The C-terminal part of VIP is important for receptor binding and activation, as evidenced by chimeric constructs of VIP/secretin

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Abstract The structural requirements of vasoactive intestinal polypeptide (VIP) for receptor binding and cAMP production were studied in a cell line stable transfected with the cDNA for rat VIP receptor 1 (rVIPR 1). Using a number of chimeric constructs of VIP and the homologue peptide secretin, it was found that the N-terminal half of VIP (1-11) can be exchanged with the corresponding sequences in secretin with only modest influence on binding and activation, whereas the opposite chimeras with N-terminal VIP and C-terminal secretin were unable to bind to the VIP receptor. The data suggest that the C-terminal region of VIP is important for receptor binding and activation.

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Key words: Vasoactive intestinal polypeptide; Secretin; Receptor binding; Chimera

1. Introduction

Vasoactive intestinal peptide (VIP) is a 28-amino-acid peptide that has a widespread occurrence in neurons of both the central and peripheral nervous system [1]. VIP seems to act as a neurotransmitter or neuromodulator in the brain, and physiological studies have provided evidence that VIP is involved in the nervous control of smooth muscle activity, blood flow, and exo- and endocrine secretion [2]. VIP belongs to the VIP/secretin family of peptides, which contains pituitary adenylate cyclase activating polypeptide (PACAP), PACAP-related peptide, peptide with N-terminal histidine and C-terminal methionine (PHM), glucagon, glucagon-like peptide-1 and -2 (GLP-1 and -2), growth hormone releasing hormone (GHRH) and gastric inhibitory peptide (GIP) as reviewed by Campbell and Scanes [3]. All these peptides have sequence homology, with the highest identity found between VIP and PACAP (68%). The sequence identity between VIP and secretin is 32%. NMR and circular dichroism studies have provided evidence that VIP can be divided into two α-helical regions (residues 9–17 and 23–28) connected by a hinge string [4,5]. The structure of VIP is in good correlation with structural NMR studies on other peptides in the family such as secretin, glucagon, GLP-1, PACAP and GHRH [6–12].

The VIP receptors are seven transmembrane G-protein-coupled receptors belonging to a family consisting of the receptors for VIP, secretin, PACAP, GHRH, GIP, glucagon, GLP-1, calcitonin, parathyroid hormone and corticotropin releasing factor. Until now three types of the rat VIP receptor have been cloned, which are able to bind VIP with different affinity [13–16].

A number of structure–function studies have previously been performed using VIP analogues and various tissue preparations [17–21]. It was found that both the N- and C-terminal ends of the peptides are important for binding and function. Thus deletion of a single amino acid from the N-terminal part resulted in a considerable decrease in binding affinity and receptor activation. Likewise deletion of two C-terminal amino acids caused a significant loss of binding and functional activity. Furthermore, it was demonstrated by Ala-scan that the residues Asp^3, Phe^6, Thr^7, Tyr^{10}, Tyr^{22} and Leu^{23} were important for the interaction with the receptors [20].

Cloning of the receptors has made it is possible to examine the structure–function relationship in mammalian cell lines expressing specifically the receptor of interest, thereby circumventing problems arising from the study of tissues that express more than one VIP receptor type. In order to elucidate which residues in VIP that are important for the interaction between the peptide and the rat VIP receptor 1, a number of chimeric peptides between VIP and secretin were made and the pharmacology of these analogues was studied in a stable transfected cell line expressing the receptor.

2. Materials and methods

2.1. Receptor constructs

The VIP receptor 1 cDNA was obtained from Shigekazu Nagata (Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan). The cDNA was subcloned as a *Hin*dIII fragment into the expression vector pBW120 resulting in pBW190. pBW120 is a derivative of pBW87 [22] where the *Hin*dIII site 3′ to the ubiquitin promoter has been deleted.

2.2. Peptide synthesis

Synthetic VIP was purchased from Peninsula Lab., Inc. (USA). ^{125}I-labelled VIP was prepared by the chloramine-T method to a specific radio activity of 34 Bq/fmol. The sequences of rat VIP, rat secretin and the VIP/secretin chimeric peptides shown in Fig. 1 were synthesised as previously described [23].

2.3. Cell culture and stable transfection

Chinese hamster oocyte (CHO) 591 cells were grown at 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum, 50 IU penicillin and 50 μg/ml streptomycin. The cell were cotransfected with 20 μg pBW190 and 2 μg pSV_2Neo by the calcium phosphate procedure as previously described [23]. Stable clones were selected in CHO medium containing 0.8 mg/ml G418. The clones were analysed for VIP binding and one clone (CHO-60-1A6) was selected for further analysis.

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Abbreviations: rVIPR 1, rat vasoactive intestinal polypeptide receptor 1; CHO, chinese hamster oocyte(s); G-protein, GTP-binding protein

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2.4. Binding of VIP and VIP/secretin analogues

The cells (2×10^5 cells/well in 24-well culture dishes) were washed 3 times in 500 μl of washing buffer (Krebs-Ringer-HEPES, pH 7.4, with 2.5 mM $CaCl_2$, 0.1% bovine serum albumin (BSA), 1 mg/ml bacitracin and 1 mM β-mercaptoethanol). The cells were incubated for 90 min at 37°C with 0.1 nM ^{125}I-labelled VIP in incubation buffer (=washing buffer but with 1% BSA) in the presence of increasing amounts of unlabelled VIP or the chimeric peptides. The incubation was terminated by washing the cells twice in ice-cold washing buffer, after which the cells were harvested by 0.2 M NaOH and counted in a gammacounter. The amount of unspecific bound ^{125}I-labelled VIP was determined as bound ^{125}I-labelled VIP in the presence of 1 μM unlabelled VIP. Binding data were fit to the following 4-parameter 1-step equation: $Y=A+(B-A)/(1+X/IC_{50})$) to obtain the actual IC_{50}, where A is non-specific binding, B is the maximum binding and $X=\log$ VIP(M).

2.5. Intracellular cAMP assay

cAMP assays were performed as previously described [23]. The cells (10^5 cells/well) were stimulated with increasing concentrations of VIP in the presence of 0.1 mM 1-methyl-3-isobuthylxanthine (IBMX). The amount of cAMP produced by the cells was quantified using a cAMP radioimmunoassay kit from Amersham (UK).

3. Results and discussion

3.1. Characterisation of the binding of VIP, secretin and VIP/secretin chimeric peptides

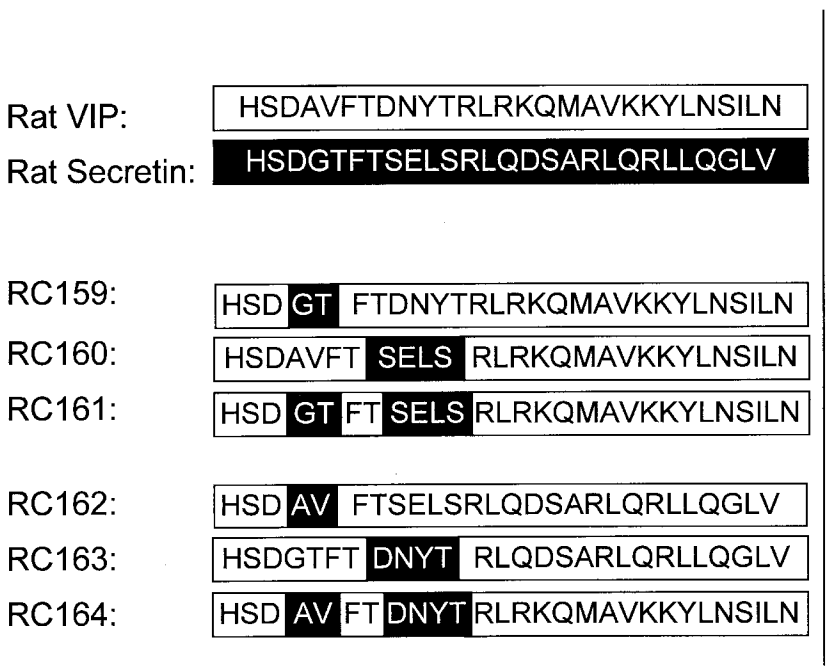
Neither secretin nor the three secretin analogues (RC162, RC163, RC164) could displace radiolabelled VIP from its receptor (Fig. 1). In a previous study [17], secretin was found to displace VIP in membrane preparation from rat cerebral cortex, although at rather high concentrations ($IC_{50}=250$ nM). In another study on a human glioma cell line [19], secretin could only partially displace the VIP radioligand at the highest concentration used (1 μM). These discrepancies could be explained by the inhomogeneous nature of the cortical tissue. Thus the tissue preparation most likely contains other receptor types from the VIP/secretin receptor family that could bind VIP with low affinity. In contrast, the recombinant cell lines (and probably also the glioma cell line) express only one VIP receptor type and this could explain the differences in the ability of secretin to displace the VIP radioligand. The VIP receptor affinity for VIP ($IC_{50}=7.8$ nM) corresponds to previously published results on the cloned VIP receptor 1 expressed in cell lines [13,14].

The binding of VIP and of the three VIP analogues (RC159, RC160, RC161) is shown in Fig. 2. The exchange in VIP of the two amino acids Ala(A)^{4}Val(V)^5 with Gly(G)^{4}Thr(T)^5 from secretin results in a marked decrease in affinity ($IC_{50}=392$ nM, see Fig. 1). The affinity, however, can almost be normalised by introducing four more residues from secretin Ser(S)^{8}Glu(E)^{9}Leu(L)^{10}Ser(S)^{11}. Thus the affinity for this VIP analogue in which the N-terminal half is secretin ($IC_{50}=40$ nM) was found to be only 5-fold lower than that of VIP. Exchanging these four amino acids alone reduced the affinity by 2.5-fold as compared to VIP.

3.2. Adenylate cyclase activation by the peptide analogues

VIP stimulated the formation of cAMP in a dose-dependent manner with a EC_{50} of 0.16 ± 0.02 nM (mean ± SEM). The three VIP analogues stimulated the adenylate cyclase with maximal efficacy demonstrating that they are full agonists in this system (Fig. 3). RC161 and RC160 showed either no changes or only a slight decrease in potency (EC_{50} 0.22 ± 0.07 nM and 0.40 ± 0.12 nM) as compared to VIP, whereas the potency of RC159 was decreased by almost a decade ($EC_{50}=1.0\pm0.5$ nM; see Fig. 3). The observed rank order of functionality corresponds to the rank order of the binding affinities. The observation that for all peptides the IC_{50}s are much lower than the corresponding EC_{50} values can be explained by the presence of spare receptors, which has also been reported by Ciccarelli et al. [24].

Ala-scan of VIP [20] has indicated that Tyr^{10} is an impor-



	IC^{50} ± SEM (nM)	Hill coeffiecient ± SD
Rat VIP:	7.84 ± 1.39 (n=5)	0.97 ± 0.15
Rat Secretin:	ND	
RC159:	392 ± 70 (n=5)	0.93 ± 0.12
RC160:	22.8 ± 5.7 (n=5)	1.02 ± 0.19
RC161:	40.4 ± 6.7 (n=8)	1.02 ± 0.15
RC162:	ND	
RC163:	ND	
RC164:	ND	

Fig. 1. The sequences of rat VIP, rat secretin and the chimeric peptides are shown. VIP residues are presented with black letters on a white background and secretin residues are presented with white letters on a black background. Mean binding affinities (IC_{50}) and Hill coefficients for VIP and VIP analogues to the VIP receptor 1 expressed in CHO cells are given. ND≈no displacement by competitor.

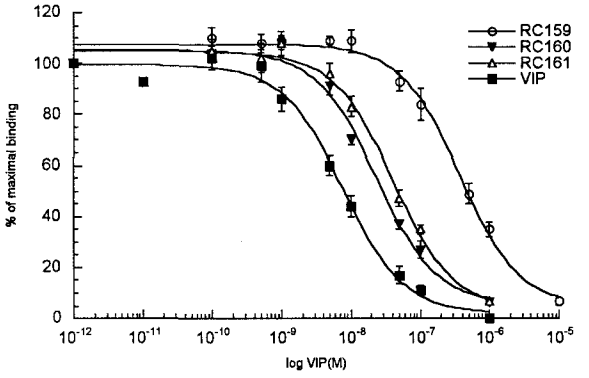


Fig. 2. Competitive binding analysis of VIP, RC159, RC160 and RC161 to VIP receptor 1, stably expressed in CHO cells (CHO-60-1A6). The binding profiles represent the mean ± SEM of 5–8 separate experiments performed in duplicate. IC_{50} values and Hill Coefficients are shown in Fig. 1.

tant residue for the interaction of VIP with its receptor. Our data demonstrate, however, that four amino acids in this area, including Tyr^{10} could be substituted with only a modest effect on binding and activation of the receptor. In contrast the substitution of residues 4 and 5 with Gly and Thr resulted in a pronounced decrease in both binding affinity (50-fold) and potency (10-fold). This loss in activity could be restored by introducing residues 8–11 from secretin, thereby generating an analogue which from residue 1–11 is secretin and from 12–28 is VIP. The finding that the secretin/VIP analogue binds the VIP receptor with only 5-fold lower affinity could indicate that the introduction of Gly and Thr in positions 4 and 5 interferes with the structure of the molecule. The introduction of secretin residues from 8–11 in combination with Gly and Thr restores the structural determinant, which apparently is important for binding. The finding could be explained as follows: by exchanging Val with Thr a hydroxyl-group is introduced, and if the hydroxyl group is unexposed to the solvent, it has to be hydrogen bound with a hydrogen donor in the peptide. The further introduction of secretin sequences $Ser(S)^8Glu(E)^9Leu(L)^{10}Ser(S)^{11}$ satisfies this possible hydrogen binding demand, and the overall structure of the peptide is therefore regenerated, resulting in high affinity for the VIP receptor. Thus disturbances in structural epitopes of the peptides are important elements in the definition of pharmacophoric elements of chimeric peptide studies.

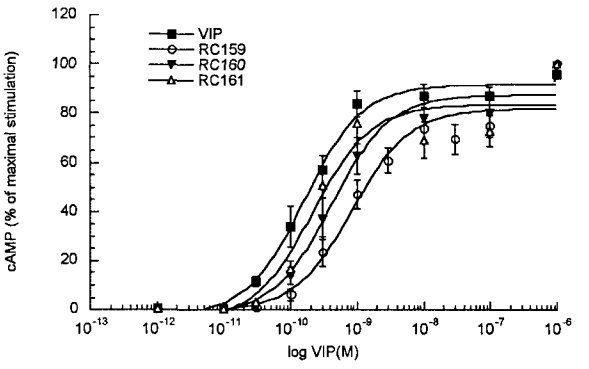


Fig. 3. Activation of adenylate cyclase by VIP, RC159, RC160 and RC161 in intact CHO-60-1A6 cells. Data represent the mean ± SEM of 6–7 experiments.

While the N-terminal half of VIP (residues 1–11) can be exchanged with the corresponding sequence in secretin with only modest influence on binding and functionality, the opposite chimera with N-terminal VIP and C-terminal secretin is unable to bind to the VIP receptor. This finding strongly suggests that the binding epitopes for VIP to its receptor are located in the C-terminal half (residue 12–28). Similar data were obtained using chimeric secretin and PACAP peptides to identify residues involved in binding [25]. Also in GLP-1 the binding epitope is located in the C-terminal half and the entire N-terminal half of GLP-1 could be exchanged with glucagon with only minor decreases in the binding affinity [26].In glucagon, on the contrary, the N-terminal part of the peptide contains the binding epitopes emphasising the importance of studying each member of the VIP/secretin family separately [26].

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References

[1] Hökfelt, T., Schultzberg, M. and Lundberg, L.M. (1989) in: Said, S.I. (Ed.), Vasoactive Intestinal Peptide, Raven Press, New York, pp. 65–90.
[2] Fahrenkrug, J. (1993) Pharmacol. Toxicol. 72, 353–363.
[3] Campbell, R.M. and Scanes, C.G. (1992) Growth Regul. 2, 175–191.
[4] Fry, D.C., Madison, V.S., Bolin, D.R., Greeley, D.N., Toome, V. and Wegrzynski, B.B. (1989) Biochemistry 28, 2399–2409.
[5] Theriault, Y., Boulanger, Y. and St.-Pierre, S. (1991) Biopolymers 31, 459–464.
[6] Clore, G.M., Nilges, M., Brünger, A. and Gronenborn, A.M. (1988) Eur. J. Biochem. 171, 479–484.
[7] Braun, W., Wider, G., Lee, K.H. and Wuthrich, K. (1983) J. Mol. Biol. 169, 921–948.
[8] Thornton, T. and Gorenstein, D.G. (1994) Biochemistry 33, 3532–3539.
[9] Inooka, H., Endo, S., Kitada, C., Mizuta, E. and Fuijno, M. (1992) Int. J. Pept. Prot. Res. 40, 456–464.
[10] Wray, V., Kakoschke, C., Nokihara, K. and Naruse, S. (1993) Biochemistry 32, 5832–5841.
[11] Clore, G.M., Martin, S.R. and Gronenborn, A.M. (1986) J. Mol. Biol. 191, 553–561.
[12] Brünger, A.T., Clore, G.M., Gronenborn, A.M. and Karplus, M. (1987) Prot. Eng. 1, 399–406.
[13] Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K. and Nagata, S. (1992) Neuron 8, 811–819.
[14] Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P.H. and Journot, L. (1993) Nature 365, 170–175.
[15] Lutz, E.m., Sheward, W.J., West, K.M., Morrow, J.A., Gink, G. and Harmar, A.J. (1993) FEBS Lett. 334, 3–8.
[16] Inagaki, N., Yoshida, H., Mizuta, M., Mizuno, N., Fujii, Y., Gonoi, T., Miyazaki, J.-I. and Seino, S. (1994) Proc. Natl. Acad. Sci. 91, 2679–2683.
[17] Staun-Olsen, P., Ottesen, B., Gammeltoft, S. and Fahrenkrug, J. (1986) Peptides 7, 181–186.
[18] Robberecht, P., De Neef, P., Gourlet, P., Cauvinn, A., Coy, D.H. and Christophe, J. (1989) Regul. Pept. 26, 117–126.
[19] Nielsen, F.C., Gammeltoft, S., Westermark, B. and Fahrenkrug, J. (1990) Peptides 11, 1225–1231.
[20] O'Donnell, M., Garippa, R.J., O'Neil, N.C., Bolin, D.R. and Cottrell, J.M. (1991) J. Biol. Chem. 266, 6389–6392.
[21] Fishbein, V.A., Coy, D.H., Hocart, S.J., Jiang, N.-Y., Mrozinski, J.E., Mantey, S.A. and Jensen, R.T. (1994) Peptides 15, 95–100.

[22] Wulff, B.S., Georg, B. and Fahrenkrug, J. (1994) FEBS Lett. 341, 43–48.
[23] Adelhorst, K., Hedegaard, B.B., Knudsen, L.B. and Kirk, O. (1994) J. Biol. Chem. 269, 6275–6278.
[24] Ciccarelli, E., Vilardaga, J.-P., De Neef, P., Paolo, E.D., Waelbroeck, M., Bollen, A. and Robberecht, P. (1994) Regul. Pept. 54, 397–407.
[25] Gourlet, P., Vilardaga, J.-P., De Neef, P., Vandermeers, A., Waelbroeck, M., Bollen, A. and Robberecht, P. (1996) Eur. J. Biochem. 239, 349–355.
[26] Hjorth, S.A., Adelhorst, K., Brogaard, B., Kirk, O. and Schwartz, T.W. (1994) J. Biol. Chem. 269, 30121–30124.