gastric inhibitory polypeptide (GIP), vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM), GLP-1 7–36 NH_2, GLP-1, GLP-2, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), and substance P (SP) were obtained from Peninsula Laboratories (Merseyside, England). Oxyntomodulin was custom synthesized by Institute of Armand Frappier (Quebec, Canada) and pure porcine glucagon was obtained from Novo Research Institute (Copenhagen, Denmark). Tween 20 (polyoxyethylene-sorbitan monolaurate) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO); protein assay reagent and BSA standards were obtained from Pierce (Rockford, IL). Carrier free Na^{125}I was obtained from Amersham International (Buckinghamshire, England).

2.2. *Tissue preparation*

Homogenization of tissues from Wistar rats was carried out in a 10-fold higher volume of ice cold 50 mM Tris-HCl buffer

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(pH 7.4) containing 0.32 M sucrose using an Ultra-Turrax homogenizer. The homogenate was centrifuged at $1000 \times g$ for 20 min and the resulting supernatant centrifuged at $50\,000 \times g$ for 20 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl. This was recentrifuged and the pellet resuspended in Tris-HCl buffer at a final protein concentration of 1–3 $\mu g/\mu l$. Protein content was estimated by the Coomassie blue dye method with BSA as standard [12].

2.3. *Preparation and analysis of the radioligand [^{125}I]GLP-1 7-36 NH_2*

GLP-1 7-36 NH_2 was iodinated by the chloramine T method [13] and purified by HPLC. In a typical experiment the following were added sequentially: 10 μg of GLP-1 7-36 NH_2 in 50 μl of 0.4 M sodium phosphate buffer (pH 7.4); 10 μl of 1 mCi $Na^{125}I$ solution; 10 μl of chloramine T (0.45 mM). After 10 s the reaction was stopped by adding 25 μl sodium metabisulphite (1 mM). The reaction mixture was diluted with 20% (v/v) aqueous acetonitrile (ACN) containing 0.1% (v/v) TFA and loaded on a C18 reverse-phase column which was equilibrated with the same solution. Optimal separation of [^{125}I]GLP-1 7-36 NH_2 was achieved by elution with an isocratic gradient of 42% ACN. Specific activity of the tracer was checked by radioimmunoassay as described previously [6].

2.4. *Binding assays*

Under standard conditions, the binding assays were performed in a final volume of 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing BSA (0.3% w/v), $MgCl_2$ (5 mM), EDTA (2 mM), Tween 20 (0.05% v/v), membranes (200 μg), [^{125}I]GLP-1 7-36 NH_2 (60 fmol) and unlabelled competing peptides as specified. Membranes were incubated at 18°C for 90 min and the separation of bound and free radioactivity was carried out by centrifugation at $12\,000 \times g$ for 2.5 min in a microfuge. The supernatant was removed and the radioactivity in the pellet counted. Specific (saturable) binding was calculated as the difference between the amount of [^{125}I]GLP-1 7-36 NH_2 bound in the absence (total binding) and presence of 0.1 μM unlabelled GLP-1 7-36 NH_2 (non-saturable binding). Scatchard plots were analysed by linear regression to determine dissociation constant (K_d) and the binding capacity (B_{max}).

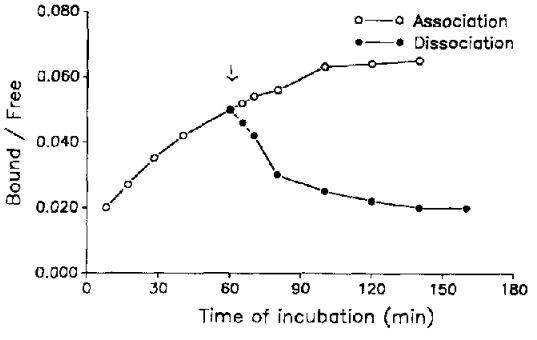

Fig.1. Time course of [^{125}I]GLP-1 7-36 NH_2association and dissociation to brain stem membranes. Dissociation was initiated by addition of native GLP-1 7-36 NH_2 to give a final concentration of 0.1 μM (indicated by arrow).

3. RESULTS

Chloramine T iodination and HPLC purification of the label consistently gave a single peak of radioactivity. This peak had a specific activity of 500–1500 Ci/mmol. Initial experiments showed that the label was adsorbed strongly to the reaction tubes (polypropylene, Eppendorf). This was minimised by adding 0.05% Tween 20 to the incubation buffer as described by Paul et al. [14].

In preliminary experiments we observed high levels of binding in the lung and brain and very low levels of binding in the liver, pancreas, adipose tissues, stomach mucosa, and spleen. No binding could be detected in the thyroid gland, adrenal gland, kidney, skeletal muscle and ileal smooth muscle.

Specific binding of [^{125}I]GLP-1 7-36 NH_2 to rat brain membranes reached a maximum after 90 min and was maintained for the next 60 min. Addition of 0.1 μM cold peptide to membranes, in equilibrium with the label, caused a rapid displacement of the bound label (fig.1).

The stability of the ligand upon incubation with membranes was investigated by studying its elution characteristics on HPLC. After 90 min incubation with membranes at 18°C nearly all the radioactivity eluted in the same position as [^{125}I]GLP-1 7-36 NH_2.

The binding of [^{125}I]GLP-1 7-36 NH_2 to brain and lung membranes was pH dependent and maximum binding was observed at pH 7.4.

A survey of the regional distribution of [^{125}I]-

Table 1

Regional distribution of [^{125}I]GLP-1 7-36 NH_2 binding sites in the brain (mean ± SE, n = 5)

Region	Specific binding (% of total [^{125}I]GLP-1 7-36 NH_2 added)
Hypothalamus	1.85 ± 0.25
Medulla oblongata	1.32 ± 0.31
Midbrain	1.28 ± 0.19
Pons	1.02 ± 0.20
Pituitary gland	0.78 ± 0.11
Cerebellum	0.18 ± 0.04
Cerebral cortex	0.12 ± 0.04
Olfactory bulb	0.05 ± 0.04

Binding is expressed as % of tracer that is specifically bound per 50 μg of protein

GLP-1 7-36 NH_2-binding sites in the brain (table 1) was performed. These values represent determinations at a single ligand concentration (60 fmol) rather than B_{max}. High specific binding in the brain was observed in the hypothalamus and the brain stem region whereas the cortex and cerebellum showed negligible binding.

To determine the specificity of GLP-1 7-36 NH_2-binding sites, a variety of peptides were tested for their ability to inhibit [^{125}I]GLP-1 7-36 NH_2 binding to brain stem and lung membranes. The patterns of displacement were similar in the brain stem and lungs (fig.2A and B). CGRP, SP, NPY, VIP, PHM, GIP did not interact with the GLP-1 7-36 receptor at concentrations up to 1 μM. The order of potency of the peptides from the glucagon gene was oxyntomodulin > GLP-1 and GLP-2 > glucagon.

The concentration dependence of GLP-1 7-36

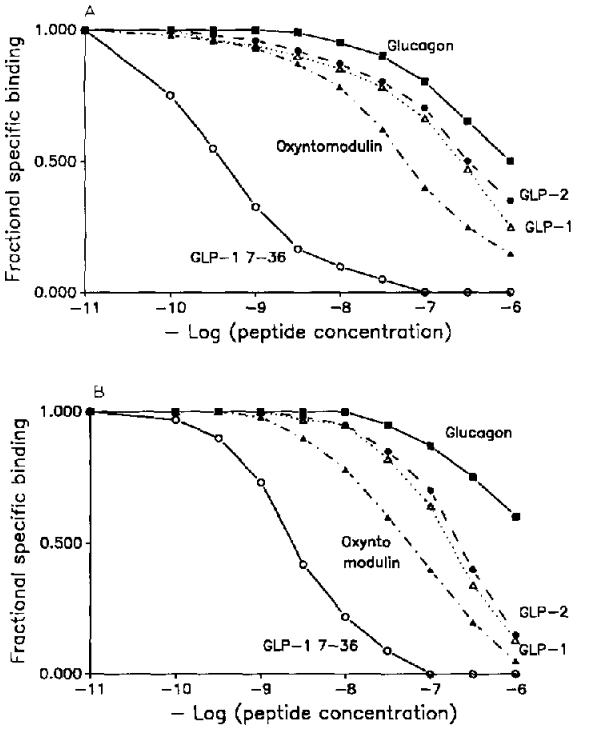

Fig.2. Inhibition of [^{125}I]GLP-1 7-36 NH_2 binding in the brainstem (A) and lung (B) by increasing concentrations of various peptides. Binding is expressed as a fraction of specific binding obtained in the presence of [^{125}I]GLP-1 7-36 NH_2 alone. Results show the mean of three experiments in triplicate (SE< 10%).

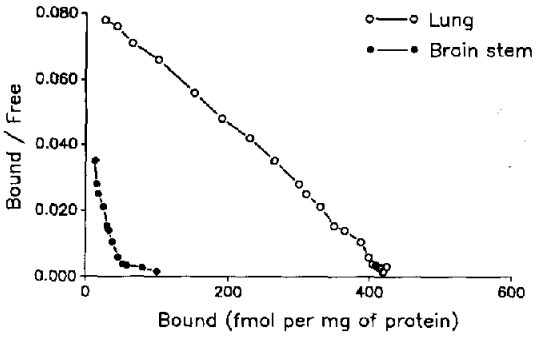

Fig.3. Scatchard analysis of GLP-1 7-36 NH_2 binding in the brain stem and the lung. Dissociation curve data obtained with increasing concentration of native GLP-1 7-36 NH_2 as in fig.2A and B were transformed to give a Scatchard plot.

NH_2 binding to rat brain and lung membranes was determined by adding increasing concentrations of unlabelled GLP-1 7-36 NH_2. Scatchard analysis of these data revealed a curve indicating two populations of binding sites in the brain stem with a dissociation constant (K_d) of 0.47 ± 0.08 and 15 ± 5.2 nM and a maximum binding capacity (B_{max}) of 35 ± 9 and 130 ± 32 fmol/mg of protein. There was one class of binding sites in the lungs with a K_d of 2.5 ± 0.9 nM and a B_{max} of 560 ± 46 fmol/mg of protein (fig.3).

4. DISCUSSION

The interaction of GLP-1 7-36 NH_2 with rat brain and lung membranes satisfied the generally accepted criteria for a peptide hormone and receptor interaction.

The high density of binding sites for GLP-1 7-36 NH_2 observed in the hypothalamus correlates well with the high density of GLP-1- and glucagon-binding sites at this location [4]. Glucagon and Glp-1 are also localized in this particular region of the brain [4,10,15]. In the hypothalamus peptides from the preproglucagon gene may play a role in the regulation of pancreatic hormone release [16].

The order of potency of related peptides in competing with GLP-1 7-36 NH_2 was similar in the brain stem and lung suggesting that the receptor-binding sites in these tissues are similar. GLP-1 and GLP-2 were equipotent in displacing GLP-1 7-36 NH_2 from its receptor at a 100-fold lower potency than GLP-1 7-36 NH_2. Oxyntomodulin showed

higher crossreactivity than glucagon for the binding site. The conformation of glucagon and oxyntomodulin is different from that deduced from competition data [17,18] and this could also account for their different interactions with the GLP-1 7-36 receptor. This property of oxyntomodulin and GLP-1 to interact with the GLP-1 7-36 NH_2 receptor could account for their weak insulinotropic activity [19] and also their effect on cAMP production in mucosal cells [11].

The presence of GLP-1 7-36 NH_2 receptors at multiple locations in the body increases the possibility of other functions for this newly discovered hormone.

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