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Biotinyl analogues of amylin as biologically active probes for amylin/CGRP receptor recognition

Andrew Chantry[1]*, Elizabeth A. Foot[2], Brendan Leighton[2], Anthony J. Day[1] and Antony C. Willis[1]

[1]*MRC Immunochemistry Unit, Department of Biochemistry, and* [2]*Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK*

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Biotinyl analogues of rat amylin were synthesised with sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-Biotin). Biotinylated amylin peptides were purified by HPLC, quantitated, and the presence of the biotin group at Lys-1 confirmed by peroxidase-labelled avidin and FAB mass spectroscopy. Amylin-biotin retained a similar affinity for binding to rat liver plasma membranes compared with rat amylin and also completely inhibited insulin-stimulated glycogen synthesis in rat soleus muscle incubated in vitro. These biologically active amylin probes will enable a complete analysis of amylin/CGRP receptor expression in various cell types and facilitate the isolation and characterisation of the hormone–receptor complex.

Amylin; CGRP; Receptor; Biotinylation; Non-insulin-dependent diabetes mellitus

1. INTRODUCTION

Amylin is a 37-amino acid polypeptide which shares about 50% primary amino acid sequence identity with calcitonin gene-related peptide [1]. Amylin, which was originally isolated from the amyloid mass in the pancreases of type 2 diabetics, has all the hallmarks of a novel glucoregulatory hormone which counteracts insulin action [2,3]. Interestingly, the neuropeptide CGRP is now known to have a high degree of functional, as well as structural, similarity with amylin. Both amylin and CGRP are affective inhibitors of insulin-stimulated glycogen synthesis in skeletal muscle incubated in vitro [4] and amylin also counter-regulates the effect of insulin on hepatic glucose production in vivo [5].

Receptors for CGRP have been identified on a number of cell lines and tissues which are often of vascular origin, consistent with the role of CGRP as a potent vasodilator [6]. High affinity CGRP receptors have also been localized to the major sites of glucose metabolism, the liver [7,8] and skeletal muscle [8,9]. Recently, we have shown that amylin and CGRP cross-react with the ^{125}I-labelled CGRP binding site in both liver and skeletal muscle, based both on equilibrium binding analysis and covalent cross-liking techniques [8]. Therefore, it is becoming increasingly apparent that any explanation of the combined physiology of amylin and CGRP must encompass differential localization of these peptides and/or the presence of receptor sub-types distributed on specific target tissues.

An important pre-requisite for the analysis of putative receptor sub-types are labelled peptides which retain receptor recognition activity. Iodination of human CGRP on the His-10 residue does not appear to reduce its receptor binding capacity. 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 reduced its binding affinity [8]. The introduction of the biotin moiety via the primary amine groups on amylin could provide useful functional ligands for the study of amylin/CGRP receptor expression and the distribution of receptor sub-types in various cells or tissues, and also facilitate the isolation of the amylin– and/or CGRP–receptor complex(es). Here, we investigated the relative binding of biotinylated analogues of rat amylin to rat liver plasma membranes and also demonstrate the retention of biological activity of these derivatives, based on their ability to inhibit insulin-stimulated glycogen synthesis in rat muscle in vitro.

2. MATERIALS AND METHODS

2.1 *Materials*

Synthetic rat amylin (Camebridge Research Biochemicals, UK) was dissolved in 3 mM HCl, aliquoted, and stored at −20°C until used. ^{125}I-labelled human CGRP-1 (ca. 2000 Ci/mmol) was obtained from Amersham, UK. Sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-Biotin) and *N*-succinimidyl-3(4-hydroxyphenyl) propionate (Bolton-Hunter reagent; SHPP) were from Pierce Chemical Co., UK. Peroxidase-labelled avidin was from Sigma.

2.2. *Synthesis and isolation of biotinylated peptides*

Synthesis of biotinylated peptides was performed by reacting 50 μl (1 mg/ml) of rat amylin in 3 mM HCl with 100 μl (0.4 mg/ml) NHS-SS-Biotin or SHPP in 0.1 M sodium borate, pH 7.5. After 30 min, 100

**Present address:* Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 8033 Martinsreid, Germany.

Correspondence address: A. Chantry, MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK. Fax: (44) (865) 275 729.

μl 0.1% trifluoroacetic acid was added, reaction mixtures were centrifuged (10 000 × g; 10 min) and then immediately fractionated by high-performance liquid chromatography (HPLC).

Samples were applied to a Whatman Partisil-10 ODS-3 column (250 × 4.6 mm) and eluted with a gradient of 5–90% with 0.1% trifluoroacetic acid in water (solvent system A) and 0.09% trifluoroacetic acid, 80% acetonitrile in water (solvent system B) at a flow rate of 1 ml/min. Main proteinaceous peaks as determined by absorbance at 215 nM were aliquoted, dried with a centrifugal evaporator and stored desiccated under argon at -20°C.

2.3. *Dot-blot assay*

Dried peptides were resuspended in 3 mM HCl and 3 μl aliquots of 200, 67 and 22 μg/ml solutions applied to nitrocellulose. Blots were then incubated with 1% bovine serum albumin in phosphate buffered saline (PBS) for 1 h at room temperature to block non-specific binding sites and then with peroxidase-labelled avidin (2 μg/ml) for 1 h at room temperature. After washing extensively with PBS, blots were developed with 1 mg/ml diaminobenzidine/0.02% hydrogen peroxide in PBS.

2.4. *FAB mass spectrometry*

Electrospray mass spectra were measured on a VG BIO Q triple quadruple atmospheric pressure mass spectrometer equipped with an electrospray interfere (VG Biotech, Tudor Road, Altrincham, Cheshire). Samples (10 μl) were injected into an electrospray source via a loop injector (Rheodyne 5717) as a solution, typically 25–50 pmol/μl, in water/methanol (1:1) containing 1% acetic acid at a flow rate of 2 μl per min (Applied Biosystems model 140A dual syringe pump). The mass spectrometer was scanned over the mass range 700–1500 Da. The instrument was calibrated with myoglobin (20 pmol/μl, mol. wt. 16951.5 Da).

2.5. *Receptor binding and soleus muscle assays*

The methodology for the rat liver plasma membrane binding assay has been described previously [8]. ^{125}I-labelled human CGRP-1 was used as a radiolabelled tracer (25 pM) and receptor recognition activity of peptides and biotinylated analogues assessed by their ability to compete for ^{125}I-CGRP binding. The rat soleus muscle assay has been described previously [4]. Concentrations of all unlabelled peptides were accurately determined in all experiments by amino acid analysis using a Waters Pico Tag system [10].

3. RESULTS

In rat amylin there are 2 iodinatable residues, Lys-1 (α-amino and ε-amino groups) at the N-terminal and also the C-terminally amidated Tyr-37. Tyr-37 amidation is known to be important for biological activity [11] and, therefore, interfering with this region of the molecule will affect receptor binding activity. Introduction of ^{125}I into the 2 primary amine groups on the N-terminal Lys via the Bolton-Hunter reagent, produced a radiolabelled peptide which failed to specifically bind to rat liver plasma membranes [8]. The reagent used in the iodination study has a short spacer length, and the affect on receptor binding activity may be due to steric hindrance. We therefore chose to derivatize the Lys-1 group with NHS-SS-Biotin which has a longer spacer and would introduce the versatile biotin label with the additional advantage of a thiol-cleavable group.

Fractionation by HPLC of rat amylin after reacting with NHS-SS-Biotin yielded 1 major peak A.2 and a minor peak A.1 (Fig. 1). When the reaction is performed at pH 7.0, instead of pH 7.5, the relative level of protonation of the α- and ε-amino groups is reduced, consequently the amount of peak A.1 relative to peak A.2 was substantially increased (data not shown). The likely explanation for this is that the more hydrophobic peak A.2 represents amylin with 2 attached biotin moieties. The presence of the biotin group in both peaks was confirmed by qualitative dot-blot analysis (Fig. 1, insert).

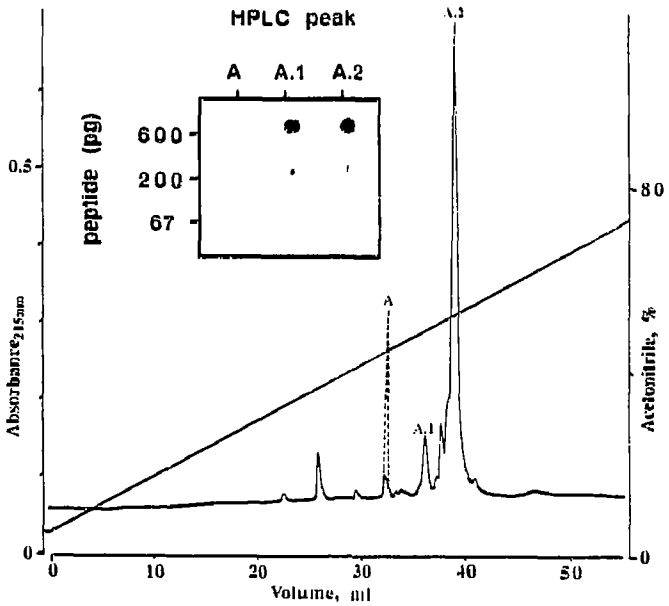

Fig. 1. Isolation of biotinylated analogues of rat amylin by HPLC. Biotinylated rat amylin was separated on a Partisil-10 ODS-3 (250×4.6 mm) with a gradient of 5–90% with 0.1% trifluroacetic acid in water and 0.09% trifluroacetic acid, 80% acetonitrile in water at a flow rate of 1 ml/min. The major biotinylated products are indicated (A.1 and A.2) and the migration of synthetic rat amylin (A) is shown by the dotted line. The major peak at about 40% acetonitrile is derived from the reagent NHS-SS-biotin. (Insert) Detection of biotinylated peptides by dot blot assay and avidin-labelled peroxidase.

By FAB mass spectrometry, rat amylin (peak A) migrates as a main peak with a mol. wt. of 3920.1 Da which is in very good agreement with the predicted value of 3920.5 Da (Fig. 2). Peaks B and C in Fig. 1 represent peptide with bound sodium and potassium ions, respectively, and Peak D is an unknown contaminant. The biotinylated peptides (peak A1 and A2) migrate as major species with mol. wts. of 4309.4 and 4699.25 Da, respectively (Fig. 2). The calculated mol. wt. of an attached biotin moiety is 390.1 Da, therefore, peak A1 represents amylin with a single biotin and peak A2 is amylin with 2 attached biotins.

The binding affinities of amylin relative to the biotinylated analogues isolated by HPLC as shown in Fig. 1 were examined by equilibrium binding analysis. We have shown previously that both amylin and CGRP can compete for ^{125}I-CGRP binding to rat liver plasma membranes [8]. Derived IC_{50} values for the ^{125}I-CGRP displacement from rat liver plasma membranes by amy-

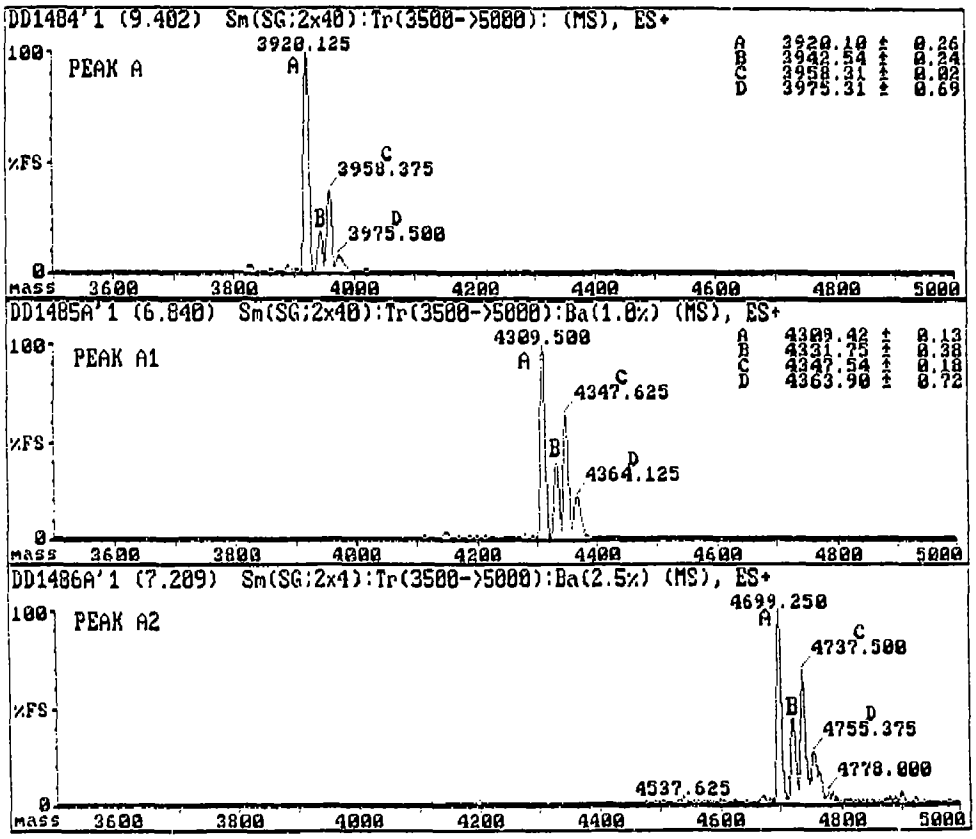

Fig. 2. FAB mass spectroscopy of amylin and amilyn-biotin derivatives A, A.1 and A.2 described in Fig. 1.

lin and amylin-biotin are summarized in Table I. In these experiments, rat amylin gave an IC_{50} of 2.5×10^{-8} M, and both biotinylated analogues, peaks A.1 and A.2, gave only marginally higher IC_{50} values of 3.2×10^{-8} M and 4.0×10^{-8} M, respectively.

Biotinylated analogues of rat amylin were also tested for their ability to elicit a biological response. For this measurement we monitored the effects of amylin and di-biotin-amylin (peak A.2) on insulin-stimulated rates of glycogen synthesis and lactate formation in isolated incubated rat soleus muscle preparations (Table I). Soleus muscles were pre-incubated for 30 min in the presence of insulin (100 μU/ml) and [U-^{14}C]glucose. After 30 min some muscles were removed and these served as the control 1 values (Table I). Other muscles were incubated for a further 30 min, either with insulin and ^{14}C-labelled glucose alone (control 2) – to obtain steady state values for stimulation of glucose metabolism in the isolated incubated soleus muscle preparations, or in addition with 100 nM underivatised/biotinylated amylin peptides (see Table I). Both rat amylin and di-biotin-amylin completely inhibited the stimulation of glycogen synthesis by insulin over the last 30 min of the incubation. Amylin and CGRP are known to increase the rates of glycogenolysis, and hence increase the rate of lactate formation in this system [3]. In the present study, there were equivalent increases in the rates of lactate forma-

Table I

Biological activity of amylin and biotin-amylin

Additions	Rates (μmol/g wet wt./unit time)			
	Glycogen synthesis		Lactate formation	
		Δ^c		Δ^d
Control 1[a]	2.20±0.31	–	6.81±0.33	–
Control 2[b]	5.04±0.41	2.84±0.41	13.21±0.77	6.40±0.77
Amylin[b]	2.31±0.28*	0.11±0.28*	16.49±0.80*	9.68±0.80*
Di-biotin-amylin[b]	2.85±0.17*	0.65±0.17*	16.70±1.47*	9.89±1.47*

Results are presented as means ± SEM. Mono-biotin amylin was not tested.

[a] Muscles were pre-incubated for 30 min with insulin (100 μU/ml) and [U-^{14}C]glucose (0.4 μCi/ml).

[b] Muscles were incubated for a further 30 min either in the presence of insulin and ^{14}C-labelled glucose only (Control 2), or in addition with 100 nM amylin or di-biotinylated amylin.

[c] Δ is the response of glycogen synthesis to insulin over the last 30 min of the incubation.

[d] Δ is the response of lactate formation to insulin over the last 30 min of the incubation period. Statistically significant differences (non-paired Student's t-test) from Control 2 values are indicated by * ($P<0.05$).

tion by amylin and the di-biotin-amylin, which suggests that there is an increase in the rate of glycogenolysis.

4. DISCUSSION

The present study demonstrates for the first time a labelled amylin analogue which can be adequately and routinely detected and retains both biological and receptor binding activity (Table I). The introduction of the biotin moiety at the N-terminal Lys residue provides a probe for the study of amylin receptor expression in various cell types and will enable isolation and characterization of amylin receptors. Recently, it has been reported that ^{125}I-amylin labelled with the Bolton-Hunter reagent can be used to identify high affinity amylin binding sites in hepatic non-parenchymal cells [12]. However in our studies using rat liver plasma membranes, in which we have shown both here and previously [8] that unmodified rat amylin is a binding-competent ligand, we find that amylin labelled with non-iodinated Bolton-Hunter reagent has a reduced (5–10-fold) receptor binding activity (A. Chantry, unpublished).

Amylin labelled at Lys-1 with Bolton-Hunter reagent may retain sufficient binding affinity for preliminary analysis of amylin receptor expression in some systems. However, it seems likely that an optimal spacer length between the peptide and the bulk of the attached derivative is required to fully prevent steric hindrance of receptor–ligand interactions. In this regard we have found that the N-terminal region of amylin and CGRP plays a role in receptor binding since reduction of the 2–7 disulphide bridge abolishes receptor recognition (C. Bacon and A. Chantry, unpublished observations) and biological activity [11]. The biotinylated analogues described here have a spacer with a disulphide bridge and 8 atoms between the biotinyl carboxyl group and the N-terminal Lys. However, the Bolton-Hunter derivative has only 2 atoms and is presumably not flexible enough or of sufficient length to avoid impinging on the adjacent N-terminal residues of amylin. One solution therefore might be to introduce an iodinatable Bolton-Hunter reagent with a longer spacer arm.

In addition to the use of biotinylated ligands for the analysis of receptor expression, the very high affinity of biotin for egg white avidin and bacterial streptavidin ($K_d \sim 10^{-15}$ M [13]) permits the use of these biotinylated ligands as probes for isolating and localizing their corresponding cell-surface receptors. The success of this approach is critically dependent upon the ability of the biotinyl-substituted hormone ligand to bind simultaneously to both avidin and the receptor. For example, the hydro-osmotic activity of biotinylated analogues of vasotocin was reversed following the addition of avidin [14]. However, the spacer arms of the molecules used in this study were very short. The length of the spacer arm in amylin-SS-Biotin is significantly greater and, assuming these chains to be fully extended, it is very unlikely that the avidin–biotin interaction would interfere with the formation of the receptor–ligand–Biotin–avidin complex. Indeed, we have recently optimized conditions for using biotinylated amylin to specifically remove and isolate amylin/CGRP receptors from detergent solubilized liver plasma membranes using avidin-agarose chromatography in a single step procedure [15]. We have shown previously that binding of ^{125}I-CGRP to rat liver plasma membranes is reversed by the addition of an excess of unlabelled amylin and CGRP [8]. Therefore, the same conditions can be used to specifically elute amylin/CGRP receptors from agarose–avidin–Biotin-SS-ligand–receptor complexes. The slightly reduced receptor affinities of the biotinylated analogues (1–2-fold lower) are still high enough to maintain the receptor–ligand interaction but, at the same time, will facilitate the reverse binding process in the presence of excess unlabelled ligand with a higher affinity than the corresponding biotinylated derivative. The presence of the thiol-cleavable disulphide bridge within the biotinylated derivatives also provides alternative receptor elution conditions. The biotinylated analogues described here may also be used to directly obtain the amylin/CGRP receptor cDNA sequence by screening transfected COS cells transiently expressing rat liver cDNA [16].

The combined physiology of amylin and CGRP has been extensively studied in recent years and a great deal of confusion has arisen over the site of action, functional overlap and relative contributions of these peptides to normal metabolic processes. The biologically active biotinylated amylin analogues described in this study will help to resolve many of the current inconsistencies.

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