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The preparation of biotinyl-ε-aminocaproylated forms of the vasoactive intestinal polypeptide (VIP) as probes for the VIP receptor

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Vasoactive intestinal polypeptide (VIP) was biotinyl-ε-aminocaproylated using sulfosuccinimidyl-6-(biotinamido) hexanoate thereby producing a series of products that were separated by high performance liquid chromatography (HPLC). Seven VIP-derivatives were isolated and the number and location of biotinyl-ε-aminocaproylation was determined by a combination of enzymatic degradation and plasma desorption mass spectrometry (PDMS). Receptor binding experiments with the VIP biotinyl-ε-aminocaproylated derivatives revealed IC_{50} values for the monobiotinyl-ε-aminocaproylated peptides that were 1.3–3.2 times higher than for natural VIP. All isolated biotinyl-ε-aminocaproylated derivatives possess VIP-like bioactivity as shown by an assay measuring pancreatic juice secretion in cat, VIP biotinyl-ε-aminocaproylated in position lysine[15] being almost equipotent with natural VIP.

Vasoactive intestinal polypeptide; Plasma desorption mass spectrometry; Biotinylated analog; Pig liver membrane

1. INTRODUCTION

Biotinylated derivatives of polypeptide hormones have proven to be useful tools for studying the interaction between hormones and their receptors in living cells [1–3] and also for purifying hormone receptors with avidin affinity columns [4–9]. Especially promising are biotinylated derivatives in which the biotin is linked to the hormone over a spacer arm. Vasoactive intestinal polypeptide (VIP) is a 28-amino acid peptide first characterized from porcine duodenum [10] and has been demonstrated to be involved in numerous biological events [11]. The amino acid sequence of pig VIP [12] is: His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys[15]-Gln-Met-Ala-Val-Lys[20]-Lys[21]-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2. VIP has subsequently been isolated from several other species of vertebrates. With the exception of guinea-pig VIP all mammalian forms, including human, have been found to have the same sequence [11]. Studies of truncated peptides suggest that the biological activity is fully preserved only in the intact peptide [13]. Calculation by Fournier et al. [14] has suggested that an α-helix domain spans residues 13–28 segregating the sequence in an amphiphilic manner. By substituting either the hydrophobic or hydrophilic surface it has been suggested that the hydrophilic surface does not participate strongly in receptor binding [15]. The biotin marker can be readily introduced into VIP via the ε-amino group of lysine residues at positions 15, 20 and 21, residues that are hypothesized to be located in the hydrophilic area. The high affinity ($K_d = 10^{-15}$ M) of biotin for egg white avidin and streptavidin [16] provides an important experimental strategy for the qualitative and quantitative study of membrane receptors. In this report we have tested several biotinyl-ε-aminocaproylated VIP derivatives as potential receptor-avidin cross-linking agents.

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2. MATERIALS AND METHODS

2.1. *Materials*

VIP was isolated from pig intestine as described previously [10]. Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) was from Pierce (Europe BV), endoproteinase Lys-C is a product of Boehringer Mannheim (Germany), phenylmethylsulfonylfluoride (PMSF) of Merck (Germany), Tris-HCl, bovine serum albumin (BSA) Fr.V and bacitracin of Sigma (USA), Placidyl was obtained from Abbot (USA), and (3-[^{125}I]iodotyrosyl[10])VIP was obtained from Amersham (UK) and is referred to as ^{125}I-VIP.

2.2. *Biotinyl-ε-aminocaproylation of VIP*

A mixture of 0.9 μmol VIP and 1.2 or 3.6 μmol NHS-LC-Biotin was dissolved in 2.5 ml 50 mM $NaHCO_3$. After 30 min of incubation at room temperature the reaction was stopped by the addition of 0.6 ml 1 M acetic acid and the mixture lyophilized. Separation of the reaction products was performed by reversed-phase HPLC on a TSK ODS 120-T C18 column (7.8 × 300 mm; LKB, Sweden) at a flow rate of 1.5 ml/min in 0.1% trifluoroacetic acid and a linear gradient of 30–45% acetonitrile/0.1% TFA in 50 min. Eluted fractions, named B0–B7, were lyophilized and stored at −20°C.

2.3. *Analysis of biotinyl-ε-aminocaproylated VIP-derivatives*

HPLC-purified fractions B1–B7 (20–40 μg) were digested with endoproteinase Lys-C at a substrate-to-enzyme ratio of 25:1 (wt/wt) in 300 μl of 0.1% ammonium bicarbonate for 4 h at 37°C. After

lyophilization the fractions were subjected to Time-of-flight plasma desorption mass spectrometry (PDMS) using a Biolon Bln 20 mass spectrometer (Biolon Nordic AB, Uppsala, Sweden). Samples were dissolved in 0.1% TFA containing 50% ethanol, loaded to aluminium foils covered with nitrocellulose and evaporated by a stream of nitrogen. Data were accumulated for 15 min at 15 kV acceleration voltage. Hydrogen and sodium were used as internal standards for calibration of the time data to mass. Molecular weights of the fragments in the different digest were obtained from the single-charged or double-charged protonated molecules (MH^+, MH^{2+}). Some fragments, especially from the C-terminal peptide region, were also detected as cationized adduct ions (MNa^+). Natural VIP and its derivatives were similarly hydrolyzed and analyzed by analytical HPLC, Vydac C18 (218TP546, 4.6 × 250 mm; The Separations Group, Hesperia, CA, USA) at a flow rate of 1 ml/min with a linear gradient of 0–50% in 30 min using the same solvents as described above.

2.4. *Receptor binding studies*

Experiments were performed with pig crude liver cell membranes prepared essentially as described by Couvineau et al. [17]. Briefly, fresh pig liver was homogenized with a Waring blender in 4 vols 10 mM triethanolamine pH 7.5, 0.25 M sucrose, 5 mM EDTA and 0.1 mM PMSF. The homogenate was filtered through 2 layers of gauze and centrifuged at 2600 × *g* for 10 min. The supernatant was then filtered through gauze and centrifuged at 40000 × *g* for 20 min. The resulting pellet was washed twice with 20 mM Tris-HCl, pH 7.4, and resuspended in this buffer at a protein concentration of 25 mg/ml, aliquoted and stored at −80°C. Protein concentrations were measured with the BCA assay (Pierce) using BSA as standard.

Binding experiments were performed as previously described [18] with some minor modifications. Samples were incubated at a protein concentration of 0.8 mg/ml in 25 mM Tris-HCl, pH 7.5, 0.5% BSA, 0.15 mg/ml bacitracin and 20 pM ^{125}I-VIP in a final volume of 250 μl in the absence or presence of increasing concentrations of natural VIP or HPLC-purified fractions B0–B7. Ligand concentrations were corrected for molecular weight. Nonspecific binding was determined in the presence of 1 μM VIP and represented less than 10% of total binding.

2.5. *Bioassay*

The ability of VIP to increase pancreatic bicarbonate juice secretion in anesthesized (Placidyl) cat was performed as described earlier for secretin [19]. The VIP-derivatives and a reference sample (natural porcine VIP) were injected intravenously and pancreatic juice secreted from the pancreas was analyzed for alkali content.

3. RESULTS

3.1. *Biotinyl-ε-aminocaproylation of VIP*

The reaction of porcine VIP with NHS-LC-Biotin resulted in a mixture of biotinyl-ε-aminocaproylated products. These were separated by reversed-phase HPLC into several discrete components (Fig. 1) designated B0–B7. In the order of their elution, B0 most probably represents the underivatized VIP since it elutes in the same position as natural VIP, and B1–B7, all less polar than VIP, the biotinyl-ε-aminocaproylated derivatives. The reaction performed at a 1:1.3 ratio of VIP/NHS-LC-Biotin resulted mainly in a production of the components B1–B3, while a higher ratio of NHS-LC-Biotin produced relatively more of the components B4–B7.

The possible reaction sites in VIP for NHS-LC-Biotin are the reactive amino groups of the 3 lysine residues and the amino terminal of the peptide. In order to determine the degree and position of biotinyl-ε-aminocaproylation in the derivatives, each peptide (B1–B7) was digested with endoproteinase Lys-C. This enzyme cleaves a peptide at the carboxyl side of a lysine residue only, and if the lysine is biotinyl-ε-aminocaproylated cleavage does not occur. Mass spectra of each digested peptide mixture were recorded and the results for B1, B2 and B3 show *m/z* peaks corresponding to VIP 21–28-, VIP 16–21- and VIP 1–20-monobiotinyl-ε-aminocaproylated respectively (Fig. 2 and Table I). B4–B7 were similarly analyzed and the data are consistent with dibiotinyl-ε-aminocaproylation in B4–B6 and tribiotinyl-ε-aminocaproylation of B7 (Table II). The B1–B3 peptide digests were also analyzed by analytical HPLC and the pattern obtained compared to that of enzyme-treated natural VIP (Fig. 3). Since biotinyl-ε-aminocaproylation of a lysine eliminates one potential cleavage site one would, in the monobiotinyl-ε-aminocaproylated derivatives, expect to see both a disappearance of 2 (B1 and B2) or 3 (B3) fragments relative to the HPLC profile of natural VIP-fragments and also the appearance of a new product. The disappearance of VIP 21–28 and 22–28 in the digest of B1, VIP 16–21 and 16–20 in that of B2, and VIP 1–15, 16–20 and 16–21 in that of B3 are in agreement with the PDMS data and are consistent with biotinylation sites of Lys^{21} in B1, Lys^{20} in B2 and Lys^{15} in B3. Among the analyzed peptides no biotinyl-ε-aminocaproylation was observed at the potentially reactive amino terminal.

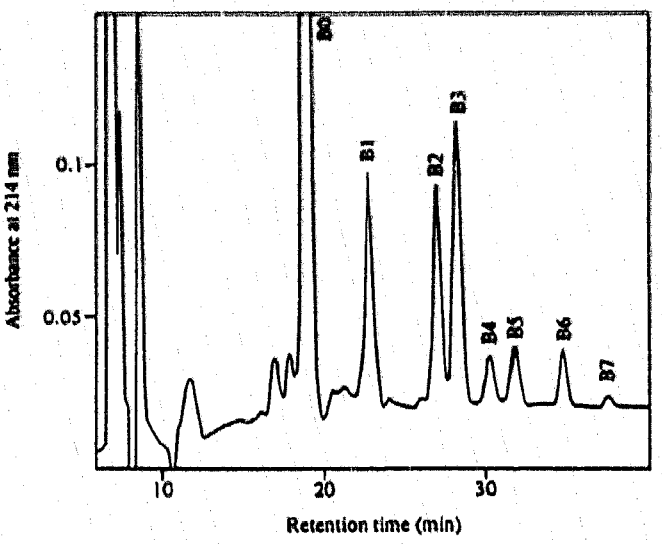

Fig. 1. HPLC-purification of biotinyl-ε-aminocaproylated VIP.

3.2. *Receptor binding properties of bioVIPs*

Biotinyl-ε-aminocaproylated peptides B1–B7 were tested for their ability to specifically displace ^{125}I-VIP

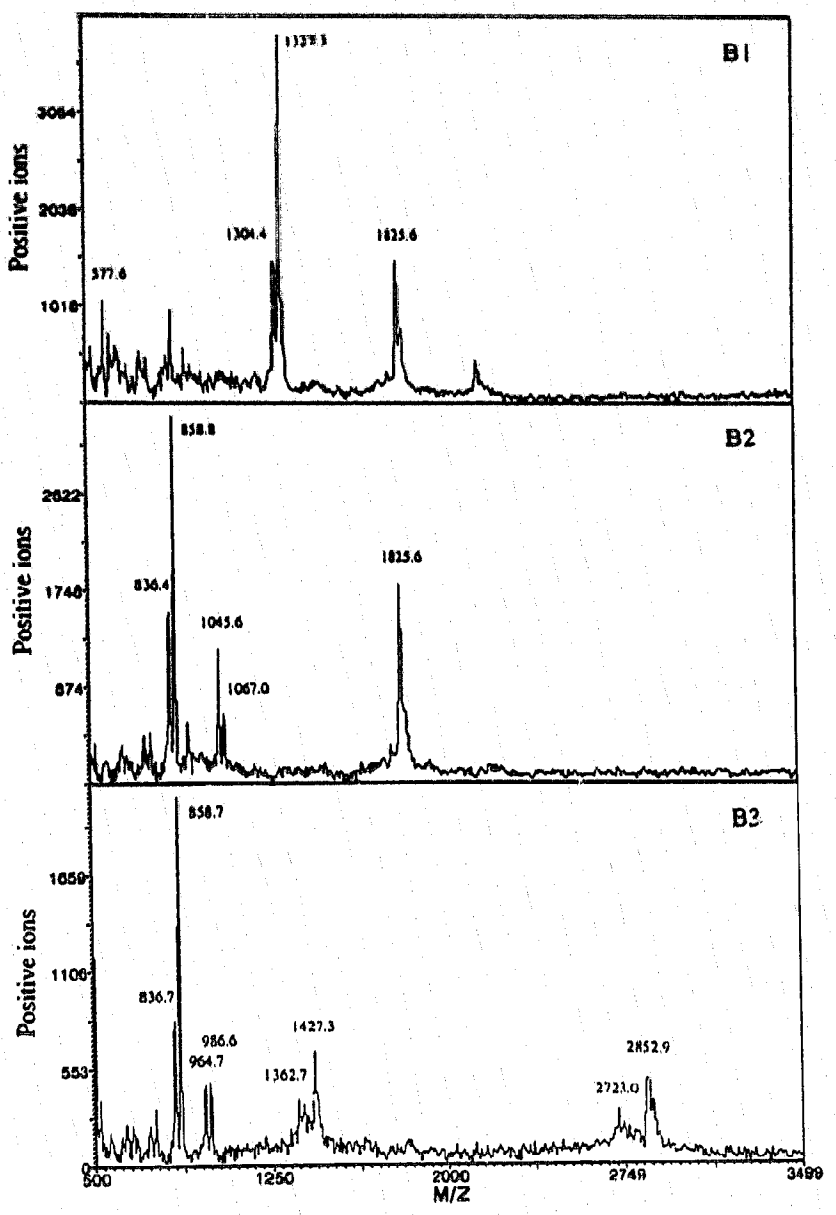

Fig. 2. Mass spectra from biotinyl-ε-aminocaproylated VIP-derivatives B1, B2 and B3 after endoproteinase Lys-C treatment. The background has been subtracted in all spectra. The peaks corresponding to the various peptides are summarized in Table I.

binding to pig liver membrane (Fig. 4). VIP has been shown [17] to bind with high affinity to a single site in this tissue. Natural VIP displaced ^{125}I-VIP-binding in a dose-dependent manner with an IC_{50} value of 3.05 ± 0.15 nM. Biotinyl-ε-aminocaproylated VIP-peptides displaced ^{125}I-VIP and the order of potency as compared to VIP is: VIP > B3 > B1 > B2 > B5 > B4 > B6 ≫ B7. The IC_{50} values of the biotinylated derivatives compared to that of natural VIP show that the analogs B1, B2 and B3 are potent VIP-receptor ligands with 1.3–3.2 times higher IC_{50} values than VIP and that the derivative monobiotinyl-ε-aminocaproylated in position Lys^{15} is the most potent ligand (Table II).

3.3. *Biological properties of bioVIPs*

VIP has been shown to stimulate pancreatic juice secretion in the anesthetized cat [20]. Monobiotinyl-ε-aminocaproylated VIP-derivatives gave a rapid VIP-like response, similar to that of natural VIP, when tested. B3 was the most potent derivative with an almost equipotent response as compared to VIP while B2 seems to be biotinyl-ε-aminocaproylated at the least favorable position giving a response less than half of VIP itself (Fig. 5). The bis- and tribiotinyl-ε-aminocaproylated derivatives also stimulated pancreatic juice secretion but with a decrease in dose response following an increase in biotinyl-ε-

Table I

Results of mass spectral analysis from enzyme-treated biotinyl-ε-aminocaproylated VIP-derivatives B1, B2 and B3

Peptide	Calculated (*m/z*)	B1	B2	B3
		Obtained (*m/z*)		
16–20	575.7	577.6[a]	–	–
22–28	835.0	–	836.4[a], 858.8[b]	836.7[a], 858.7[b]
21–28	963.1	–	–	964.7[a], 986.6[b]
1–15	1823.0	1825.6[a]	1825.6[a]	–
21–28 MB	1302.5	1304.4[a], 1327.3[b]	–	–
16–21 MB	1043.5	–	1045.6[a], 1067.0[b]	–
1–20 MB	2720.1	–	–	2723.0[a], 1362.7[c]
1–21 MB	2848.3	–	–	2852.9[a], 1427.3[c]

[a] MH^+, [b] MNa^+, [c] MH^{2+}

Calculated molecular weights of VIP and monobiotinyl-ε-aminocaproylated (MB) VIP-fragments after endoproteinase Lys-C treatment compared to obtained values from mass spectra of B1, B2 and B3

Table II

IC_{50} values, relative potency and biotinyl-ε-aminocaproylated position(s) of VIP and VIP-derivatives

Analog	IC_{50} (nM)	Potency	Position of biotinyl-ε-aminocaproylation
VIP	3.0 ± 0.2	+ + +	–
B1	8.6 ± 0.5	+ + +	21
B2	9.9 ± 0.3	+	20
B3	4.0 ± 0.2	+ + +	15
B4	27.3 ± 7.3	+[a]	20, 21[a]
B5	16.1 ± 0.9	+ +[a]	15, 21[a]
B6	29.3 ± 3.2	+[a]	15, 20[a]
B7	> 1 μM	(+)[a]	15, 20, 21[a]

[a] Data not shown

IC_{50} values represent the mean ± SEM from 2–3 separate experiments and the potency of VIP-derivatives is compared to VIP at the same dose tested. Positions of biotinyl-ε-aminocaproylation were from mass spectral analysis

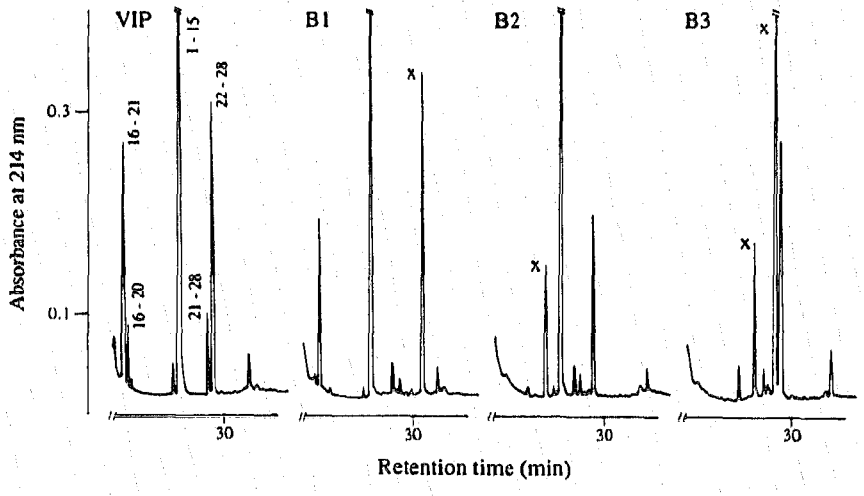

Fig. 3. HPLC chromatograms of endoproteinase Lys-C digest of VIP and the biotinyl-ε-aminocaproylated derivatives B1, B2 and B3. VIP fragments were determined after total amino acid determination. (X) denotes the appearance of a new product.

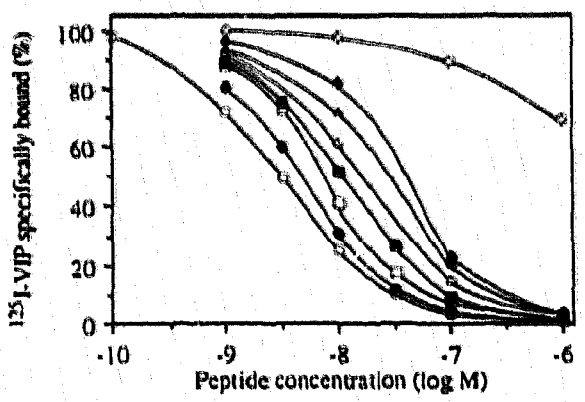

Fig. 4. Competitive inhibition of ^{125}I-VIP binding to pig liver membranes by VIP (○), B1 (□), B2 (■), B3 (●), B4 (▲), B5 (△), B6 (◆) and B7 (◇). Results are expressed as percentage of maximum specific binding in the absence of unlabeled peptides. Data are the mean of 2–3 experiments, and ± SEM values, omitted for clarity, are less than 2%.

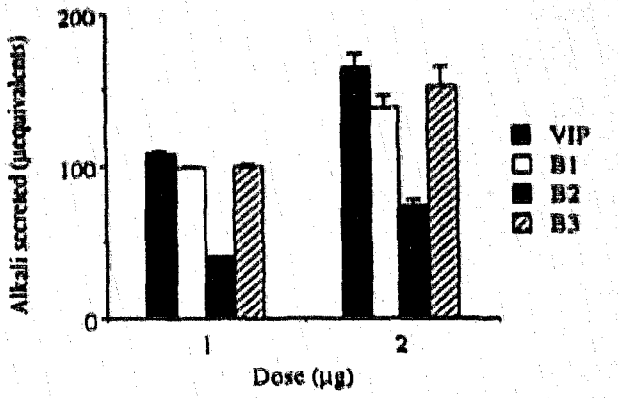

Fig. 5. Stimulation of pancreatic bicarbonate secretion in cat by VIP (■), B1 (□), B2 (▩) and B3 (▨). Data are the mean ± SEM from one (of 2) experiments with 2 observations.

aminocaproylation number (data not shown) indicating that none of the lysine residues is an absolute requirement for a biological response.

4. DISCUSSION

The products of random biotinyl-ε-aminocaproylation of VIP were characterized by combined HPLC and mass spectral analysis that revealed distinct products of VIP mono-, bis-, or tribiotinyl-ε-aminocaproylated at lysine residues. The lack of any detectable biotinyl-ε-aminocaproylation at the amino terminal remains unclear but has also been observed in the biotinylation of human β-endorphin [21] while a parathyroid hormone analog is readily biotinylated at the N-terminal position [22]. The lower reactivity of the amino terminal histidine residue might indicate that this residue is involved in forming the folded structure of the peptide as has been suggested for VIP [23] and for β-endorphin [24]. The ε-amino groups of lysine in VIP are readily biotinyl-ε-aminocaproylated producing analogs that are recognized by the VIP-receptor in pig liver and exert biological activity in the cat pancreas. The position of biotinyl-ε-aminocaproylation at Lys^{15} and Lys^{21} appears to have less influence on VIP–receptor binding as compared to position Lys^{20}. Indeed, when the binding affinity of monobiotinyl-ε-aminocaproylated (B1, B2, B3) and the bisbiotinyl-ε-aminocaproylated (B4, B5, B6) are compared separately, the higher IC_{50} values obtained are attributed to the derivatives biotinylated on Lys^{20} (B2, B4, B5). It is interesting that VIP B1 and B3 both stimulated the cat pancreas with a potency only slightly lower than VIP itself while B2 showed a clearly lower potency suggesting that the Lys^{20} residue is somewhat more critical for binding to the receptor and initiating activity. Whether this is a result of lower affinity for the receptor or an inability to stimulate activation of the second messenger cannot be concluded from these results since it has been suggested that heterogeneity exists for VIP-binding sites in different tissues [25,26]. The difference in binding affinity and biological activity observed can be attributed directly to the modification of lysine residues of VIP. However, it cannot be excluded that these differences result as a consequence of creating a larger lipophilic biotin moiety rather than masking the positively charged ε-amino group of lysine.

The most promising derivative for studies of VIP–receptor interaction is that in which VIP is biotinyl-ε-aminocaproylated in position Lys^{15}, a part of the molecule that seems not to interact with the receptor since it has also been shown that Arg^{14} [27] and Met^{17} [28] are accessible for modification. The biotinylated ligands with a spacer arm of 6 carbons, making them more accessible for a high affinity and stable interaction with avidin, should be useful for the study of receptor-mediated endocytosis using detectable avidin conjugates. Another important use of biotinylated analogs is in the purification of receptor protein(s) via avidin–biotin affinity chromatography.

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