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Expression and characterization of VIP and two VIP mutants in NIH 3T3 cells

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Abstract

Prepro-vasoactive intestinal peptide (preproVIP) was expressed in NIH 3T3 cells, and the preproVIP-derived peptides produced by the cells were analyzed by chromatography combined with sequence-specific radio-immunoanalysis. In accordance with what has previously been reported on processing in non-endocrine cell lines, the VIP precursor was processed poorly in these non-endocrine cells. Mainly an extended form of VIP could be detected in the media from the cells, and no immunoreactivity specific for amidated VIP was found. However, by changing the dibasic cleavage site positioned N-terminal to the VIP sequence in the precursor into the consensus sequence (Arg,X,Lys/Arg,Arg) for the ubiquitous processing enzyme furin, thought to process, e.g. insulin receptors, factor VII, and by deleting residues 156–170 in the VIP precursor, expression of amidated VIP was obtained in this fibroblast cell line. Peptides from the wild-type VIP precursor as well as peptides from the mutated VIP precursor were found to be able to stimulate the adenylate cyclase in cells expressing the VIP receptor.

Key words: Vasoactive intestinal peptide; Prohormone processing; NIH 3T3; Amidation

1. Introduction

Vasoactive intestinal polypeptide (VIP) is a 28 amino sacid neuropeptide with a broad range of biological activities. The precursor for VIP (preproVIP) is synthesized as a 170 amino acid polypeptide, which after removal of the signal peptide is proteolytically processed at mono and dibasic cleavage sites into a N-terminal flanking peptide (preproVIP 22–79), PHM (peptide with N-terminal His and C-terminal Met-amide), preproVIP 111–122, VIP, and a C-terminal flanking peptide (preproVIP 156– 170), as shown in Fig. 1. The dibasic amino acids C- terminal to the VIP and PHM sequences are removed with a carboxypeptidase H-like activity and the C-terminal glycine residue in PHM and VIP is converted by peptidyl-glycine α-amidating monooxygenase (PAM) to amidated PHM and VIP. In some instances the dibasic cleavage site C-terminal to PHM is left uncleaved resulting in a C-terminal extension of PHM to a 42 amino acid peptide, PHV (peptide with N-terminal His and C-terminal Val) (Fig. 1). The ratio of PHM to PHV is 1:1 in gastric antrum and 9:1 in colon and brain, and thus this alternative processing is tissue specific. [1,2]. In the present paper, we report the expression of cDNA encoding preproVIP in a non-endocrine cell line, NIH 3T3. These cells lack secretory granules, and, as expected, the precursor was poorly processed. In an attempt to obtain efficient processing of the cleavage site positioned N-terminal to the VIP sequence in the precursor, the residues at position 121 and 122 were both changed into Arg, resulting in a tetrabasic cleavage site. This corresponds to the consensus cleavage site (Arg,X,Lys/Arg,Arg) for the ubiquitous processing enzyme furin, which processes a number proproteins e.g. the insulin receptor, factor VII and factor IX [3–5]. Previously it was reported that when a C-terminal truncated NPY (NPY-Gly,Lys,Arg) is expressed in non-endocrine cells, the cells synthesize amidated NPY [6]. In order to probe the possibility of expressing amidated VIP in NIH 3T3 cells, a truncated VIP precursor with residues 156–170 deleted was constructed. This truncation was made on the tetrabasic VIP mutant precursor resulting in a VIP precursor with a tetrabasic cleavage site N-terminal to the VIP sequence and with only Gly,Lys,Arg as the C-terminal extension of VIP. With this mutant it was possible to obtain expression of amidated VIP in the NIH 3T3 cell line, indicating that non-endocrine cell lines might be a promising alternative to chemical synthesis for production of amidated or otherwise modified peptide hormones and neuropeptides.

2. Experimental

2.1. DNA construction

The VIP cDNA, (kindly provided by Professor H. Okamoto, Tohuko University School of Medicine, Sendai 980, Japan), lacked the se-

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quences encoding the precursor signal sequence. In order to obtain a full-length VIP cDNA the missing part was obtained by PCR on human genomic DNA. After verification of the PCR fragment by sequence analysis, the fragment was subcloned into the VIP cDNA resulting in a full length cDNA. The 5′ PCR primer introduced at the same time a *Bam*HI restriction enzyme site 5′ to the initiation codon.

To obtain expression of the VIP cDNA in mammalian cells, an expression vector containing the human growth hormone polyadenylation signal and a human ubiquitin promoter was constructed in the following way: the promoter from the human ubiquitin C gene [7] was subcloned as a *Hin*dIII fragment into *Hin*dIII-digested pUC19 resulting in pHD183. A Klenow-treated *Sma*I–*Eco*RI fragment from pHD184 [8] containing the human growth hormone polyadenylation signal was subcloned into a blunt-ended *Eco*RI-digested pHD183. This resulted in the expression vector pBW87. A 1,130 bp *Bam*HI–*Dra*I VIP cDNA fragment was rendered blunt by Klenow polymerase and subcloned into blunt-ended *Bam*HI-digested pBW87 resulting in the preproVIP expression vector pBW91. By using the *Dra*I restriction enzyme site in the VIP cDNA, the CG tailing which arose from the original cloning of the VIP cDNA [9] was excluded. The two mutants: [PV121,122RR]preproVIP (tetrabasic mutant) and [PV121,122RR, Δ156–170]preproVIP (tetrabasic, 156–170-deletion mutant) were introduced by site-directed mutagenesis by the Altered sites in vitro mutagenesis system (Promega, Wisconsin, USA) and the mutagenic primers 5′-AGT GAC GTT TGC GAC GTA CAG GGT CT-3′ for the introduction of arginine in residues 121 and 122, and 5′-TCT TTT TCA TCA CCT CTT TCC ATT-3′ for deletion of residues 156–170.

The deletion was performed on the cDNA encoding the mutant [PV121,122RR]preproVIP, thereby resulting in a double mutant. The introduced mutations were verified by sequence analysis. The mutated cDNAs were subcloned as *Xba*I–*Eco*RI fragments into *Xba*I–*Eco*RI-digested pBW91 resulting in pBW125 encoding PV121,122RR]preproVIP and pBW135 encoding [PV121,122RR;Δ156–170]preproVIP.

2.2. Cell culture and transfection

NIH 3T3 cells from ATCC (MD, USA) were grown at 10% CO_2 in Dulbecco's modified Eagles medium supplemented with 10% newborn calf serum (NCS), 50 IU penicillin and 50 μg/ml streptomycin. The cells were transfected with 20 μg of the peptide expression vector and cotransfected with 2 μg of pSV2neo using the calcium phosphate precipitate procedure [10]. Stable clones were selected in NIH 3T3 medium supplemented with 0.6 mg/ml G418 (Sigma).

24 h cell culture media were collected from a confluent 100 mm Petri dish receiving fresh cell culture media containing only 0.5% newborn calf serum 24 h before harvest.

2.3. Radioimmunoassays

Four different antisera recognizing different peptides in the VIP precursor were used: antiserum Ab 5598 (the general VIP antiserum) recognizing an internal region of VIP and reacting with both C- and N-terminal extended VIP forms; antiserum Ab 5603 which is specific for the amidated C-terminus of VIP, as it does not react with glycine-extended VIP or VIP with a carboxy group at the C-terminus [11,12]; antiserum Ab 7314 recognizing preproVIP 156–170; and antiserum Ab 3668 recognizing both PHM and PHV [1] (see also Fig. 1 for the recognition sites of the antisera).

2.4. Chromatography

2.4.1. Gel filtration. 24 h cell culture media from stable clones were applied to a Sephadex G 50 Superfine column (11 × 1,000 mm), as described [13]. The columns were calibrated with synthetic VIP and preproVIP 156– 170. The fractions were freeze-dried and reconstituted in assay buffer prior to radioimmunoassay. DB 2000 and vitamin B_{12} were used as void volume marker and total volume marker, respectively.

2.4.2. HPLC. The peak fractions of VIP immunoreactive material in the gel filtration profiles of media from NIH- 9-1-E4 or NIH-20-2-E2, were pooled (fractions 51–57 and 52–60 from the gel filtrations of NIH-9-1-E4 and NIH-20-2-E2, respectively). The pools were freeze-dried and reconstituted in H_2O with 0.1% TFA and analyzed by HPLC as described previously [1]. The media from clone NIH-23-3-C1 were applied directly to the column. Gradient elution was done at 30°C with 99% ethanol (Lichrosolv, Merck, Germany) containing 0.1% TFA (solvent B). The elution gradient was isocratically 10% for 2 min, 10–25% solvent B for 4 min, 25–70% for 50 min, 70–80% for 4 min and isocratically for 10 min. The flow rate was 0.5 ml/min, and fractions of 0.5 ml were collected, freeze-dried in a speed vacuum concentrator, reconstituted in assay buffer and analyzed by radioimmunoassay. The column was calibrated in separate runs with synthetic VIP and preproVIP 156–170.

2.4.3. cAMP assay. For cAMP assays a stable CHO clone, CHO-60-1-A6 expressing the rat VIP receptor was used (the rat VIP receptor cDNA was kindly provided by Prof. Nagata, Osaka Bioscience Institute, Osaka, Japan). 10^5 cells/well were seeded in 24-well cell culture dishes, and the following day the cells were washed twice in fresh NIH 3T3 cell culture media containing only 0.5% NCS, stimulated with 0.5, 5 or 50 μl 24-h media from either NIH 3T3, NIH-9-1-E4 or NIH-23-3,C1 cells in the presence of 0.1 mM 1-methyl-3-isobutylxanthine. The cells were stimulated in 0.5 ml NIH 3T3 cell culture media containing 0.5% NCS for 45 min at 37°C. After the incubation the cells were washed twice in Kreb's–Ringer- HEPES buffer, pH 7.4, containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 g bovine serum albumin/100 ml, 1% Bacitracin and 1 mM β-mercaptoethanol. cAMP was extracted by incubating the cells for 20 min on ice in 100 μl 0.4% perchloric acid. After centrifugation, cAMP in the supernatant was quantified using a cAMP radioimmunoassay kit from Amersham (UK).

3. Results

Stable NIH 3T3 clones expressing preproVIP, [PV121,122RR]preproVIP and [PV121,122RR,Δ156–170]preproVIP were established, and the preproVIP derived peptides found in the media were characterized by gel filtration combined with radioimmunoassays specific for preproVIP 156–170 (Ab 7314), and VIP (Ab 5598). Fractions from gel filtrations of the [PV121,122RR,Δ156–170]preproVIP clones were also analyzed with the amide specific VIP antiserum (Ab 5603). In addition, the VIP immunoreactive material was characterized by HPLC combined with radioimmunoassay. For each construct, the gel filtrations were performed on 2–3 different clones, and the HPLC characterizations on at least two different clones.

3.1. Processing of preproVIP in NIH 3T3 cells

Fig. 2 (upper panel) shows a gel filtration profile of the peptides found in the media from a stable clone expressing preproVIP (NIH-9-1-E4). In the preproVIP 156–170 profile, a part of the immunoreactive material eluted as the synthetic standard. The VIP immunoreactivity was eluting earlier than the standard, indicating that VIP is extended, probably at the C-terminus as this peak also reacted with the radioimmunoassay detecting preproVIP 156–170.

To further characterize the VIP immunoreactive material eluting from the gel filtration column, the peak fractions were pooled (peak I, fractions 51–57, Kd 0.36–0.52) and analyzed by HPLC (Fig. 2, lower panel). The material eluted as a single peak reacting both with the VIP and the preproVIP 156–170-specific antisera, indicating that this peak consists of C-terminally extended VIP. No immunoreactive material for PHM or PHV (Ab 3668) could be detected (data not shown).

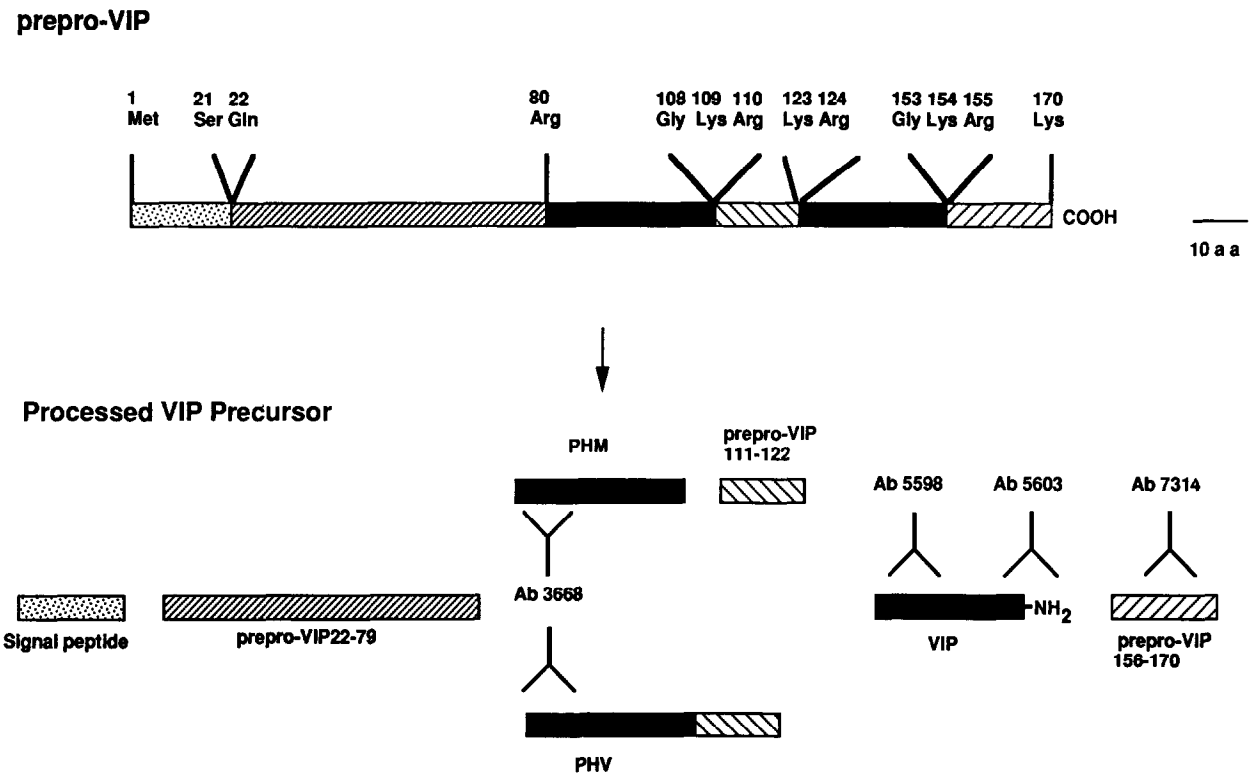

Fig. 1. Schematic representation of preproVIP and the preproVIP derived peptides. The recognition sites of the antisera used are indicated.

3.2. *Processing of [PV121,122RR]preproVIP in NIH 3T3 cells*

The gel filtration profile of a stable clone expressing [PV121,122RR]preproVIP is shown in Fig. 3 (upper panel). The VIP immunoreactive material eluted as one major peak at the same position as the wild-type peak (Fig. 2). However, with the mutant no earlier eluting VIP immunoreactivity was found, indicating that the introduced tetrabasic cleavage site was completely cleaved. As for the wild-type, a part of the preproVIP 156–170 immunoreactive material eluted as the standard. The peak (fractions 52–60, Kd 0.34–0.49) reacting with both VIP (Ab 5598) and preproVIP 156–170-specific antisera were pooled and characterized by HPLC (Fig. 3, lower panel). The elution profiles were similar to the HPLC profiles from the wild-type preproVIP (Fig. 2, lower panel).

3.3. *Processing of[PV121,122RR,Δ156–170]preproVIP in NIH 3T3 cells*

In the gel filtration profiles from clones expressing this mutant, VIP immunoreactive material eluted as the synthetic standard. (Fig. 4, upper panel). Some of this material also reacted with the amide-specific VIP antiserum, demonstrating that a fraction of the VIP immunoreactive material was amidated. As expected no preproVIP 156–170 immunoreactivity was found (data not shown).

To further characterize the VIP immunoreactivity found in the media, the media were applied to HPLC, and the fractions were analyzed with the two VIP antisera, Ab 5598 and Ab 5603 (Fig. 4, lower panel). The Ab 5598 immunoreactive material consisted of two peaks, one co-eluting with the standard and also reacting with the antiserum specific for amidated VIP, and a peak eluting as a more hydrophilic peptide reacting only with the Ab 5598 antiserum. This peak probably consists of VIP C-terminally extended with the residues Gly-Lys-Arg.

3.4. *Biological activity of the expressed peptides*

The biological activity of the VIP immunoreactive material produced by a clone expressing preproVIP (NIH-9-1-E4) and by a clone expressing [PV121,122RR,Δ156–170]preproVIP (NIH-23-3-C1) was studied. The concentrations of immunoreactive material (both Ab 5598 and Ab 5603) in the media from control cells, NIH-23-3,C1 cells and NIH-9-1-E4 cells are shown in Table 1. The control cells expressed no detectable amounts of either Ab 5598 or Ab 5603 immunoreactivity. The two clones produced comparable concentrations of Ab 5598 immunoreactive material (3.6 nM vs. 4.3 nM), whereas the media from NIH-23-3,C1 cells contained approximately a 100-fold higher amount of Ab 5603 immunoreactivity (specific for amidated VIP) than the NIH-9-1-E4 cells (2.7 nM vs. 23 pM).

Addition of 0.5 μl and 5 μl control media to CHO-60-1-A6 cells gave no measurable production of cAMP, and addition of 50 μl control media resulted 1.4 pmol cAMP/10^5 cells (Table 2). In contrast addition of 50 μl media

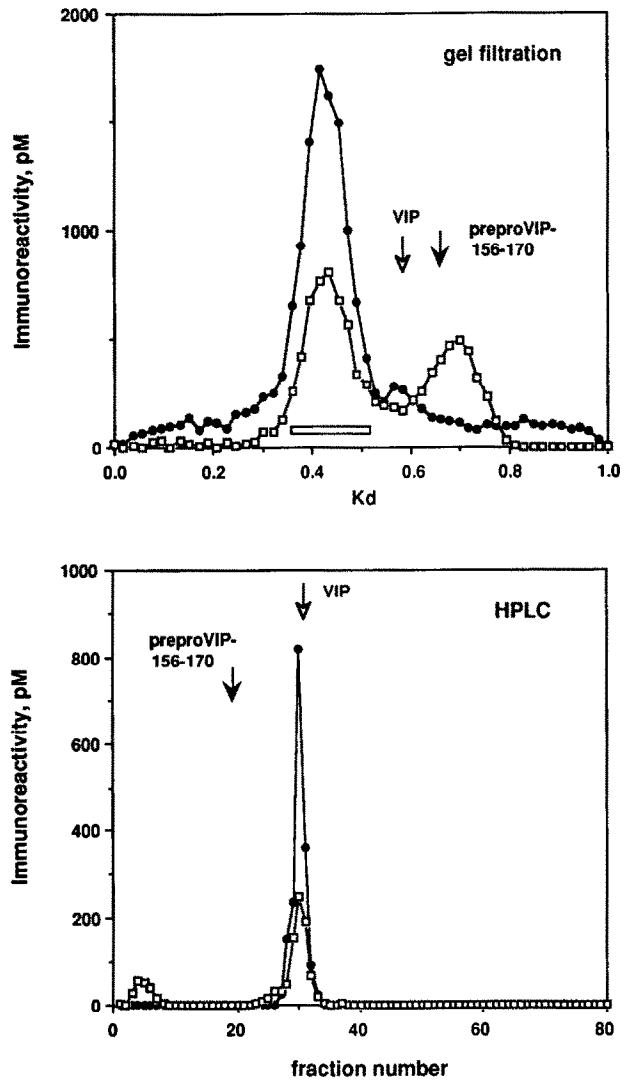

Fig. 2. (Upper panel) Gel filtration profiles of VIP (Ab 5598, ●) and preproVIP 156–170 (Ab 7314, □) immunoreativities in culture medium from cells stably expressing preproVIP. (Lower panel) HPLC analysis of the immunoreative VIP peak in the gel filtrations (fractions pooled are indicated by open rectangle in upper panel). The fractions were analyzed for VIP (Ab 5598) and preproVIP 156–170 (Ab 7314) immunoreactivities. (symbols as above). The elution positions of VIP and preproVIP 156–170 are indicated by open and closed arrows, respectively.

from both clones to the CHO-60-1-A6 cells resulted in a significant increase in cAMP (Table 2), and also 5 μl media from the two clones were able to increase the cAMP production in CHO-60-1-A6 cells.

4. Discussion

When preproVIP is expressed in NIH 3T3 cells, very low amounts of amidated VIP (Table 1) are found in the cell culture media. This demonstrates, as also found for other peptide precursors expressed in fibroblast cell lines [14–16] that these cell lines have no or only limited processing capacity. Nevertheless, when the VIP immunoreactive material is characterized by gel filtration, one major peak is found. This peak also reacts with the preproVIP 156–170 antiserum but not with the PHM/PHV antisera, indicating that the dibasic processing site N-terminal to the VIP sequence is susceptible to some kind of cleavage.

In an attempt to obtain expression of amidated VIP in NIH 3T3 cells two preproVIP mutants were expressed: (i) [PV121,122RR]preproVIP, where the dibasic cleavage site N-terminal to the VIP sequence was changed into a tetrabasic cleavage site (Arg^{121},

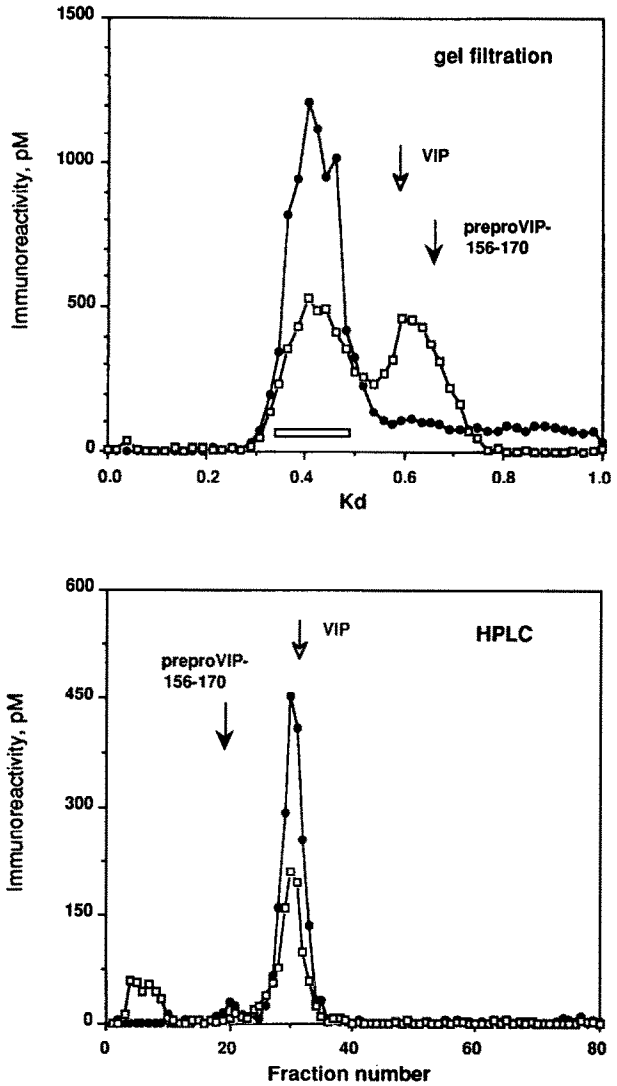

Fig. 3. (Upper panel) Gel filtration profiles of VIP (Ab 5598, ●) and preproVIP 156–170 (Ab 7314, □) immunoreativities in cell culture medium from cells stably expressing [PV121,122RR]preproVIP. (Lower panel) HPLC analysis of the immunoreactive VIP peak in the gel filtrations (fractions pooled are indicated by a rectangle in upper panel). The fractions were analyzed for VIP (Ab 5598) and preproVIP 156–170 (Ab 7314) immunoreactivities. The elution positions of VIP and preproVIP 156–170 are indicated by open and closed arrows, respectively.

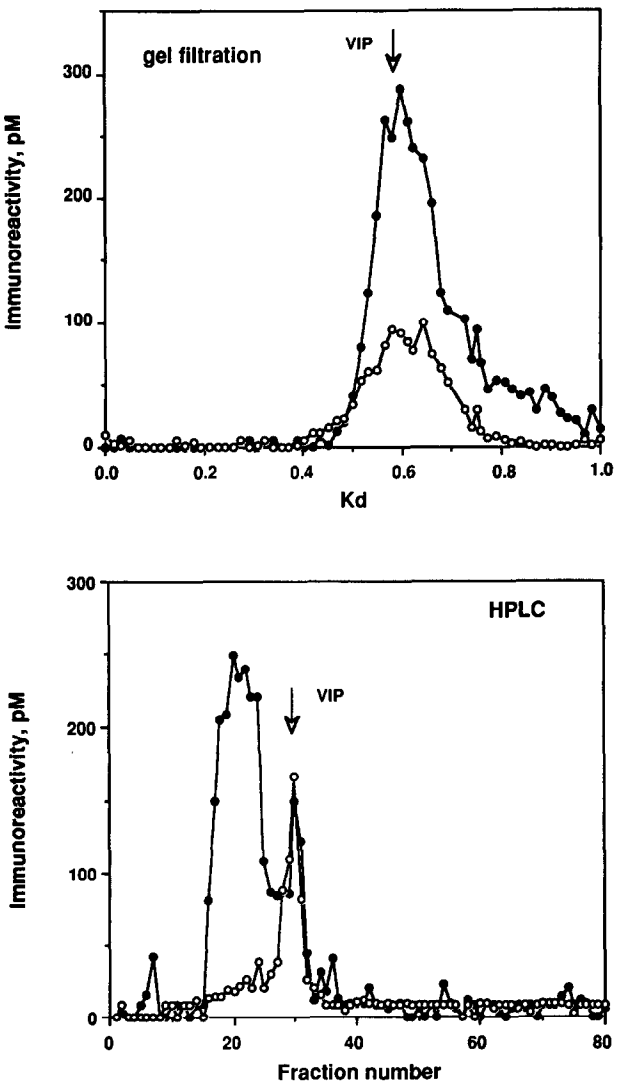

Fig. 4. (Upper panel) Gel filtration profiles of VIP (Ab 5598, ●) and amide specific VIP (Ab 5603, □) immunoreactivities in the cell culture media from cells stably expressing [PV121,122RR,Δ156–170]prepro-VIP. (Lower panel) HPLC analysis of VIP (Ab 5598) and amide-specific VIP (Ab 5603) immunoreactivities in the cell culture media.

Arg^{122},Lys^{123},Arg^{124}), which fits the consensus sequence Arg,X,Lys/Arg,Arg for cleavage by the ubiquitous enzyme furin [17]; (ii) [PV121,122RR,Δ156–170]-preproVIP, a double mutant containing the tetrabasic sequence and with residues 156–170 deleted.

Table 1
VIP immunoreactive material in NIH 3T3, NIH-9-1-E4 and NIH-23-3-C1 as determined by the general (Ab 5598) and the amide-specific VIP antisera

Media from	AB 5598 (pM)	Ab 5603 (0M)
NIH 3T3	< std*	< std*
NIH-9-1, E4	4,337	23
NIH-23-3, C1	3,550	2,650

* Below detection limit.

Table 2
Induction of cAMP production by media from NIH 3T3, NIH-9-1-E4 and NIH-23-3-C1 cells

Media added from	cAMP (pM/10^5 cells) produced after addition of 0.5 μl media ($n = 4$) mean ± S.E.M.	cAMP (pM/10^5 cells) produced after addition of 5.0 μl media ($n = 4$) mean ± S.E.M.	cAMP (pM/10^5 cells) produced after addition of 50 μl media ($n = 4$) mean ± S.E.M.
NIH 3T3	< std	< std	1.4 ± 0.3
NIH-9-1, E4	1.0 ± 0.1	5.2 ± 2.6	23.5 ± 0.5
NIH-23-3, C1	1.1 ± 0.2	12.6 ± 3.3	36.5 ± 3.4

The amount of cAMP produced after addition of, respectively 0.5, 5 or 50 μl media from the three cell lines to cell culture wells containing CHO-60-1-A6 cells in 500 μl media was measured (< std, below detection limit.

The expression of [PV121,122RR]preproVIP in NIH 3T3 cells resulted in efficient cleavage at the cleavage site N-terminal to VIP (Fig. 3 upper panel), but still no VIP immunoreactive material eluting as the synthetic standard on gel filtration was found. The major part of the VIP immunoreactive material from cells expressing [PV121,122RR,Δ156–170]preproVIP eluted as the synthetic standard on gel filtrations, and part of the immunoreactivity also reacted with the amide-specific VIP antiserum (Fig. 4). HPLC analysis of the media showed that the amide-specific immunoreactive material eluted as the synthetic VIP standard. Consequently this cell line contains both carboxypeptidase H-like activity and amidation enzyme. The expression of the amidation enzyme mRNA in NIH 3T3 cells has previously been shown [18].

Although amidated VIP is expressed, the processing is incomplete and the major part of the VIP immunoreactivity detected with Ab 5598 elutes from the HPLC column as a more hydrophilic peptide. This indicates that the peptide exists in an extended form, probably C-terminally extended with Gly-Lys-Arg.

Nevertheless, we have shown that by introducing the appropriate mutations in the peptide precursor, it is possible to synthesize amidated VIP in a non-endocrine cell line.

A minimum length of about 65 amino acids is required for entering the secretory pathway [19,20], and for production of small amidated or otherwise modified peptides in non-endocrine cells, a C- or N-terminal extension of the peptides are necessary. As also showed for the protein C precursor [21], we found that it is possible to alter the dibasic cleavage site in the VIP precursor in such a way that the resulting precursor is efficiently cleaved in non-endocrine cells. The same was found when a tetrabasic mutant of human insulin was expressed in four different non-endocrine cell lines [22]. Besides, we could, by deleting the C-terminal peptide (residues 156–170), obtain amidation of the expressed VIP. It has been suggested that expression of C-termi-

nally deleted peptide precursors could be used for the expression of small amidated peptides in non-endocrine cells [6]. However, this concept could not be directly applied to the expression of VIP as the C-terminally truncated VIP is only partially processed N-terminal to the VIP sequence. Furthermore the length of VIP plus the signal peptide is probably less than the required length to enter the secretory pathway [19], and consequently a N-terminal extension of the peptide is required. Efficient cleavage at this site was obtained by changing the cleavage site N-terminal to VIP into a consensus cleavage site for the ubiquitous processing enzyme furin. Using this method it will be possible to express small modified peptides, e.g. peptides with disulfide bridges, amidated peptides and glycosylated peptides (e.g. endothelin, calcitonin and vasopressin that all contain at least one disulfide bridge), in non-endocrine cell lines, which are generally used for large-scale production. Possibly the processing in these cells can be optimized by engineering the cell line to over-express furin and carboxypeptidase E. Over-expression of furin in COS-7 cells was found to increase the cleavage efficiency in a mutated proinsulin with a tetrabasic cleavage site [23]. Another possibility is to screen a number of non-endocrine cell lines for the expression of furin, carboxypeptidase H and amidating enzyme, and choose a cell line with a suitable enzyme combination.

Media from cells expressing [PV121,122RR,Δ156–170]preproVIP and from cells expressing preproVIP were able to activate the VIP receptor as demonstrated by induction of cAMP production in CHO cells expressing the VIP receptor. The highest amounts of cAMP was produced after addition of 50 μl media from cells expressing [PV121,122RR,Δ156–170]preproVIP (36 pmol/10^5 cells). However, media from the cells expressing the wild-type precursor also significantly increased the cAMP production, (50 μl media from preproVIP expressing cells produced 23 pmol cAMP/10^5 cells). This indicates that VIP extended C-terminally by residues 156–170 is able to bind to and activate the receptor. Although this extended VIP form is not found in tissue, the observation corresponds to the finding that both PHM and its C-terminally extended form, PHV has biological activity [13] and to the finding that both PACAP 27 and PACAP 38 are biologically active [24].

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