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Preparation and Characterization of Two LysB29 Specifically Labelled Fluorescent Derivatives of Human Insulin

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Abstract: The preparation and characterization of two novel LysB29 selectively labelled fluorescent derivatives of human insulin are described. Two probes were chosen: 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD) and 7-methoxycoumarin-4-acetic acid (MCA), which have a relatively small, compact structure and are able to react with amino groups to form highly fluorescent derivatives. The combination of solid phase peptide synthesis and enzymatic semisynthesis was chosen for preparation of these fluorescent derivatives. Using two different protocols of solid-phase peptide synthesis, two fluorescent octapeptides were prepared corresponding to the position B23–B30 of human insulin, each with a different fluorescent label, NBD or MCA, on the ε-amino group of lysine. Then, the fluorescent octapeptides were coupled to desoctapeptide-(B23–B30)-insulin by a trypsin catalysed reaction. The receptor binding affinities of two novel fluorescent derivatives of human insulin with NBD and MCA (HI-NBD and HI-MCA) were determined on rat adipose tissue plasma membranes. Both fluorescent insulins, HI-NBD and HI-MCA, had only slightly reduced binding affinity and will be used for studying the interaction of insulin with its receptor. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: human insulin; NBD; MCA; fluorescence; solid-phase peptide synthesis; enzymatic semisynthesis

Abbreviations: CZE, capillary zone electrophoresis; DBU, 1,8-diazabicyclo[5,4,0]undec-7-ene; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DOI, desoctapeptide-(B23-B30)-insulin; FAB, fast atom bombardment; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HI, human insulin; HI-MCA, human insulin with the MCA probe attached to the ε-amino group of LysB29; HI-NBD, human insulin with the NBD probe attached to the ε-amino group of LysB29; MALDI-TOF, matrix assisted laser desorption and ionization-time of flight; MCA, 7-methoxycoumarin-4-acetic acid; Mtt, 4-methyltrityl; NBD, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; NMP, 1-methyl-2-pyrrolidinone; PMSF, phenylmethylsulfonyl-fluoride; RP-HPLC, reverse phase-high performance liquid chromatography; tBu, tert-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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INTRODUCTION

At least 155 million people in the world have diabetes mellitus and almost all (90%–95%) these patients suffer from type 2 diabetes [1], which is probably caused by a dysfunction of the insulin receptor or by defects in the insulin receptor downstream signalling events [2].

A better understanding of the insulin–insulin receptor interaction would undoubtedly be helpful in the treatment of diabetic patients. Because of the difficulties in elucidating the structure of the insulin receptor by crystallography or NMR spectroscopy [3,4], the current view of insulin receptor interaction is derived both from the properties of insulin mutants and from recent knowledge about insulin receptor structure.

The regions in the insulin molecule responsible for interaction with the insulin receptor are the *N*-terminus of the A chain, the *C*-terminal β-strand of the B-chain, the central α-helix of the B-chain, and the residues close to these regions [5–9]. Despite this large amount of information the active conformation of insulin is still unknown. Several studies [10–13] have addressed the hypothesis that insulin must undergo conformational changes upon binding to its receptor. Recently, much attention has been paid to the elucidation of these insulin movements in close proximity to the insulin receptor. Fluorescently labelled peptides represent a useful tool for investigation of the relationship between structure, function and activity [14–17].

One of the most common fluorescent probes is fluorescein isothiocyanate (FITC) which was used for the localization of insulin in different tissues [18]. The detailed preparation and properties of fluorescein isothiocyanate labelled insulin analogues were dealt with in several studies [19–21]. Whereas the mono-FITC-insulin labelled on LysB29 retained about 30% biological activity [21], the substitution of all three amino groups showed no biological activity relative to the natural insulin [19,20]. Wollmer and his colleagues [22] labelled insulin with less bulky groups than FITC. No interference on dimerization of insulin labelled on the ε-amino group LysB29 was observed by fluorescence resonance energy transfer which is used for the analysis of insulin association.

The aim of this study was to develop a relatively simple synthetic protocol for the preparation of new sufficiently potent fluorescent insulin derivatives specifically labelled on LysB29. A semisynthetic approach was used enabling the versatile synthesis of insulin analogues differing in substitution in the *C*-terminus of the B-chain [23]. The resulting fluorescent insulin analogues could be useful for studying conformation changes occurring during the binding of insulin to its receptor.

From a number of available fluorescent probes, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD, Figure 1A) and 7-methoxycoumarin-4-acetic acid (MCA, Figure 1B) were chosen for the preparation of two novel selectively labelled fluorescent derivatives of human insulin. The NBD and MCA probes have relatively small, compact structures and are able to react with amino groups to form highly fluorescent derivatives. It seems important to employ rather small fluorescent probes, because bulky residues on LysB29 could negatively affect the insulin–insulin receptor interaction [20,21]. Additionally, the suitable fluorescent probe close to the receptor binding site could be useful for investigating the interaction of the hormone with its receptor. The spectral properties of NBD fluorescent derivatives offer high sensitivity and higher fluorescence with an increase in hydrophobic environments [24]. Consequently, NBD might be a convenient and reliable fluorescent indicator of changes in conformation or in the environment on the surface of protein [14]. Both excitation and emission spectra of NBD show peaks in the visible region. For example, NBD used in the labelling of Na^+/K^+-ATPase helped to gain key information about the accessibility or depth of the

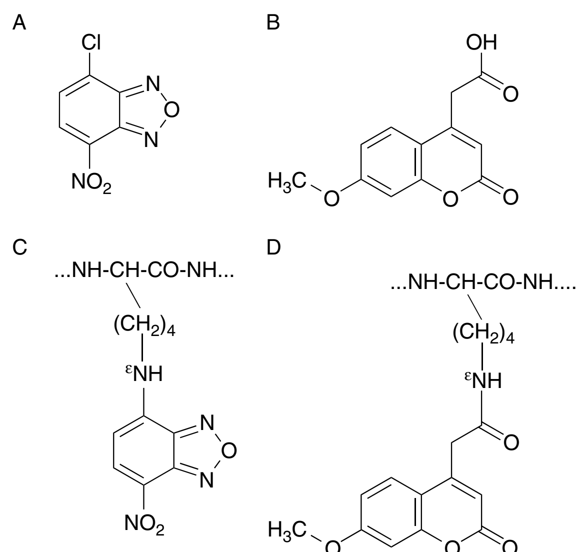

Figure 1 Structure of NBD (A), MCA (B) and the structures of NBD (C) and MCA (D) attached to the ε-amino group of lysine.

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two ATP-binding sites [25]. The fluorescent probe MCA is suitable for the preparation of fluorescent peptides by solid-phase peptide synthesis [26]. The stable amide bond formed with the amino group of an amino acid is a great advantage of this probe. The spectral properties of MCA fluorescent derivatives are shifted more to the UV region. Moreover, MCA is a highly fluorescent and chemically stable probe and also can be quenched efficiently by the dinitrophenyl group [27].

Two different fluorescent derivatives of human insulin labelled selectively on the ε-amino group of LysB29 either with NBD (HI-NBD, Figure 1C) or with MCA (HI-MCA, Figure 1D) were prepared using the combination of solid-phase peptide synthesis and enzymatic semisynthesis. Each of the fluorescent derivatives of human insulin was synthesized in a different chemical way. Their fluorescent spectral properties and receptor binding affinities in rat adipose tissue plasma membranes were compared with that of human insulin.

MATERIALS AND METHODS

All Fmoc amino acid derivatives and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Nova Biochem (Laufelfingen, Switzerland). The solid-phase peptide synthesis of octapeptides was carried out on Fmoc-Thr(tBu) pre-loaded Wang resin from Nova Biochem (Laufelfingen, Switzerland). Fluorescent labels 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD) and 7-methoxycoumarin-4-acetic acid (MCA) were from Sigma (St Louis, MO USA). TPCK-trypsin and human insulin were purchased from Sigma (St Louis, MO, USA), porcine insulin from Spofa a.s. (Prague, Czech Republic). The other chemicals, i.e. *N*,*N*-diisopropylethylamine (DIPEA), *N*-methylpyrrolidone (NMP), dimethylformamide (DMF), trifluoroacetic acid (TFA), triisopropylsilane (TIS) were purchased from Aldrich (Milwaukee, WI, USA) and Fluka (Buchs, Switzerland). All chemicals used were of analytical grade. Gel chromatography was performed on Sephadex G-50 F (Pharmacia, Uppsala, Sweden). Analytical RP-HPLC was performed on a Watrex (Nucleosil 120, C18, 5 μm, 250 × 4 mm) column (Prague, Czech Republic) and the preparative RP-HPLC was done on a Vydac (218TP51, 250 × 10 mm) column (Columbia, MD, USA) using a Waters LC 625 System (Milford, MA, USA). Different gradients of acetonitrile from 8% to 72% (v/v) in water with 0.1% (v/v) TFA were used for the elution of peptides and proteins. The mass spectra of products were measured using both the FAB technique, on a three-sectored mass spectrometer ZAB-EQ with BEQQ geometry (VG Analytical, Manchester, UK), and the MALDI-TOF technique, on a mass spectrometer from Bruker Daltonics (Leipzig, Germany). The excitation and emission spectra of fluorescent peptides were performed on a Perkin Elmer 50B-Luminiscence spectrometer (Beaconsfield, Bucks, UK). Capillary zone electrophoresis (CZE) analyses were performed on a home-made instrument [28] which included a quartz capillary, UV detector and a hydrostatic sample application device.

Solid-phase Peptide Synthesis of Octapeptides with Fluorescent Labels

The synthesis of the peptide chain GFFYTPKT (*C*-terminal octapeptide of human insulin B-chain) was carried out manually by a stepwise solid-phase procedure using Fmoc-protected amino acids and Fmoc-Thr(tBu)-Wang resin [29]. Typically, 3 equiv of Fmoc-amino acid, 3 equiv of HBTU and 6 equiv of DIPEA in NMP were added to the resin. The coupling reaction was allowed to proceed for 1 h. Then the coupling efficiency was checked by the Kaiser ninhydrin test [30] for the presence of primary amino groups or by the chloranil colour test [31] for the presence of secondary amino groups. In most cases, an additional 2 equiv of the same Fmoc-amino acid, 2 equiv of HBTU and 5 equiv of DIPEA in NMP were added to the resin for 1 h. The tBu groups were used for the protection of the side chain hydroxy groups of threonine and tyrosine and the Mtt protection group for the ε-amino group of lysine. The Fmoc α-amino protection group was removed with 30% piperidine in DMF.

The method of attachment of the fluorescent probe to the ε-amino group of LysB29 of GFFYTPKT was different for each probe as follows.

Synthesis of GFFYTPK(NBD)T. After the completion of the synthesis of the resin-bound GFFYTPK octapeptide, the *N*-terminal Fmoc group of glycine was not removed. The Mtt protection group of the ε-amino group lysine was cleaved with a TFA/DCM/TIS (2% : 96% : 2%) mixture and the resin with octapeptide was neutralized with 5% solution of DIPEA in DMF. Then 3 equiv of NBD and 5 equiv of DIPEA in DMF were added to the resin. The mixture was stirred for 15 h at room temperature. For complete labelling, another 3 equiv of NBD

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and 5 equiv of DIPEA in DMF were added several times for shorter coupling periods. Nα-deprotection of glycine was accomplished by treatment of the resin with a solution of 2% (v/v) DBU and 4% (v/v) piperidine in DMF [32]. The cleavage of the fluorescent octapeptide from resin, simultaneously with the cleavage of tBu side-chain protecting groups, was accomplished using 96% (v/v) TFA containing 2% (v/v) TIS and 2% (v/v) water. The octapeptide GFFYTPK(NBD)T was isolated by RP-HPLC and recovered by lyophilization. The purity of the product was checked by analytical RP-HPLC, CZE and mass spectroscopy. The fluorescent properties of the octapeptide were analysed in the UV/VIS region.

Synthesis of GFFYTPK(MCA)T. After attachment of lysine, the *N*-terminal Fmoc group was not removed. The Mtt protecting group of the ε-amino group of lysine was cleaved as described above. Three equivalents of fluorescent label MCA, 3 equiv of HBTU and 5 equiv of DIPEA in NMP were added to the resin. The mixture was stirred at room temperature for 15 h. For complete labelling, another 3 equiv of MCA, 3 equiv of HBTU and 5 equiv of DIPEA in NMP were added to resin, and this process was repeated until a negative Kaiser test was obtained (usually five couplings). Nα-deprotection of lysine was accomplished by treatment with a solution of 30% (v/v) piperidine in DMF. Attachment of the next six residues, final TFA cleavage, peptide recovery and characterization were performed as described above.

Preparation of Desoctapeptide-(B23–B30)-insulin (DOI)

Zinc-free porcine insulin [33] was dissolved in 0.05 M Tris/HCl pH 9.0, and TPCK treated trypsin in 0.1 M $CaCl_2$ was added (enzyme/insulin weight ratio 1/50) [34]. This solution was left for 20 h at room temperature. The reaction was stopped by adjustment of pH to 5.4 by 1 M HCl. The solution was centrifuged at 2000 × **g** for 20 min, the supernatant was poured off, and the sediment was dissolved in 10% (v/v) acetic acid. Desoctapeptide-(B23–B30)-insulin was isolated by gel chromatography.

Enzymatic Semisynthesis of Fluorescent Derivatives of Human Insulin

Enzymatic semisyntheses of the derivatives of human insulin with a fluorescent label (NBD or MCA) on the ε-amino group of lysine were performed according to the previously published methods [35,36] employing several modifications. The respective fluorescent octapeptide (150 mM) and DOI (30 mM) were dissolved in a solution containing 55% (v/v) DMF, 50 mM $CaCl_2$ and TPCK-trypsin. The molar ratio of enzyme/substrate was 1/50. The pH value of 6.9–7.1 was adjusted by N-methylmorpholine. The mixture was stirred for 22 h at room temperature and the formation of products was monitored by analytical RP-HPLC. After the completion, the reaction was stopped by the addition of acetone. The sediment was diluted with 10% (v/v) acetic acid and the products were isolated by semipreparative RP-HPLC using gradient elution with acetonitrile. The product purity was checked by analytical RP-HPLC and by CZE. Finally, the fluorescent spectra and mass spectrum of the products were measured.

Determination of Binding Affinity of New Fluorescent Derivatives of Human Insulin

Isolation of rat adipose tissue plasma membranes. Plasma membranes were prepared from epididymal fat of male Wistar rats weighing 210–250 g. The fat (5–6 g) was homogenized using an ultra-turrax with 10 ml of buffer consisting of 250 mM sucrose, 1 mM PMSF, 1 mM benzamidine and 10 mM Tris/HCl (pH 7.4). The suspension was centrifuged at 3000 × **g** for 15 min at 4 °C and then the supernatant was centrifuged again at 17 000 × **g** for 15 min at 4 °C. The pellet was re-homogenized with 1 ml of 0.05 M Tris/HCl buffer (pH 7.6) and the protein concentration was determined according to Bradford [37].

Receptor binding studies. An assay according to Zorad *et al.* [38] was used to determine the relative receptor binding affinities to rat adipose tissue plasma membranes. Purified ^{125}I-iodotyrosyl^{A14}-porcine insulin [39] was used as a reference native hormone in the binding experiments. Plasma membranes (50 µg of protein) were incubated in 3 ml tubes with ^{125}I-insulin (2×10^{-10} M, ~70 000 cpm) and various concentrations of human insulin or fluorescent derivatives of human insulin (range 10^{-13}–10^{-6} M) in buffer composed of 2 mM N-ethylmaleimide, 13.2 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin and Tris/HCl (pH 7.6) in a total volume of 250 µl. The solution was incubated for 21 h at 4 °C and then the reaction was terminated by the addition of ice-cold 120 mM

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NaCl followed by a quick filtration on a Brandell cell harvester (Gaithersburg, MD, USA). Bound radioactivity was determined by γ-counting (Gamma Counter Minigamma 1275, LKB-Wallac, Sweden). The total binding (the binding in the absence of the unlabelled insulin) was about 10% of the total added radioactivity. The non-specific binding (binding in the presence of 10^{-5} M insulin) was determined to be less than 15% of the total binding. The receptor binding potencies of fluorescent derivatives were determined as the concentration of the derivative causing half-maximal inhibition of labelled insulin binding. Competitive binding curves were plotted using Graph-Pad Prizm 3 Software (San Diego, CA, USA).

RESULTS AND DISCUSSION

Synthesis of Peptides

The fluorescent octapeptides GFFYTPK(NBD)T and GFFYTPK(MCA)T were synthesized by solid-phase methodology using Fmoc/tBu strategy and Wang resin as polymeric support. An Fmoc-Thr(tBu) preloaded Wang resin was used to prevent complications with attachment of the first amino acid such as racemization, dipeptide formation and particularly, low yield of coupling. Furthermore, the cleavage of peptides from Wang resin must be achieved by concentrated TFA. This allows the use of mild (2% v/v TFA) cleavage of the Mtt protection group of the ε-amino moiety of lysine and does not affect the attachment of peptide to the resin. Yields of 86%–95% were achieved during coupling reactions with individual amino acids. Fluorescent labelling was the most crucial and problematic step of the synthesis. The free ε-amino group of lysine, after cleavage of the Mtt protecting group, was labelled with fluorescent probe, either NBD or MCA. Labelling with NBD was achieved after the synthesis of the whole GFFYTPKT peptide chain, whereas labelling with MCA was carried out immediately after the attachment of the second amino acid, lysine. Both coupling reactions were performed under different conditions. Labelling with highly reactive NBD was easily accomplished in DMF alone while the labelling with MCA was performed under the same coupling conditions (HBTU/DIPEA in NMP) as for other amino acids. In contrast to NBD, which is alkali- and acid-labile, MCA forms a very stable amide bond. Both fluorescent octapeptides, GFFYTPK(NBD)T and GFFYTPK(MCA)T, were purified by RP-HPLC whereby the yields of the pure octapeptides were 52% and 46%, respectively. The purities of the octapeptides were 81%–98% as determined by analytical RP-HPLC and CZE and each of the individual impurities represented less than 2%. Both fluorescent octapeptides were characterized by FAB mass spectroscopy (MH^+ found 1124.0 and MH^+ expected 1124.2 for GFFYTPK(NBD)T, MH^+ found 1177.0 and MH^+ expected 1177.3 for GFFYTPK(MCA)T) and fluorescent spectroscopy. The fluorescent octapeptide GFFYTPK(NBD)T showed an excitation maximum around 480 nm, emission maximum around 535 nm, and the spectra were characteristic for NBD fluorescent derivatives. The spectral values observed with the GFFYTPK(MCA)T fluorescent octapeptide were shifted to the UV region with an excitation maximum around 330 nm and an emission maximum around 390 nm.

Preparation of desoctapeptide-(B23–B30)-insulin. Desoctapeptide-(B23–B30)-insulin was obtained by tryptic digestion of porcine insulin and isolated by gel chromatography (the final yield was 88%). The product was characterized by FAB mass spectroscopy (MH^+ found 4867.7; MH^+ expected 4866.4). The purity was about 88%–90% as determined by analytical RP-HPLC and CZE.

Enzymatic semisynthesis of fluorescent derivatives of human insulin. Synthetic fluorescent octapeptides GFFYTPK(NBD)T and GFFYTPK(MCA)T were tryptically coupled to desoctapeptide-(B23–B30)-insulin in DMF/water mixture at pH_{app} 7.0 without protection of *N*-terminal α-amino groups of desoctapeptide-(B23–B30)-insulin. Moderate yields of the enzymatic semisynthesis of about 10% were probably caused by the rather low solubility of fluorescent octapeptides in the reaction mixtures. The fluorescent derivatives of human insulin (HI-NBD and HI-MCA), isolated from reaction mixtures, were identified by MALDI-TOF mass spectroscopy (MH^+ found 5971.7 and MH^+ expected 5972.6 for HI-NBD, MH^+ found 6025.4 and MH^+ expected 6025.7 for HI-MCA). The relative purities of HI-NBD and HI-MCA were estimated by both analytical RP-HPLC and CZE and were between 80% and 90%. HI-NBD showed an excitation maximum around 475 nm and an emission maximum around 530 nm (Figure 2A). HI-MCA gave an excitation maximum around 330 nm and an emission maximum around 390 nm (Figure 2B). Spectra of HI-NBD and HI-MCA were in a close agreement with those of the respective fluorescent

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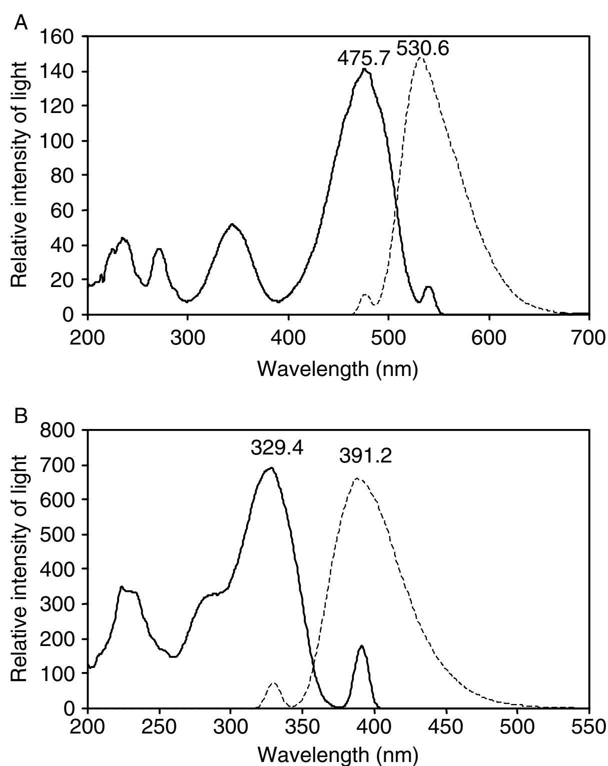

Figure 2 Excitation (———) and emission (- - - -) spectra of HI-NBD (A) and HI-MCA (B).

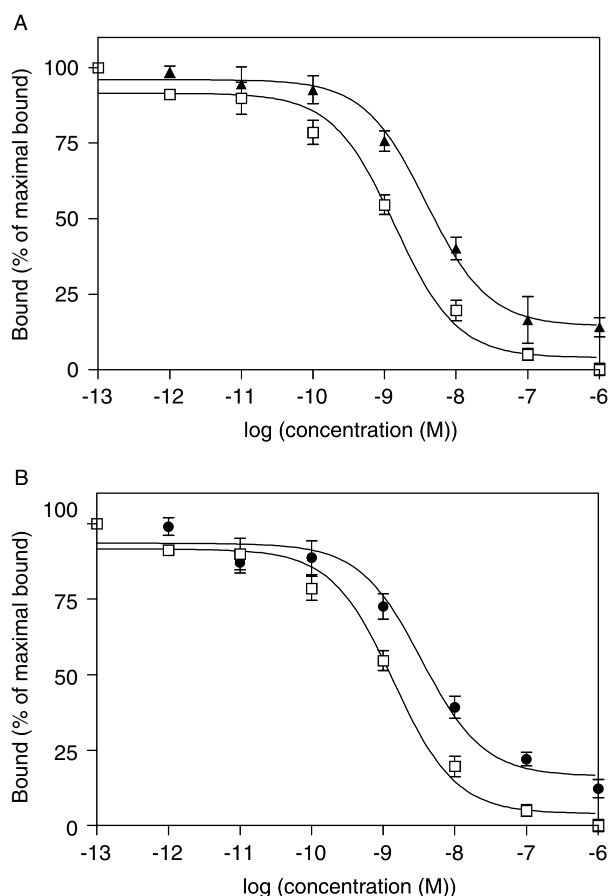

Figure 3 Inhibition of binding of ^{125}I-insulin to adipose tissue plasma membranes by human insulin and fluorescent derivatives of human insulin. See Methods for detail. Quantitative information is provided in Table 1. Values are mean ± SEM (standard error of measurement). Panel A: human insulin (□); HI-NBD (▲). Panel B: human insulin (□); HI-MCA (●).

octapeptides GFFYTPK(NBD)T and GFFYTPK(MCA)T (not shown).

Binding Studies

Receptor binding affinities of two of the fluorescent derivatives of human insulin, HI-NBD and HI-MCA, were carried out on rat adipose tissue plasma membranes and were related to that of human insulin. The IC_{50} values were determined from competition binding curves (Figure 3). The IC_{50} values of HI-NBD and HI-MCA were found to be weaker than human insulin (47%–46%, Table 1), nevertheless their ability to bind effectively to the insulin receptor was largely retained. Our data are consistent with Bailey *et al.* [21]. Their LysB29 mono-FITC labelled insulin retained about 30% of biological activity which is slightly less than our analogues (about 47%). The reason for this difference may be the fact that the FITC moiety is a relatively larger group than NBD or MCA. A similar result was observed with human insulin having a phenylacetyl group on LysB29 [35]. This analogue retained 69% binding affinity. On the other hand, an FITC-trisubstituted analogue of human insulin was observed to have its binding affinity severely diminished [20]. We suppose the main reason was a simultaneous blockage of A1 and B29 amino groups.

Our group was not the first to prepare a fluorescent insulin derivative with an NBD label on B29 lysine. There are, however, several major differences between the reported approach [40] and ours that was dictated by the need to develop a more versatile synthetic method for the preparation of LysB29-labelled insulin analogues. Schlein *et al.* [40] prepared a similar insulin analogue with an NBD label attached to B29 lysine via a hexanoate spacer. This insulin derivative retained 50% ability to incorporate glucose into lipids in isolated mouse adipocytes compared with human insulin. The

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Table 1 Values of IC_{50}[a] and Receptor Binding Affinities[b] of Human Insulin and Fluorescent Derivatives of Human Insulin

Analogue	$IC_{50} \pm$ SEM (nM)	(*n*)	Affinity (%)
HI	1.78 ± 0.98	(9)	100
HI-NBD	3.76 ± 1.73	(6)	47.3
HI-MCA	3.88 ± 1.54	(6)	45.9

[a] IC_{50} values represent concentration of insulin or fluorescent derivatives of human insulin causing half maximal inhibition of binding of ^{125}I-insulin to insulin receptor. Each value represents the mean ± SEM (standard error of measurement) of multiple determinations; (*n*) the number of separate determinations is shown in parentheses.
[b] Relative receptor binding potency is defined as (IC_{50} of human insulin/IC_{50} of analogue) ×100. See methods for details.

analogue bound reversibly to the soluble insulin receptor fragment with a K_d of about 11 nM, and exhibited about 40% enhancement of fluorescence quantum yield upon binding. The authors [40] used a different method to ours for the preparation of their analogue. Thus, they labelled (A1,B1)-Boc-protected human insulin with the activated NBD-hexanoate fluorescent label in solution. The product was isolated by RP-HPLC. Consequently, Boc protecting groups were removed from the precursor and the final product was purified again by HPLC.

These proposed methods for the preparation of B29 lysine labelled fluorescent derivatives of insulin offer several advantages. In our case, it was intended to present two relatively simple ways for the preparation of selectively labelled human insulin analogues, avoiding the preparation and use of A1,B1-protected insulin. As these methods include solid-phase synthesis of the *C*-terminal octapeptide, they provide high versatility for the preparation of fluorescent insulin analogues with substitutions in this part of insulin, which is highly important for the receptor recognition. The combination of specific insulin analogues and various fluorescent probes could be a very interesting tool in the further investigation of insulin function and the metabolic pathway.

It has been suggested [6] that the B24–B29 stretch of insulin is intimately involved in the binding of hormone to its receptor, and hence human insulins with fluorescent labels in this stretch were prepared. It is hoped that these new fluorescent derivatives of human insulin could be valuable in studying conformational changes by fluorescent techniques during the binding of insulin to its receptor. Moreover, our proposed method of preparation is relatively simple and offers high versatility for the preparation of different fluorescent insulin analogues with substitutions in the *C*-terminal octapeptide of the B-chain of human insulin.

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