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Cross-reactivity of amylin with calcitonin-gene-related peptide binding sites in rat liver and skeletal muscle membranes

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This study examines whether the high degree of sequence identity between amylin and calcitonin-gene-related peptide (CGRP) is reflected in their cross-reactivity at the level of membrane receptor binding. Rat liver plasma membranes contain a specific saturable binding site for ^{125}I-labelled human CGRP-1. Binding reached equilibrium within 30 min and was rapidly reversed by re-incubating membranes in the presence of 1 μM human CGRP. In addition, the presence of 50 mM- or 500 mM-NaCl lowered specific binding by 30% and 77% respectively. Scatchard analysis was consistent with a single high-affinity site with a dissociation constant (K_d) of 0.125 nM and binding capacity ($B_{max.}$) of 580 fmol/mg of membrane protein. Specific binding of ^{125}I-labelled human CGRP-1 to both liver and skeletal muscle membranes was inhibited by human CGRP-1 [IC_{50} (concn. causing half-maximal inhibition of binding) 0.1–0.3 nM], and rat amylin (IC_{50} 10 nM), but not by human calcitonin. Covalent cross-linking of ^{125}I-CGRP to its binding site in rat skeletal muscle and liver membranes resulted in labelling of a major species of about 70 kDa under reducing conditions and about 55 kDa under alkylating conditions, as visualized on SDS/PAGE. These radiolabelled species were absent in the presence of CGRP or amylin at 1 μM. These results are indicative of a common binding site for both CGRP and amylin in liver and skeletal muscle, and it is suggested that both peptides mediate their actions through the same effector system. The normal physiological importance and the relevance to the pathology of type 2 diabetes of these data are discussed.

INTRODUCTION

Calcitonin-gene-related peptide (CGRP) is a 37-amino-acid peptide which was identified by analysis of the nucleotide sequence of the calcitonin gene [1]. CGRP shares about 50% amino acid sequence identity with a recently discovered polypeptide hormone named amylin [2]. Amylin was originally isolated from amyloid deposits in the pancreases of patients with type 2 diabetes mellitus, and it has been suggested that an overproduction of amylin explains the peripheral insulin-resistance seen in this disorder [3]. This hypothesis is supported by the fact that amylin is a potent inhibitor of insulin-stimulated glycogen synthesis in rat skeletal muscle preparations *in vitro* [3,4], and amylin also causes insulin-resistance *in vivo* [5]. CGRP is equally effective at inducing insulin-resistance in these experimental systems; however, it is not clear whether the responses to amylin and CGRP are mediated through the same or separate effector systems.

Binding sites for CGRP have been identified in a number of peripheral and central nervous system tissues [6]. There is also a degree of overlap between the distribution of CGRP-binding sites and CGRP-immunoreactive structures in many tissues. In muscle, CGRP-immunoreactive material has been localized in both motor-end plates and sensory nerves [7]. Corresponding high-affinity CGRP-binding sites have been identified in sarcolemmal membranes [8]. However, immunoreactive CGRP could not be detected in liver [9], where recent studies have demonstrated a high-affinity CGRP-binding site [10], and only a minimal amount of CGRP is distributed in the sensory fibres of the hepatic vasculature [11].

The site of amylin biosynthesis and secretion into the bloodstream is the β-cell in the islet of Langerhans of the pancreas. The low abundance of endogenous CGRP in the liver suggests that the putative glucoregulatory role of these peptides in hepatic metabolism may be mediated primarily by amylin. Therefore, the main aim of this study was to examine the binding characteristics of amylin and CGRP in the liver and skeletal muscle and to correlate this specificity of binding with the known tissue distribution of these peptides.

MATERIALS AND METHODS

Materials

Human CGRP-1 was obtained from Peninsula Laboratories (Belmont, CA, U.S.A.) and rat amylin was from Cambridge Research Biochemicals (Cambridge, U.K.). Human thyrocalcitonin was from Sigma and ^{125}I-human CGRP-1 (approx. 2000 Ci/mmol) was obtained from Amersham. All non-radiolabelled peptides were dissolved in 3 mM-HCl, divided into portions and stored at −20 °C until used. The concentration of peptide was accurately determined by amino acid analysis using a Waters PicoTag system [12].

Membrane preparations

Rat liver and hind-limb skeletal muscle were obtained from overnight-starved male Wistar rats (140–160 g) purchased from Harlan-Olac (Bicester, U.K.). Freshly dissected skeletal muscle was chopped with scissors, homogenized (Ultra-Turrax) in 10 vol. of 20 mM-Hepes (pH 7.4)/0.1 mM-phenylmethanesulphonyl fluoride/2 mM-$MgCl_2$/0.6 mM-$CaCl_2$ (buffer H), filtered through muslin and centrifuged (250 *g*, 5 min, 4 °C) to remove nuclei. The supernatant was centrifuged (100000 *g*, 40 min, 4 °C), and membranes were washed twice by centrifugation (100000 *g*, 40 min, 4 °C) in buffer H and finally resuspended in buffer H.

Liver plasma membranes were prepared essentially according

Abbreviations used: CGRP, calcitonin-gene-related peptide; IC_{50}, concentration causing 50% inhibition of binding; DSS, disuccinimidyl suberate; G-protein, guanine-nucleotide-binding protein.

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to the method of Pilkis [13]. Briefly, about 20 g of fresh rat liver was homogenized in 200 ml of buffer H and filtered through muslin. Membranes were centrifuged (100000 *g*, 40 min, 4 °C) and resuspended in a minimal volume of buffer H (16–20 ml) followed by 180 ml of 70.7% (w/v) sucrose in buffer H. The suspension was homogenized briefly and layered at the bottom of a 48.2% and 42.5% discontinuous sucrose gradient. Following centrifugation (100000 *g*, 60 min, 4 °C), plasma membranes were collected from the 42.5/48.2% sucrose interface and washed twice in buffer H (100000 *g*, 30 min, 4 °C). Membranes were resuspended in about 40 ml of buffer H and portions were stored frozen at −70 °C. Membrane protein content was estimated by the method of Lowry [14] using BSA as a standard.

Membrane binding assay

The methodology of the binding assay used was as follows. Membranes were rapidly thawed, washed twice by centrifugation (10000 *g*, 10 min, 4 °C) with buffer A [buffer H containing 0.1% (w/v) BSA] and resuspended to 0.8 mg of protein/ml. Membranes (0.4 mg of protein/ml) were exposed to various concentrations of ^{125}I-human CGRP in a final volume of 500 μl. All dilutions were performed in buffer A. Non-specific binding was determined by including 1 μM unlabelled human CGRP-1. After a specified time at 4 °C, 50 μl was removed for determination of total c.p.m. and the remaining membranes were centrifuged (10000 *g*, 10 min, 4 °C), the supernatant carefully aspirated and tips of the Eppendorf tubes cut off, and the amount of bound radioactivity was determined.

Photoaffinity cross-linking

The methodology was essentially that described by Larose *et al.*, [15]. It does not require the use of a specific cross-linking reagent and relies upon the spontaneous cross-linking of adjacent proteins in the presence of lipid and u.v. light. Rat liver or muscle membranes (0.8 mg of protein/ml) were incubated in the dark (4 °C, 40 min) with 100 pM-^{125}I-CGRP with or without unlabelled CGRP or amylin at 1 μM in a total volume of 1 ml of buffer A. Reaction mixtures (900 μl) were then carefully layered on to 300 μl of 0.3 M-sucrose in buffer A and membranes were pelleted by centrifugation (10000 *g*, 10 min). Pellets were resuspended in 200 μl of buffer H, transferred to a 6 mm × 50 mm Pyrex glass tube and then exposed to u.v. light (302 nm) for 5 min. Membranes were transferred back to Eppendorf tubes, pelleted by centrifugation and then washed twice by centrifugation with 0.5 ml of ice-cold acetone. Pellets were then resuspended in 50 μl of 0.1 M-Tris/HCl (pH 8)/1% SDS/4 M-urea. Proteinaceous samples were then alkylated to protect free thiol groups by incubation with iodoacetamide (42 mM, 30 min, 37 °C) or first reduced with dithiothreitol (20 mM, 30 min, 37 °C) to break disulphide bridges, and then alkylated with iodoacetamide (42 mM, 30 min, 37 °C). Samples were then separated on SDS/7.5%-PAGE according to the method of Laemmli [16]. Gels were stained with 0.05% (w/v) Coomassie Blue/40% (v/v) methanol/10% (v/v) acetic acid, destained, dried under vacuum and then exposed to X-ray film for 6 days.

RESULTS

The availability of biologically active radiolabelled peptide ligand was an important consideration in this study. Preliminary binding experiments indicated that when ^{125}I-labelled human CGRP-1 was used as a tracer, amylin was found to compete for binding of this radiolabelled peptide. Therefore, as no biologically active ^{125}I-labelled amylin is available and, at least in our hands, iodination of amylin on either Tyr-37 or Lys-1 substantially decreased its binding affinity (results not shown), all binding experiments were performed with commercially available ^{125}I-labelled human CGRP-1 as a tracer.

The association of ^{125}I-CGRP to rat liver plasma membranes reached equilibrium within 30 min at 4 °C, and was rapidly decreased, almost to the level of non-specific binding, by removing free radiolabelled peptide and re-incubating membranes in the presence of 1 μM human CGRP-1 (Fig. 1*a*). Binding of ^{125}I-CGRP in a buffer containing 20 mM-Hepes was sensitive to ionic strength and was decreased by 30% in the presence of 50 mM-NaCl and by 77% in the presence of 500 mM-NaCl (Fig. 1*b*). We cannot rule out the possibility that the effect of NaCl on binding might be due to a specific effect of Na^+ or Cl^- ions. In addition, it is notable that a high salt concentration does not lower peptide binding entirely to the level of non-specific binding.

Binding of ^{125}I-CGRP was concentration-dependent and reached saturation (Fig. 2*a*). Adsorption isotherm analysis reveals a single high-affinity binding site with a dissociation constant (K_d) of 0.125 nM and binding capacity ($B_{max.}$) of 580 fmol/mg of protein (Fig. 2*b*). This ^{125}I-CGRP binding site was inhibited by human CGRP-1 with an IC_{50} value (concn. causing 50% inhibition of binding) close to the K_d (about

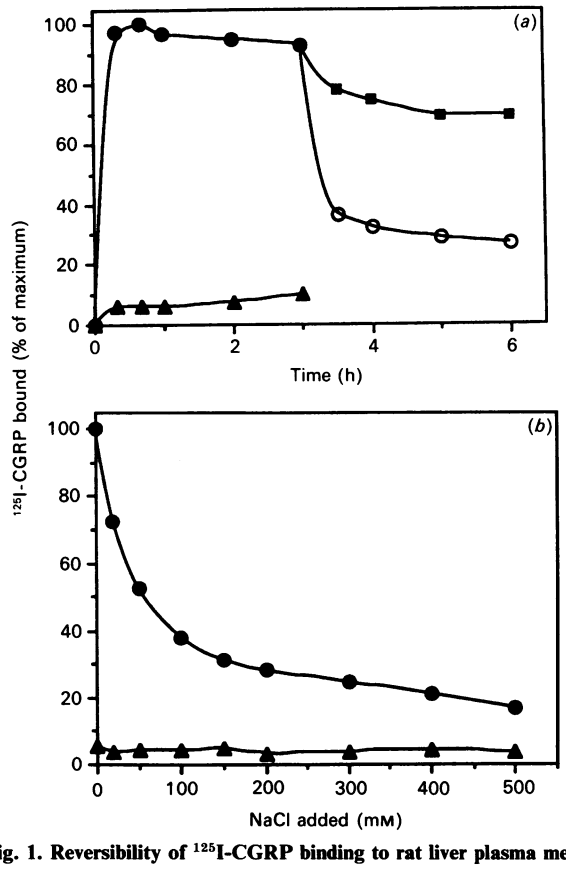

Fig. 1. Reversibility of ^{125}I-CGRP binding to rat liver plasma membranes

(*a*) Rat liver plasma membranes were incubated at 4 °C with 25pM-^{125}I-CGRP (●, total binding) or in the presence of 1 μM excess CGRP (▲, non-specific binding) and media were sampled over the time course indicated. After 3 h, membranes were centrifuged (10000 *g*, 10 min) and reincubated in medium alone (■) or medium with 1 μM-CGRP (○). (*b*) Membranes were incubated (4 °C, 40 min) with 25pM-^{125}I-CGRP in the presence of increasing amounts of added NaCl as indicated (●, total binding; ▲, non-specific binding). Data are expressed as a percentage of total bound counts in the absence of inhibitor. Values are duplicate points and are representative of four independent experiments.

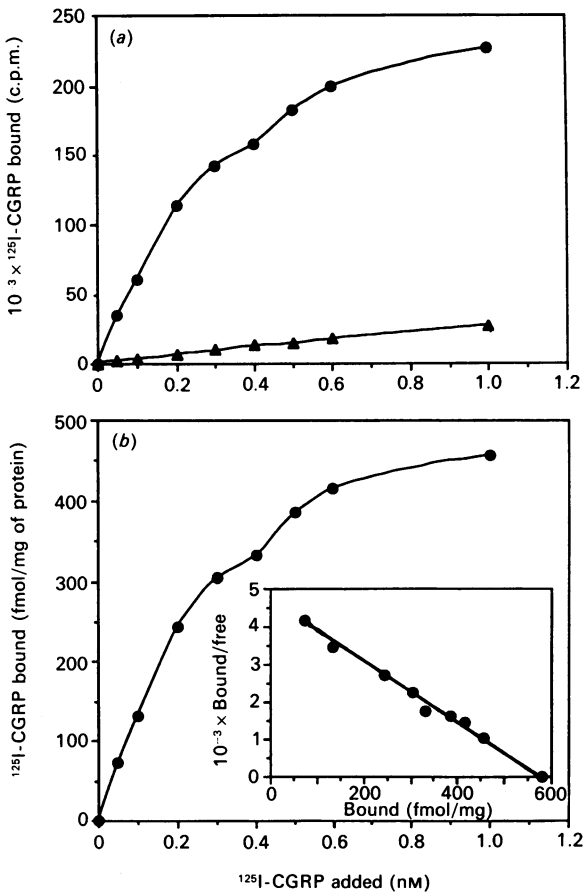

Fig. 2. Saturation isotherm analysis of ^{125}I-CGRP binding to rat liver plasma membranes

(*a*) Liver membranes were incubated (4 °C, 40 min) with increasing concentrations of ^{125}I-CGRP alone (●, total binding) or in the presence of 1 μM-CGRP (▲, non-specific binding). (*b*) Specific binding (●) was defined as the difference between total and non-specific binding. Values are duplicate points and are representative of four independent experiments. The inset is the Scatchard analysis of the specific binding data.

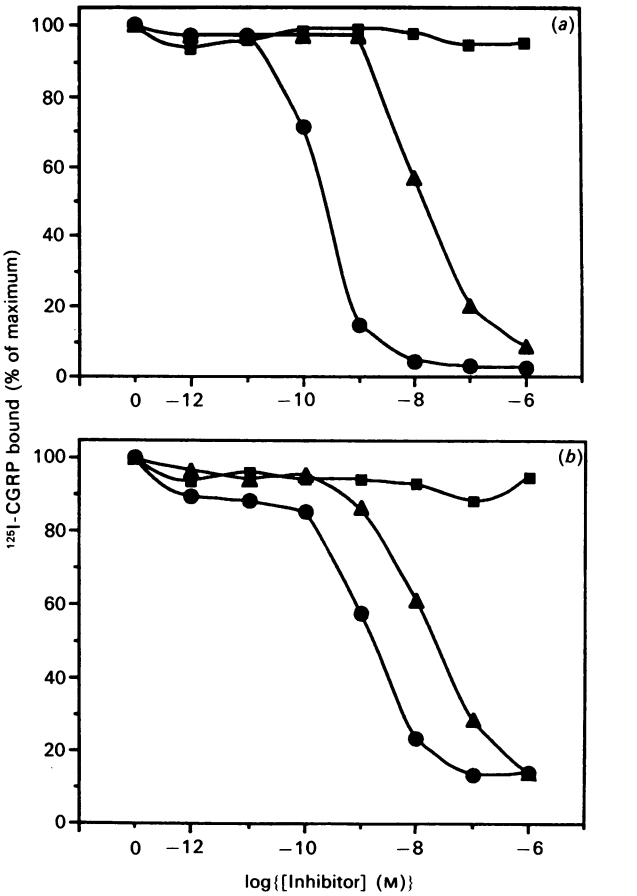

Fig. 3. Inhibition of ^{125}I-CGRP binding to rat liver plasma membranes and skeletal muscle membranes

Rat liver plasma membranes (*a*) or rat skeletal muscle membranes (*b*) were incubated (4 °C, 40 min) with 25pM-^{125}I-CGRP in the presence of increasing amounts of human CGRP-1 (●), rat amylin (▲) or human thyrocalcitonin (■). Data are expressed as the percentage of ^{125}I-CGRP bound in the absence of added peptide. Values are duplicate points and are representative of four independent experiments.

0.1 nM), whereas human thyrocalcitonin at 1 μM had no effect (Fig. 3*a*). ^{125}I-CGRP binding was also inhibited in a dose-dependent manner by rat amylin, with an IC_{50} value of 10 nM (Fig. 3*a*).

In crude skeletal muscle membranes, both amylin and CGRP compete for ^{125}I-CGRP binding with respective IC_{50} values of 0.3 nM and 10 nM (Fig. 3*b*). The overall level of specific binding is about 10% of that found in purified liver plasma membranes. Non-specific binding in muscle is slightly higher, 20% of the total compared with 5% in the liver, and probably reflects the relative purity of the two membrane preparations. It was for this reason that the ^{125}I-CGRP binding was characterized more fully in this study using purified liver plasma membranes.

The molecular sizes of the ^{125}I-CGRP binding sites were examined by photoaffinity labelling of ^{125}I-CGRP bound to membranes followed by SDS/PAGE analysis. Radiolabelled bands, the intensity of which were diminished when samples were reduced prior to SDS/PAGE, were identified with a molecular mass of about 70 kDa under reducing conditions and about 55 kDa under alkylating conditions in both liver plasma membranes and skeletal muscle membranes. Other minor bands were visible, one of about 44 kDa in liver, with a muscle-specific band of about 110 kDa (Fig. 4). Labelling of these bands was prevented by the inclusion of 1 μM-CGRP and amylin (Fig. 4). The specificity of this binding was confirmed by the fact that human

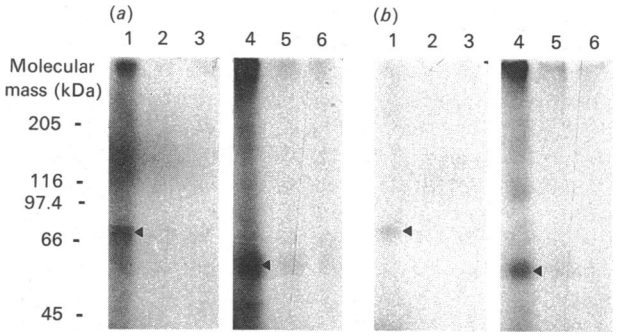

Fig. 4. Photoaffinity cross-linking of ^{125}I-CGRP to its binding site in rat liver and sketetal muscle membranes

Rat liver (*a*) and skeletal muscle (*b*) membranes were incubated with ^{125}I-CGRP in the absence of unlabelled ligand (lanes 1 and 4) or in the presence of 1 μM-CGRP (lanes 2 and 5) or 1 μM rat amylin (lanes 3 and 6) and receptor–ligand complexes were cross-linked by exposure to u.v. light as described in the Materials and methods section. Samples were separated by SDS/7.5%-PAGE under reducing/alkylating (lanes 1–3) or alkylating (lanes 4–6) conditions and gels were stained, dried and exposed to X-ray film for 6 days. The migration of the following marker proteins is indicated: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa. The major radiolabelled species are indicated (◀).

calcitonin did not inhibit binding of ^{125}I-labelled CGRP to its binding site (results not shown).

DISCUSSION

The main finding reported in this paper is the cross-reactivity of amylin with ^{125}I-CGRP binding sites in both liver and skeletal muscle. Amylin and CGRP have a high degree of primary sequence identity, being almost totally conserved in the *N*- and *C*-terminal regions, and also, qualitatively, share many functional activities [3,4,17]. We now report that amylin and CGRP also recognize a common binding site in both liver and skeletal muscle to mediate their actions through the same effector system.

Tracer amounts of ^{125}I-CGRP were displaced by CGRP with an IC_{50} value of about 0.1 nM. This compares favourably with the dissociation constant for ^{125}I-human CGRP binding to rat liver plasma membranes of 0.125 nM. Yamaguchi *et al.* [10], using different assay conditions, have reported a K_d of 0.260 nM for ^{125}I-Tyr-O-labelled rat CGRP binding to rat liver plasma membranes. However, this K_d value does not correlate with their IC_{50} value for CGRP of 10 pM. They also reported a 10^5 higher IC_{50} value for rat amylin than for CGRP [18]. In the present study, with different assay conditions and using a different radiolabelled ligand, the IC_{50} for rat amylin is only about 10–100 times higher. Therefore it seems reasonable to predict that amylin and CGRP might be functioning through a common receptor. Commercially available synthetic amylin preparations, when compared with native pancreatic human amylin, have a significantly lower biological activity (up to 100-fold), as measured by the rat soleus muscle bioassay (A. Chantry, B. Leighton & A. J. Day, unpublished work). Therefore, we would predict that amylin cross-reacts at the ^{125}I-CGRP binding site *in vivo* at an even lower concentration. This is the first report, to our knowledge, that amylin cross-reacts with a ^{125}I-CGRP binding site in rat skeletal muscle membranes, although a specific site for CGRP alone has been described in human skeletal muscle membranes [8], rat sarcolemmal membranes [19] L6 myocytes [20] and isolated chick myotubes [21].

CGRP has many activities, which include vasodilation and positive chronotropic and ionotropic effects [6]. The effects of CGRP on the liver are not established and immunocytochemical localization studies have suggested that the role of CGRP may be restricted to sensory transmission in the hepatic vasculature [11]. In the majority of tissues examined, the presence of CGRP-like immunoreactivity normally correlates with the presence of CGRP binding sites. However, the lack of a detectable amount of CGRP in the liver [9], appears to be unrelated to the specific binding sites for CGRP found in rat liver plasma membranes (see Fig. 1).

The demonstration that the CGRP binding sites in liver plasma membranes are recognized by both CGRP and amylin suggests that both peptides could, in theory, have identical physiological roles. Distribution of these peptide ligands then becomes important when considering their selective action. Importantly, amylin, which is known to be released from the pancreatic β-cells in response to secretagogues [22], would immediately be delivered to the liver via the hepatic portal vein. Therefore we believe that amylin is likely to be the primary mediator acting via the putative CGRP/amylin effector system.

In skeletal muscle, CGRP is localized in both the motor end plate and sensory nerves [7]. However, it is not clear if there is sufficient CGRP in muscle to affect glucose metabolism, since the whole fibre would have to be innervated. Therefore it is anticipated that any effect on insulin-stimulated glycogen synthesis in skeletal muscle is primarily mediated by amylin delivered by the bloodstream.

The normal physiological role of amylin may be to modulate insulin action. Amylin is co-secreted with insulin from the pancreatic β-cells following a meal due to the subsequent rise in blood glucose levels [23]. Insulin would be expected to inhibit the gluconeogenic pathway in liver, resulting in a concomitant decrease in hepatic glucose output and/or glycogen synthesis. Amylin could exert a modulatory action in liver by decreasing the sensitivity of the gluconeogenic pathway to insulin and encouraging the conversion of metabolic intermediates such as lactate or other C_3 intermediates to glucose 6-phosphate. The glucose 6-phosphate could be stored as glycogen or released as glucose depending on the effect of amylin on glucose-6-phosphatase activity. Amylin does indeed suppress the ability of insulin to inhibit hepatic glucose output [5]. One of the consequences of such a mechanism is to facilitate redistribution and disposal of fuel molecules. Skeletal muscle has a much greater capacity for glycogen storage than liver, and any glucose passing through the liver is immediately converted to glycogen in skeletal muscle in an insulin-mediated process.

Circulating levels of amylin have not yet been accurately defined; therefore, it is not certain whether enough amylin bypasses the liver to have a significant effect on insulin-sensitivity in muscle. Amylin is known to cause an insulin-resistant state in skeletal muscle *in vitro* [4] and *in vivo* [5]. The possibility exists that it is only under conditions of hyperamylinaemia, which may be associated with disease states such as non-insulin-dependent diabetes mellitus, that amylin has a significant effect on carbohydrate metabolism in skeletal muscle.

Further evidence for a common binding site for CGRP and amylin in muscle and liver came from SDS/PAGE analysis of cross-linked ^{125}I-CGRP–receptor complexes. In liver and muscle, formation of the radiolabelled species was inhibited by both CGRP and amylin. Using a recently developed direct photoaffinity-labelling approach [15], the apparent molecular size of the CGRP–amylin receptor complex was approx. 70 kDa under reducing conditions. Under alkylating conditions, this major band migrated with a molecular mass of 55 kDa, which may indicate the presence of intrachain disulphide linkages. There is an additional radiolabelled species of 44 kDa in liver and muscle, and a muscle-specific band of 110 kDa. The same radiolabelling pattern was apparent when using disuccininimidyl suberate (DSS) as cross-linking reagent (A. Chantry, B. Leighton & A. J. Day, unpublished work).

Foord & Craig [24] have reported a molecular mass for the placental CGRP–receptor complex of 240 kDa when using DSS as a cross-linking reagent, and state that this is composed of 60–70 kDa subunits. In liver and muscle we found no evidence for cross-linking of a 240 kDa multi-subunit complex, although a large radiolabelled aggregate is apparent which is not able to penetrate the running gel.

Sano *et al.* [25] have examined CGRP binding sites in a number of tissues and have noted radiolabelled species of 70 kDa (spinal cord), 70 kDa and 90 kDa (coronary arteries), and 70 kDa and 120 kDa (left and right atria). A common feature in all studies is a component of the CGRP binding site with a molecular size of 70 kDa. The presence of other radiolabelled species may represent receptor heterogeneity/subtypes or the coupling of the CGRP/amylin receptor to guanine-nucleotide-binding protein (G-proteins). In this regard, it is known that CGRP activates adenylate cyclase, probably via G-proteins, in a number of tissues, including muscle [26] and liver [10]. The molecular sizes of G-proteins are 30–50 kDa; therefore interprotein cross-linking with the ^{125}I-CGRP–receptor complex may account for the 110 kDa band found in skeletal muscle. Variability in the labelling of this particular species has been noted between different membrane preparations and its presence may be artifactual.

Further clarification of the receptor binding characteristics of the amylin/CGRP receptor, including possible receptor sub-types and an understanding of their intracellular coupling mechanisms, will emerge from receptor purification and cloning studies.

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