FEBS Letters 349 (1994) 301–306

FEBS 14306

Synthesis and characterization of photoactivatable peptide agonists of the human thrombin receptor

Rainer Bischoff[a,*], Yves Cordier[a], Ulla B. Rasmussen[a], Yasmin Schlesinger[a], Christian Gachet[b], Michel Jaquinod[c,**], Brian Tripet[d], Pele C.S. Chong[d], Andrea Pavirani[a]

[a]*Transgene S.A., 11 rue de Molsheim, 67082 Strasbourg Cedex, France*
[b]*Centre Régional de Transfusion Sanguine, Institut National de la Santé et de la Recherche Médicale U-311, 67085 Strasbourg Cedex, France*
[c]*Université Louis Pasteur, Laboratoire de Spectrometrie de Mass Bioorganique, URA 31, CNRS, 5 rue Blaise Pascal, 67008 Strasbourg Cedex, France*
[d]*Connaught Laboratories Ltd., 1755 Steeles Avenue West, Willowdale, Ont., Canada*

Received 22 June 1994

Abstract

Chemical synthesis and biochemical analysis of modified agonist peptides of the human thrombin receptor derived from the sequence SFLLRNP containing photoactivatable azido groups and biotin for sensitive detection is described. Substitution of leucine in position three with *p*-azidophenylalanine and extension of the C-terminus with a KGGK spacer containing biotin covalently linked to the side chain of the C-terminal lysine residue resulted in an active receptor agonist as determined by intracellular Ca^{2+} mobilization in human erythroleukemia (HEL) cells. In contrast, substitution of phenylalanine in position two with *p*-azidophenylalanine reduced agonist activity significantly.

Key words: Thrombin receptor; Photoaffinity labelling; *p*-Azidophenylalanine; Biotinylation; Ca^{2+} mobilization

1. Introduction

α-Thrombin, a serine protease, plays a key role in the coagulation cascade and acts as a growth factor for a number of different cell types [1–3]. Thrombin is thus essential to hemostasis, wound healing and tissue repair. Furthermore, thrombin might also be implicated in tumor vascularization and in the formation of atherosclerotic plaques [4]. Inhibition of thrombin will affect all of these diverse activities and it would be advantageous in disease states where the growth factor activity of thrombin is involved, such as atherosclerosis, to block the respective receptor instead of inhibiting thrombin itself.

Expression of mRNA derived from both human and hamster cDNA in *Xenopus* oocytes has led to the identification of a functional thrombin receptor which is a member of the G-protein coupled receptor family containing seven membrane spanning domains [5,6]. Previous work has shown activation to occur through proteolytic cleavage by α-thrombin between residues R^{47} and S^{48} in the extracellular N-terminal region of the human receptor, thus generating a new amino-terminus which functions as the activating ligand [5,7]. Interestingly, it was also shown that short synthetic peptides corresponding to the newly generated N-terminus were effective in activating the receptor, and structure–activity relationships using synthetic peptides have subsequently shown certain residues to be critical for receptor activation [8–14] (Table 1).

Based on these results, we have synthesized two biotinylated peptide agonists containing photoactivatable groups as molecular tools to elucidate further the ligand binding site within the thrombin receptor. In the present paper, we describe the synthesis and analysis of two biotinylated *p*-azidophenylalanine-containing peptide ligands and report on their biological activities in a human erythroleukemia (HEL) cell line, which has previously been shown to respond to both thrombin and thrombin receptor agonist peptides with an increase in intracellular Ca^{2+} concentration [15].

2. Experimental

2.1. Synthesis and purification of biotinylated photoactivatable peptides

The biotinylated photoactivatable peptides were manually synthesized using standard N^{α}-tert-butyloxycarbonyl (Boc) chemistry on a methylbenzhydrylamine resin (0.2 mmol) as solid support [16], resulting in the respective C-terminal amides of the synthesized peptides. All chemicals used in the peptide synthesis were purchased from Peninsula Lab. Inc. (Belmont, CA, USA). A fourfold excess of Boc amino acids was used in each cycle of coupling. Boc-(N^{ε}-Fmoc)-lysine was the first amino acid attached to the resin and Boc-*p*-azidophenylalanine, which was prepared according to Schwyzer and Caviezel [17], was used for residues 2 and 3 of peptides AZO-1 and AZO-2, respectively. After the last cycle of coupling (Boc-Serine (*O*-benzyl)) was completed, peptide

*Corresponding author. Fax: (33) 88 22 58 07.

** *Present address:* Odense Universitet, Campusvej, DK-5230 Odense, Denmark

Abbreviations: HEL, human erythroleukemia; Fmoc, N^{α}-9-fluorenylmethyloxycarbonyl; Boc, N^{α}-tert-butyloxycarbonyl; RP-HPLC, reverse-phase high-performance liquid chromatography; ESMS, electrospray mass spectrometry; BSA, bovine serum albumin; PBS, phosphate- buffered saline; ELISA, enzyme-linked immunosorbent assay; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulf onic acid]).

SSDI 0014-5793(93)00692-O

resins were treated with piperidine to remove the Fmoc-protecting group from the C-terminal lysine residue. Peptides were subsequently biotinylated with the *N*-hydroxysuccinimide ester of biotin (Pierce, Rockford, IL, USA). Both peptides were cleaved from the resin using anhydrous HF in the presence of 10% anisole and collected by ethyl ether precipitation. Peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a semi-preparative Vydac C_4 column (The Separations Group, Hesperia, CA, USA) using a 0–40% acetonitrile gradient in 0.1% trifluoroacetic acid developed over 40 min at a flow rate of 2 ml/min.

2.2. Analysis of biotinylated photoactivatable peptides

Amino acid composition analysis was performed by hydrolysis in 6 N HCl followed by derivatisation to phenylthiocarbamyl amino acids using a model 420H analysis system coupled to an on-line model 130A HPLC (Applied Biosystems, Foster City, CA). Data acquisition and analyses of the chromatographic data were performed on a model 920A data acquisition system (Applied Biosystems) with norleucine as the internal standard.

Electrospray mass spectrometry (ESMS) was performed on a triple quadrupole mass spectrometer with a mass range of 4000 equipped with a pneumatically assisted electrospray source (Fisons Bio-Q, VG-Bio-Tech, Altrincham, UK). Samples were dissolved in a mixture of water/methanol (50:50) containing 1% acetic acid at a concentration of 15–20 pmol/μl and 10 μl of this solution were introduced at a flow rate of 4 μl/min into the ion source. Calibration was performed based on the multiply charged ions from a separate introduction of horse heart myoglobin (16951.5 Da), at a resolution so that the m/z = 998 peak was 1.2 amu wide at 50% peak height. Scanning was performed from m/z = 350 to 1550 amu in 12 s in the multi-channel acquisition mode.

2.3. Photolabelling

To evaluate the capacity of the biotinylated photoactivatable peptides to crosslink with proteins upon photolysis, bovine serum albumin (BSA) was used as a model. BSA (10 mg/ml) was mixed with individual peptides AZO-1 and AZO-2 (2 mg/ml) in phosphate-buffered saline (150 mM NaCl, 10 mM K_2HPO_4, pH 7.4; PBS) at room temperature for 1 h under N_2 gas. Half of the mixtures were stored at 4°C as controls. The photolysis tubes containing the other half of the BSA/peptide mixtures were irradiated at 4°C for 30 min using 4 RPR 300 nm and 4 RPR 350 nm lamps. The photolyzed material was removed from the photolysis tubes and analyzed for BSA-peptide conjugates by ELISA (see below).

2.4. Enzyme-linked immunosorbant assay (ELISA)

Aliquots of the photolyzed mixtures (BSA/AZO-1 and BSA/AZO-2 each equivalent to 200 ng of peptide) or 200 ng of AZO-1 or AZO-2 were coated onto microtiter wells of a 96-well ELISA plate (Nunc-Immunoplate, Nunc, Denmark) in 50 μl coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 16 h at room temperature. The plates were subsequently blocked with 0.1% (w/v) BSA in PBS for 30 min. Serially diluted streptavidin-peroxidase solutions (Jackson ImmunoResearch Labs Inc., PA, USA) were added to the wells and incubated for 1 h at room temperature. After removal of the streptavidin-peroxidase conjugate, plates were washed five times with PBS containing 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA. The plates were developed using tetramethylbenzidine in H_2O_2 as a substrate and the reaction was stopped by addition of 1 N H_2SO_4. Absorbance was read at 450 nm using a Titretek Multiskan II (Flow Labs, VI, USA). Assays were performed in triplicate.

2.5. Measurement of thrombin receptor agonist activity

HEL cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI medium supplemented with 10% fetal calf serum. For measurement of thrombin receptor agonist activity, HEL cells were harvested and washed twice in RPMI medium containing 10 mM HEPES, pH 7.3, and resuspended at 37°C in the same buffer at 5×10^6 cells per ml. Fura-2/acetoxymethyl ester (Molecular Probes Inc., Eugene, OR, USA) was added to a final concentration of 4 μM and the cells were incubated for 1 h at 37°C with gentle shaking. Cells were washed twice and resuspended at 10^6 cells per ml in HEPES/saline (10 mM HEPES, pH 7.3, 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% BSA). Two 1 ml aliquots were transferred into a quartz cuvette equipped with a magnetic stirrer which was placed in a thermostatically controlled chamber at 37°C in a PTI Delta-Scan-1 fluorescence spectrophotometer (Photon Technology International Inc., Princeton, NJ, USA). Peptides were added directly at the indicated times to a final concentration of 10 μM. During the experiments, the excitation wavelengths were altered between 340 and 380 nm and fluorescence was measured by recording the emitted light at 510 nm. Bandwidths were 4 nm on both excitation and emission monochromators.

3. Results

3.1. Design of peptides

Since the discovery of the human thrombin receptor [5] and its hamster analog [6] a number of studies have focused on the structural requirements that lead to activation of the receptor by the newly liberated N-terminus after thrombin cleavage. A compilation of the obtained results is given in Table 1 showing that positions 2 and 4 allow only conservative changes while positions 1, 3

Table 1
Consensus sequence of the human thrombin receptor agonist peptide

NH_2	Ser[1]	Phe[2]	Leu[3]	Leu[4]	Arg[5]							
	Ala	p-fluoro-Phe	Phe*	Phe	Gln							
	Met		Trp	Ile	Lys							
	Thr		Tyr		Ornithine							
	Cys		Ala		Citruline							
	Gly		Ile									
	Isoserine		Arg									
			Gly									
			Pro									
NH_2	Ser[1]	AzidoPhe[2]	Leu[3]	Leu[4]	Arg[5]	Asn[6]	Pro[7]	Lys[8]	Gly[9]	Gly[10]	BiotinLys $CONH_2$	(AZO-1)
NH_2	Ser[1]	Phe[2]	AzidoPhe[3]	Leu[4]	Arg[5]	Asn[6]	Pro[7]	Lys[8]	Gly[9]	Gly[10]	BiotinLys $CONH_2$	(AZO-2)

The natural sequence is given at the top with substitutions that do not affect activity significantly being listed below the respective amino acid residue. Sequences of the photoactivatable biotinylated peptides AZO-1 and AZO-2 are shown at the bottom.
*Phe is the naturally occuring amino acid in the hamster, rat and mouse thrombin receptor.

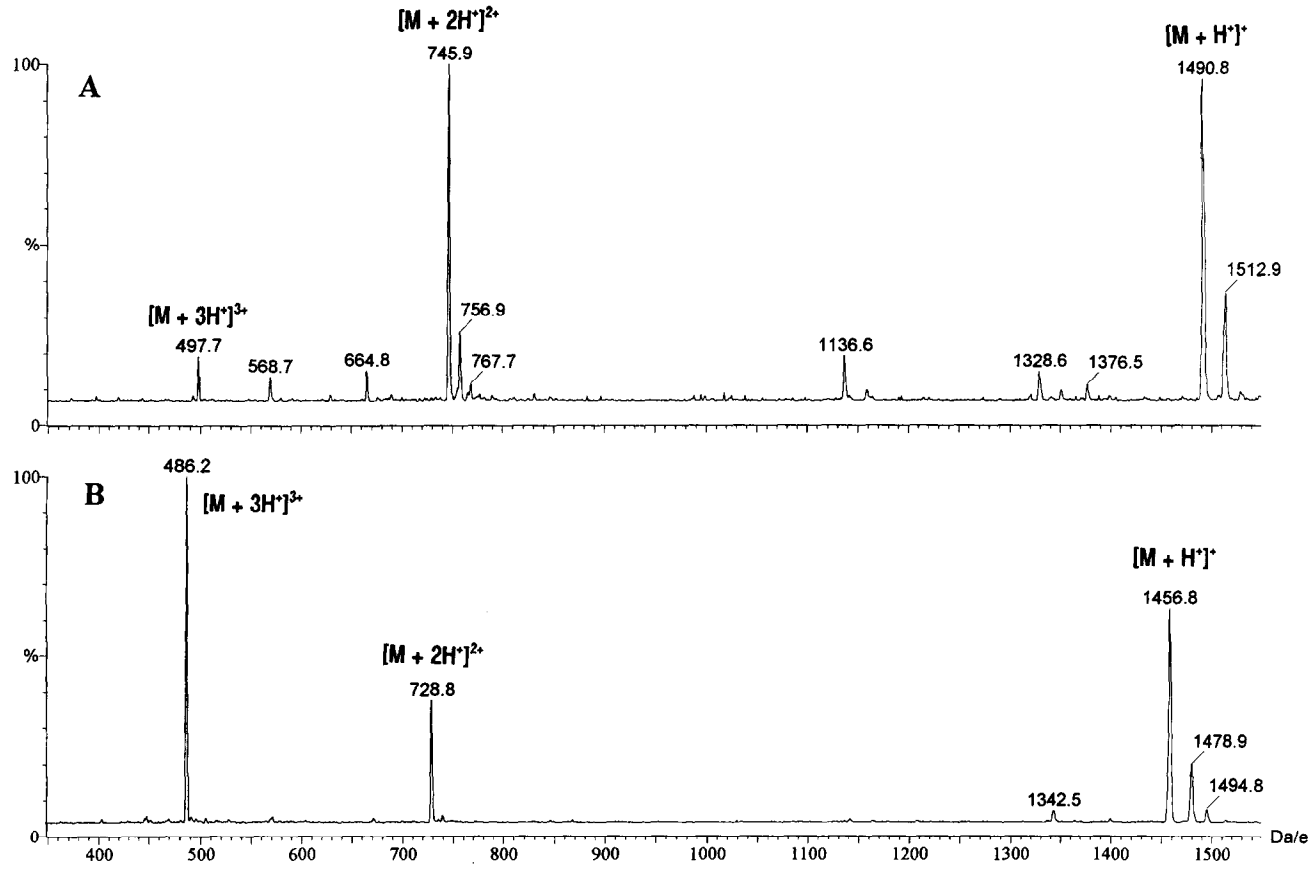

Fig. 1. Electrospray mass spectra of peptides AZO-2 (A) and AZO-1 (B). Singly and multiply protonated molecular ions are labelled as $[M+nH^+]^{n+}$. Measured molecular masses (AZO-2, 1489.9 ± 0.1 Da; AZO-1, 1455.7 ± 0.1 Da) were calculated based on a series of three molecular ions (n: 1, 2 and 3) and are given with their respective standard deviations. Signals at 1512.9 and 756.9 amu for AZO-2 (A) and 1478.9 for AZO-1 (B) relate most likely to Na^+-adducts of the respective peptides. Ions at 1376.5 amu and 1342.5 amu for AZO-2 and AZO-1, respectively, may correspond to deletion of Asn during synthesis (measured Δm = 114.4 Da [AZO-2]; 114.2 Da [AZO-1]; expected Δm = 114.1 Da). Furthermore, ions at 568.7 amu (doubly protonated) and 1136.6 amu (singly protonated) in the preparation of AZO-2 (measured mass: 1135.5 Da) may correspond to a missing biotinylated lysine (measured Δm = 354.4 Da; expected Δm = 354.5 Da). The contaminant with a measured mass of 1327.6 Da (singly and doubly protonated ions at 1328.6 and 664.8 amu, respectively) could not be correlated with simple truncations or deletions of AZO-2.

and 5 are more tolerant. Furthermore, a free N-terminal amino group appears to be important for activity. These results led us to design two photoactivatable peptides with substitutions in positions 2 (AZO-1) and 3 (AZO-2) with the aim of further probing the structural requirements for receptor activation as well as to develop molecular tools to be used in studying the architecture of the ligand binding site within the receptor. In addition, a biotin moiety was included in each peptide to allow easy and sensitive detection of the photocrosslinked peptides as well as to potentially serve as affinity handles for receptor purification. The biotin label was attached to a C-terminal KGGK extension of the agonist peptide, thus providing a spacer which should facilitate detection by streptavidin since extensions of the C-terminal part of the originally described agonist peptide SFLLRNP were shown to have little effect on receptor activation [11].

3.2. Synthesis and analysis of photoactivatable peptides

Peptides were synthesized as their C-terminal amides by solid-phase synthesis using Boc chemistry and purified by RP-HPLC. Boc-*p*-azidophenylalanine was synthesized and substituted for either Phe[2] or Leu[3]. Biotin was incorporated as a marker for purification and for identification of crosslinking sites. The coupling efficiency was >99.5% for each cycle, but the crude peptides were shown to contain multiple peaks by RP-HPLC. This may have been due to *p*-azidophenylalanine or biotin undergoing side reactions during HF cleavage. After RP-HPLC purification overall yields were 40% and 25% for AZO-1 and AZO-2, respectively. Analytical RP-HPLC showed that AZO-1 was 95% pure while AZO-2 had a purity of approximately 86% (data not shown). Both peptides were therefore re-purified by RP-HPLC and only the major components were tested for biological activities. Both peptides were further subjected to amino acid composition analysis confirming the substitution of phenylalanine in AZO-1 and leucine in AZO-2, respectively (Table 2). The *p*-azidophenylalanine residues resulted in a peak co-eluting with leucine upon

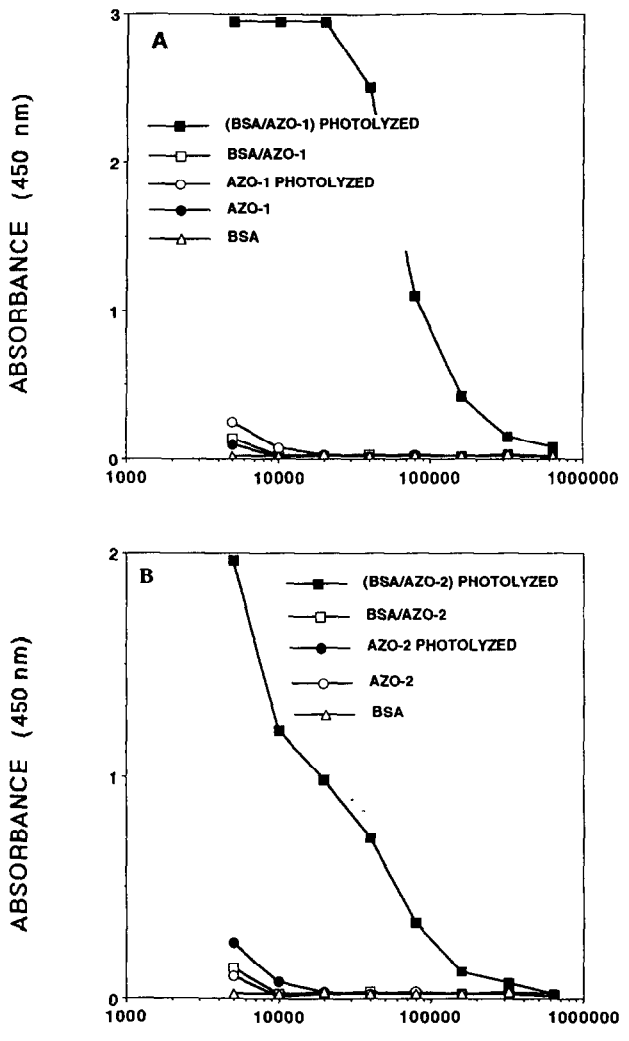

Fig. 2. Evaluation of photocrosslinking between AZO-1 (A) and AZO-2 (B) and BSA by ELISA (see section 2 for details). Filled-in squares show photocrosslinking between the peptides and BSA as detected by streptavidin-peroxidase. The other traces are controls where either irradiation was omitted (open squares) or where the peptides (irradiated or not) and BSA were coated onto the ELISA plates individually.

separation of the PTC amino acid derivatives, thus apparently augmenting the amount of leucine in each peptide. Structural analysis by automated N-terminal sequencing proved to be unsuitable, most likely due to the presence of the reactive *p*-azido group close to the peptides' N-termini which prevented Edman degradation from progressing beyond this point.

Electrospray mass spectrometry (ESMS) was thus employed to confirm the structure of the peptides and most importantly to assure that both the *p*-azidophenylalanine and the biotin modifications had been correctly incorporated. Surprisingly, both spectra showed that the major products had molecular masses which were approximately 27 Da lower than expected (Fig. 1). Fast atom bombardment mass spectrometry (FAB-MS) yielded similar results with mass differences of about 26 Da between the expected and measured values (data not shown). In view of the lability and reactivity of the azido group we assumed that N_2 had been eliminated during mass measurement, either due to the elevated temperature of the drying gas in ESMS (70°C) or to reaction with the FAB matrix (thioglycerol) transforming *p*-azidophenylalanine into *p*-aminophenylalanine (Δm = 26.0 Da). To ensure that both peptides contained photoactivatable azido groups they were photoactivated in the presence of BSA (see below).

3.3. Photolabelling

To evaluate the ability of the azido-peptides to covalently link BSA upon photolysis at 300 and 350 nm, BSA was irradiated for 30 min at 4°C in the presence of a 9-fold molar excess of either AZO-1 or AZO-2. Individual peptides (photolyzed or native), peptide/BSA mixtures (photolyzed or non-photolyzed) were coated onto microtiter plate wells. ELISAs showed that biotin was detected by a streptavidin-peroxidase conjugate in the wells containing either AZO-1 or AZO-2 photolyzed with BSA (Fig. 2). The other wells were negative due to non-crosslinked peptides binding only weakly to the microtiter plate. These results confirmed the presence of photoactivatable azido groups and supported the hypothesis that the azido groups had been converted into amino groups during mass spectrometric analysis.

Table 2
Amino acid composition analysis of photoactivatable biotinylated peptides AZO-1 and AZO-2[a]

Amino acid	Expected AZO-1	Measured AZO-1	Expected AZO-2	Measured AZO-2
D + N	1	1.2	1	1.2
E + Q	0	0	0	0
S	1	1.1	1	0.9
G	2	1.7	2	1.7
H	0	0	0	0
R	1	0.8	1	0.9
T	0	0	0	0
A	0	0	0	0
P	1	1.1	1	1.3
Y	0	0	0	0
V	0	0	0	0
I	0	0	0	0
L[b]	2	3.4	1	2.1
F[c]	0	0	1	1.1
K[d]	2	1.9	2	2.1

[a] All measured values are averaged over four determinations. Trp, Met and Cys were not included in these analyses.
[b] A derivative of *p*-azidophenylalanine gave rise to a peak that co-eluted with PTC-leucine thus increasing the apparent amount of leucine.
[c] *p*-Azidophenylalanine was not included in the expected amino acid composition since it will not give phenylalanine upon acid hydrolysis.
[d] Biotinylated lysine was included in the expected amino acid composition since the biotin moiety was linked to the lysine side chain via an amide linkage which will most likely be hydrolyzed in 6 N HCl.

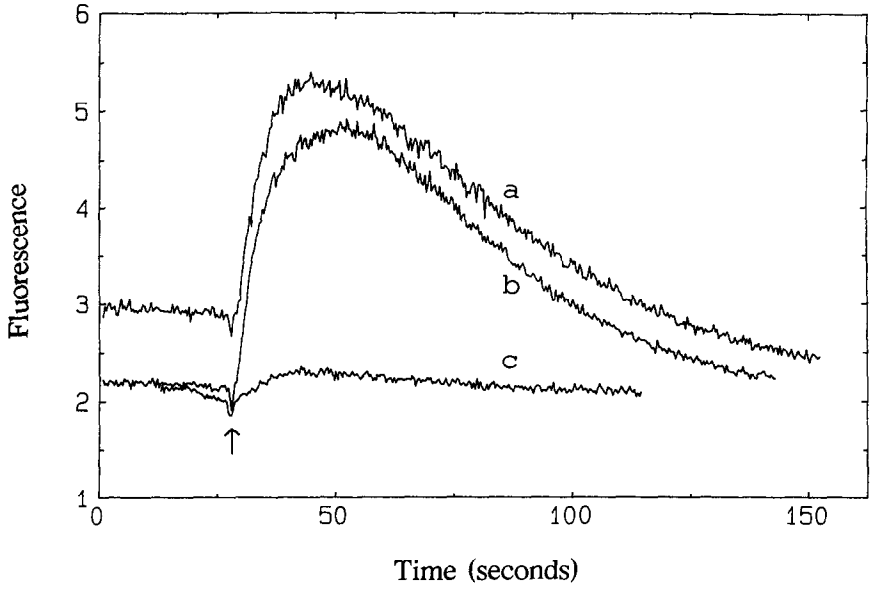

Fig. 3. Effects of photoactivatable peptides on intracellular Ca^{2+} mobilization in HEL cells. Each peptide was tested at a concentration of 10 μM (the arrow indicates the time when peptides were added) with the natural agonist SFLLRNP (a) and AZO-2 (*p*-azidophenylalanine in position 3) (b) giving identical results while AZO-1 (*p*-azidophenylalanine in position 2) (c) showing a markedly reduced response. Intracellular Ca^{2+} concentration is shown as the fluoresecence emission of the Ca^{2+}/fura2 complex at 510 nm, calculated as the ratio of emissions upon excitation at 340 and 380 nm, respectively (see section 2 for details).

3.4. Biological activity

Functional activity of the photoactivatable peptides was evaluated by measuring the increase in intracellular Ca^{2+} concentration in HEL cells that had been preloaded with the fluorescence indicator fura-2/acetoxymethyl ester. It had previously been shown that activation of the thrombin receptor resulted in an increase in the intracellular Ca^{2+} level as part of the signal transduction pathway [15]. Incubation of preloaded HEL cells with either the natural agonist SFLLRNP or with AZO-2 (*p*-azidophenylalanine in position 3, see Fig. 1) at 10 μM resulted in an identical increase in fluorescence at 510 nm indicating that both peptides were equally capable of increasing intracellular Ca^{2+} concentrations at this concentration (Fig. 3). However, incubation with 10 μM AZO-1 (*p*-azidophenylalanine at position 2, see Fig. 1) had almost no influence on the level of intracellular Ca^{2+}, indicating that AZO-1 was a much less potent receptor agonist than AZO-2 (Fig. 3).

4. Discussion

Photoaffinity labelling of proteins, including G-protein coupled receptors, has provided detailed insights into the binding sites for substrate analogs, receptor agonists and antagonists, as well as into the structural transitions related to activation and signal transduction [18]. This approach has to be considered as being complementary to site-directed mutagenesis in locating amino acid residues that are critical for ligand binding and signal transduction. Both photoaffinity labelling and site-directed mutagenesis can provide insights into the architecture of the ligand binding site and help to design new peptidic or non-peptidic agonists and antagonists in conjunction with molecular modelling techniques [19].

The present work describes the synthesis and functional analysis of modified photoactivatable peptides derived from the human thrombin receptor agonist peptide SFLLRNP. The results show that Leu^3 can be substituted with the photoactivatable amino acid derivative *p*-azidophenylalanine with little effect on biological activity (AZO-2 peptide) while *p*-azidophenylalanine in position 2 reduces activity significantly (AZO-1 peptide). These results are in agreement with earlier work where the structure of the amino acid in position 2 was shown to be of critical importance for agonist activity while position 3 was more forgiving towards structural changes ([11] and Table 1). Peptide AZO-2, an agonist of the human thrombin receptor containing a photoactivatable azido group located between two amino acid residues (positions 2 and 4, see Table 1) which have been shown to be crucial for receptor activation, is therefore an interesting candidate for photoaffinity labelling studies and may allow a more detailed view of the receptor ligand binding site which is so far unknown. Furthermore, incorporation of biotin into AZO-2 should allow sensitive detection of the labelled receptor and may in the future help in its isolation and the study of the crosslinking sites.

Acknowledgements: The cooperation of D. Colin (Institut of Microbiology, Strasbourg, France) with regard to fluorescence spectrophotometric measurements, and of B. Bouchon and C. Ebel for amino acid composition analysis, is highly appreciated. We also thank K.-U. Lentes for valuable discussions, and M. Courtney for critically reading the manuscript.

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