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Characterization of glucagon-like peptide-I(7–36)amide receptors of rat lung membranes by covalent cross-linking

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^{125}I-labelled GLP-I(7–36)amide was cross-linked to a specific binding protein in rat lung membranes using disuccinimidyl suberate. A single radiolabelled band at M_r 66000 was identified by SDS-PAGE after solubilization of the ligand-binding protein complex which is consistent with the presence of a single class of binding sites on rat lung membranes. The band was undetectable when 1 μmol/l GLP-I(7–36)amide was included in the binding assay. No change in the mobility of the band was observed under reducing conditions suggesting that the binding protein in the receptor is not part of a larger disulphide-linked protein. The intensity of the radiolabelled protein band was reduced when the incubation with ^{125}I-labelled GLP-I(7–36)amide was carried out in the presence of guanine nucleotides suggesting that the GLP-I(7–36)amide receptor is coupled to the adenylate cyclase system.

GLP-I(7–36)amide; Receptor; Lung; Covalent cross-linking

1. INTRODUCTION

Glucagon-like peptide-I(7–36)amide (GLP-1) is a posttranslational processing product of proglucagon in the mammalian intestine [1–3]. The amino acid sequence of GLP-1 was kept during evolution and is identical in different mammals including man [4–6]. This indicates an important biological meaning of GLP-1 at least in mammals. In previous studies, it has been shown that GLP-1 is released in response to oral glucose and potently stimulates the glucose-induced insulin secretion [7–9]. Thus, GLP-1 is considered to be a new incretin candidate besides the gastric inhibitory peptide (GIP). Furthermore, GLP-1 is a strong inhibitor of the pentagastrin-stimulated gastric acid secretion and therefore considered to be an enterogastrone candidate [10,11]. Corresponding to these data, receptors for GLP-1 have been identified on rat insulinoma-derived RINm5F cells and isolated gastric glands of the rat [12,13]. Recently, we demonstrated specific binding sites for GLP-1(7–36) amide on rat lung membranes [14]. The present study describes the use of a covalent cross-linking reagent to further characterize the ligand-binding component of the GLP-1 receptor on rat lung membranes.

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2. MATERIALS AND METHODS

2.1. *Reagents*

GLP-1(7–36)amide was purchased from Peninsula Laboratories (St. Helens, Merseyside, UK). Disuccinimidyl suberate was from Pierce Europe BV (Oud-Beijerland, The Netherlands). GTP-γ-S and GDP-β-S were from Sigma Chemicals (Deisenhofen, Germany). ^{125}I-labelled GLP-1(7–36) amide (spec. act. 74 TBq/mmol) was prepared as described by Göke and Conlon [12].

2.2. *Cells*

RINm5F cells were grown in plastic culture bottles under the conditions described by Praz et al. [15]. The cells were detached from the surface of the bottles before experimentation using phosphate-buffered saline (NaCl 136 mmol/l, KCl 2.7 mmol/l, Na_2HPO_4 8.1 mmol/l, KH_2PO_4 1.5 mmol/l; pH 7.3) containing 0.7 mmol/l EDTA. Cell concentrations were determined either by using a Neubauer haemocytometer or by measurement of cellular DNA [16].

2.3. *Membrane preparation*

Plasma membranes of RINm5F cells were prepared as described previously [17]. Briefly, approximately 6×10^8 RINm5F cells were suspended in 30 ml ice-cold homogenization buffer (Tris-HCl 10 mmol/l, NaCl 30 mmol/l, dithiothreitol 1 mmol/l, phenylmethylsulphonylfluoride (PMSF) 5 μmol/l; pH 7.5) and disrupted using a tight-fitting glass-glass homogenizer with six passages at 150 rpm.

Rat lung membranes were prepared as described in detail previously [14]. Male albino Wistar rats (200–220 g body weight) were killed by decapitation, the lungs were removed and homogenized in homogenization buffer with an Ultra-Turrax followed by a further homogenization using a glass-glass homogenizer.

The following steps in the preparation of RINm5F cell plasma membranes and rat lung membranes were identical. The homogenate was layered over a 41% (w/v) sucrose solution and centrifuged for 60 min at 4° at 95000 × *g*. The band at the interface of the layers represented the membranes and was collected, diluted and centrifuged for 30 min at 40000 × *g*. The pellet was resuspended in a modified Krebs-Ringer buffer (KRB) (Tris-HCl 2.5 mmol/l, NaCl 120 mmol/l,

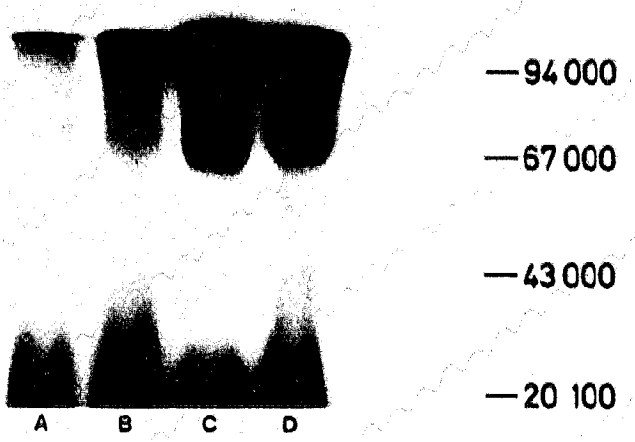

Fig. 1 Effect of glucagon-like peptide-1(7-36)amide (GLP-1(7-36)-amide on the cross-linking of ^{125}I-GLP-1(7-36)amide to its receptor in rat lung plasma membranes. Equal aliquots of membranes were incubated with tracer in the absence (D) and presence of 0.1 nmol/l (C), 10 nmol/l (B) and 1000 nmol/l (A) GLP-1(7-36)amide. Experiment was carried out as described in section 2. The numbers show the mobilities of phosphorylase *b* (M_r 94 000), human serum albumin (M_r 67 000), ovalbumin (M_r 43 000) and trypsin inhibitor (M_r 20 100).

$MgSO_4$ 1.2 mmol/l, KCl 5 mmol/l, CH_3COONa 15 mmol/l; pH 7.4) containing 1% (w/v) human serum albumin, 0.1% (w/v) bacitracin and EDTA 1 mmol/l, frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined as described by Bradford [18].

2.4 *Cross-linking experiments*

Plasma membranes (200 μg) were incubated with ^{125}I-labelled GLP-1(7-36)amide (approximately 40 000 cpm; spec. act. approx. 74 TBq/mmol) in Hepes buffer (50 mmol/l; pH 7.5) containing 0.02% (w/v) human serum albumin for 30 min at 37°C. Incubations were also carried out in the presence of a range of concentrations of unlabelled GLP-1(7-36)amide or in the presence of guanine nucleotides as indicated. Incubations were terminated by centrifugation (10 000 × *g* for 5 min at 4°C) and pellets resuspended in ice-cold Hepes buffer (10 mmol/l; pH 9.0) containing 0.02% (w/v) human serum albumin.

A solution of disuccinimidyl suberate in dimethylsulphoxide was added to give a 0.1 mM final concentration [17]. After incubation for 10 min at 4°C the reaction was stopped by addition of ammonium acetate solution (final concentration 10 mmol/l). Membranes were centrifuged (10 000 × *g* for 5 min at 4°C) and the pellets resuspended in Hepes buffer (10 mmol/l; pH 7.5) containing 0.02% human serum albumin. An incubation for 30 min at 25°C was carried out in order to dissociate non-covalently bound ligand. After centrifugation (10 000 × *g* for 5 min at 4°C), the pellets were suspended in sodium phosphate buffer (10 mmol/l; pH 7.5) containing 2% (w/v) sodium dodecylsulphate (SDS). Samples were boiled for 5 min in the presence and absence of 5% (v/v) mercaptoethanol. Electrophoresis was carried out using the discontinuous buffer system described by Laemmli [19] with a 4% acrylamide stacking gel and a 10% acrylamide separating gel. The gels were calibrated using a molecular weight marker kit from Pharmacia, Uppsala, Sweden. Gels were dried and exposed to Kodak type X-Omat AR film for 2 weeks at −80°C using an image-intensifying screen.

3. RESULTS

Incubation of lung membranes with ^{125}I-labelled GLP-1(7-36)amide followed by chemical cross-linking and solubilization has identified a single ligand-binding protein complex of M_r 66 000 ± 1000 (n = 6) (Fig. 1). The intensity of the radiolabelled band was reduced when the incubation with ^{125}I-labelled GLP-1(7-36) amide was carried out in the presence of increasing concentrations of unlabelled GLP-1(7-36)amide. At 1 μmol/l unlabelled GLP-1(7-36)amide no band was detectable anymore (Fig. 1). When experiments were carried out in the absence of rat lung membranes no radioactive labelled band was detectable in the gel. Incubations carried out in the presence of GTP-γ-S (0.1 mmol/l) or GDP-β-S (0.1 mmol/l) resulted in a reduction of the specific receptor linked tracer binding (Fig. 2).

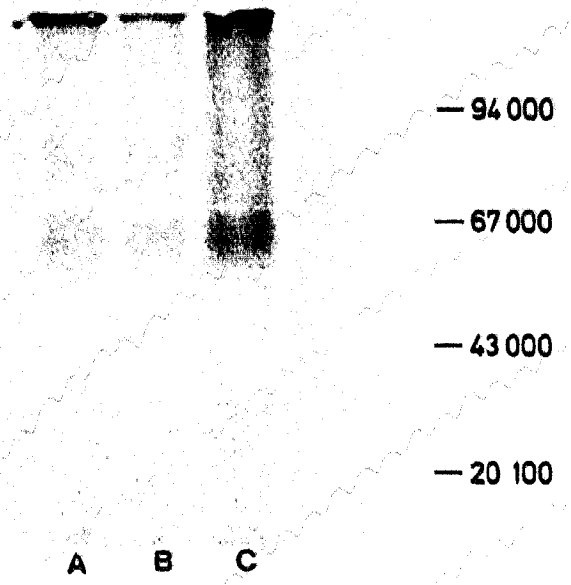

Fig. 2. Effect of guanine nucleotides on the cross-linking of ^{125}I-GLP-1(7-36)amide to its receptor in rat lung plasma membranes. Equal aliquots of membranes were incubated as described in section 2 in the absence (C) and presence (B) of 0.1 mmol/l GTP-γ-S and 0.1 mmol/l GDP-β-S (A). The numbers show the mobilities of phosphorylase *b* (M_r 94 000), human serum albumin (M_r 67 000), ovalbumin (M_r 43 000) and trypsin inhibitor (M_r 20 100).

A solubilization of the cross-linked receptor/ligand complex under reducing conditions did not lead to a change of the motility of the labelled M_r 66 000 band (Fig. 3).

Previously, we characterized GLP-1 receptors on RINm5F plasma membranes by covalent cross-linking [17]. Experiments carried out to compare cross-linked and solubilized GLP-1 receptor-ligand complexes from rat lung and RINm5F membranes revealed no significant difference in the molecular weight of the ligand-binding protein complexes (Fig. 4).

Vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) are able to displace ^{125}I-GLP-1(7-36)amide from rat lung membranes with a K_d

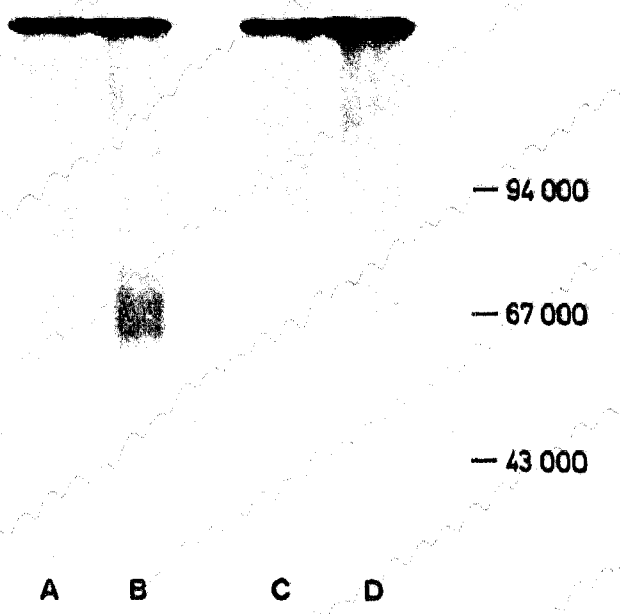

Fig. 3. Effect of solubilization under reducing conditions on the cross-linking of ^{125}I-GLP-1(7–36)amide to its receptor in rat lung plasma membranes. Solubilization was carried out in the presence (A,B) and absence (C,D) of 5% (v/v) β-mercaptoethanol. Incubations were carried out in the absence (B,D) and presence (A,C) of 1 μmol/l GLP-1(7–36)amide. The numbers show the mobilities of phosphorylase *b* (M_r 94 000), human serum albumin (M_r 67 000) and ovalbumin (M, 43 000).

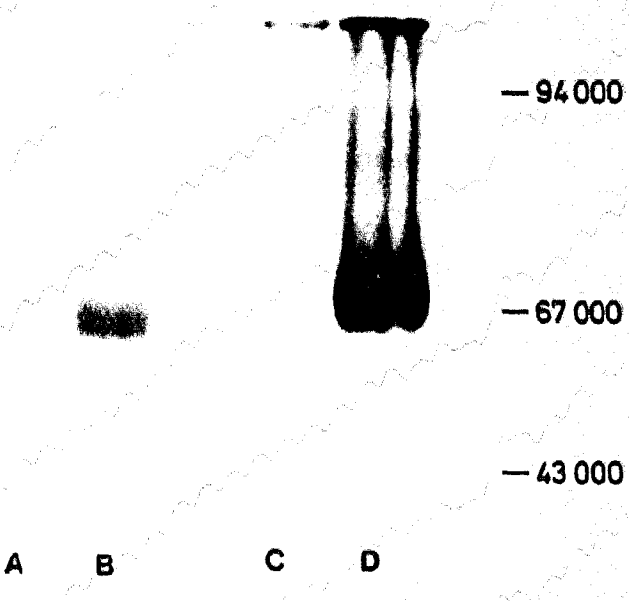

Fig. 4. Comparison of the cross-linking of ^{125}I-GLP-1(7–36)amide to its receptor in rat lung (A,B) and RINm5F (C,D) plasma membranes. Incubations were carried out as described in section 2 in the presence (A,C) and absence (B,D) of 1 μmol/l GLP-1(7–36)amide. The numbers show the mobilities of phosphorylase *b* (M_r 94 000), human serum albumin (M_r 67 000) and ovalbumin (M_r 43 000).

which is 2.5 and 4.7 times less, respectively, than that of GLP-1(7-36)amide [14]. We then incubated rat lung membranes (1 mg protein/ml) with ^{125}I-VIP in the presence and absence of GLP-1(7-36)amide. GLP-1(7-36)amide did not displace ^{125}I-VIP from its binding to rat lung membranes (data not shown).

4. DISCUSSION

Recently, we demonstrated the presence of a single class of high affinity receptors for GLP-1(7–36)amide on rat lung membranes [14]. Consistent with this result only a single protein with M_r 66000 was affinity-labelled using the cross-linking agent disuccinimidyl suberate which is reactive towards amino groups and has found widespread application in the characterization of binding proteins for neurohormonal peptides [20–25]. Evidence that the tracer bound to a component of the GLP-1 receptor was obtained by the fact that labelling of the protein was inhibited by increasing concentrations of unlabelled GLP-1(7–36)amide.

Solubilization of the receptor/ligand complex under reducing conditions did not lead to a change of the motility of the labelled band suggesting that the binding component for GLP-1(7–36)amide in rat lung membranes is not a part of a larger disulphide-linked protein.

Recently, we reported that binding of ^{125}I-GLP-1(7–36)amide to lung membranes is decreased in the presence of guanine nucleotides [14]. Confirming these data, we demonstrate now that incubations in the presence of guanine nucleotides resulted in a reduction of the specific receptor linked tracer binding. This supports the assumption that in rat lung membranes GLP-1(7–36)amide receptors are also linked to the adenylate cyclase system.

The data of this study are very similar to results we obtained with GLP-1(7–36)amide receptors from RINm5F plasma membranes in a previous study [17]. Directly compared both receptors exhibited the same molecular weight. Thus, it seems that GLP-1(7–36)-amide receptors in RINm5F and rat lung plasma membranes do not differ due to their biochemical properties.

Interestingly, the binding affinity of GLP-1(7–36)-amide on rat lung receptors is 10 times less compared to receptors on RINm5F membranes [14]. Furthermore, vasoactive intestinal peptide (VIP) and peptide histidine isoleucin (PHI) do not bind to GLP-1(7–36)amide receptors on RINm5F membranes but are able to displace ^{125}I-GLP-1(7–36)amide from rat lung membranes with a K_d which is only 2.5 and 4.7 times, respectively, less than that of GLP-1(7–36)amide [14] while ^{125}I-VIP is not displaced by GLP-1(7–36)amide from rat lung membranes.

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