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Fragments of pituitary adenylate cyclase activating polypeptide discriminate between type I and II recombinant receptors

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) analogues were tested for their ability to occupy the recombinant selective PACAP receptors (PACAP type I receptor) or the non-selective PACAP–vasoactive intestinal polypeptide (VIP) receptors (PACAP type II, VIP_1 and VIP_2 receptors) stably transfected and expressed in Chinese hamster ovary (CHO) cells. The synthetic analogues consisted of N- and/or C-terminally shortened peptides. Thus, peptides starting at amino acid 1, 2, 3 or 6 and terminating at amino acid 27, 29, 30, 32 or 38 were compared on the three receptors studied. The shortening of PACAP-(1–38) to PACAP-(1–27) was of little influence. However, in N-terminally deleted peptides the PACAP-38 derivatives were of higher affinity than the PACAP-27 fragments on PACAP type I and PACAP type II, VIP_2 receptors but not on PACAP type II, VIP_1 receptors. The presence of the sequence 28–32 was in all cases sufficient to reproduce the data obtained with the PACAP-38 analogues. PACAP-(3–32) is able to discriminate the PACAP type II, VIP_2 subtype from the other two subtypes, and PACAP-(6–30), PACAP-(6–32) and PACAP-(6–38) can discriminate the PACAP type II, VIP_1 receptors from the other two subtypes. These molecules may help in the quantitative detection of receptor subclasses in complex systems when two or more receptor subtypes are found.

Keywords: Recombinant PACAP receptor; PACAP (pituitary adenylate cyclase activating polypeptide) analog

1. Introduction

Binding and in vitro functional data (Christophe, 1993; Shivers et al., 1991) suggest a classification of the receptors for the neuropeptide, vasoactive intestinal polypeptide (VIP), and pituitary adenylate cyclase activating polypeptide (PACAP) into two main classes: (1) the PACAP type I receptors recognize PACAP-27 and PACAP-38 with an equal high affinity, and VIP with a 300- to 1000-fold lower potency (these are the selective PACAP receptors); (2) the PACAP type II receptors recognize with a similar high affinity PACAP-27, PACAP-38 and VIP (these are non-selective VIP/PACAP receptors or VIP receptors).

The PACAP type I receptors were cloned simultaneously by different groups (Hosoya et al., 1993; Pisegna and Wank, 1993). Spengler et al. (1993) and Svoboda et al. (1993) discovered independently the existence of variants resulting from alternative splicing of the mRNA. The two major forms were called the normal PACAP receptor (the shortest form) and the HOP_1 PACAP receptor (with a longer third intracellular loop connecting the transmembrane helices 5 and 6). The PACAP type II receptor was cloned from rat lung (Ishihara et al., 1992) and from human colon cells (Sreedharan et al., 1993). This receptor may be considered as the classical VIP receptor described in lung, intestine, pancreas, liver and brain and is presently known as the VIP_1 receptor. A second VIP receptor was cloned by Lutz et al. (1993) from rat brain and was called the VIP_2 receptor. The mouse (called by the authors the PACAP type III receptor (Inagaki et al., 1994)) and the human VIP_2 receptors (Svoboda et al., 1994) have also been cloned. It is likely that the human VIP_2 receptor corresponds to the so-called

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'helodermin-preferring receptor' we described in the human lymphoblastic cell line SUP-T1 (Robberecht et al., 1988).

We transfected the recombinant rat PACAP type I receptor (the normal form), the recombinant rat PACAP type II, VIP_1 receptor and the recombinant human PACAP type II, VIP_2 receptor in Chinese hamster ovary cells (CHO cells) and obtained cell lines expressing stably the receptors. As the CHO cells did not express constitutively PACAP or VIP receptors, the transfected cells constitute a unique model to compare the requirements for occupancy of each receptor type. We tested in the present study the binding properties of N- and C-terminally shortened PACAP-38 derivatives to the three classes of PACAP receptors.

2. Materials and methods

2.1. Transfection, selection and maintenance of the cell lines

The DNA coding for the PACAP type I receptor (Ciccarelli et al., 1995), the PACAP type II, VIP_1 receptor (Ciccarelli et al., 1994) and the PACAP type II, VIP_2 receptor (Svoboda et al., 1994) was cloned into a mammalian expression factor containing the selectable neomycin phosphotransferase gene. The resulting recombinant plasmid was transfected into the CHO cell line DG44 by electroporation using a gene pulser. The selection of the clones as well as their main characteristics have already been published.

Cells were maintained in α-minimal essential medium (αMEM), supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin with an atmosphere of 95% air, 5% CO_2 at 37°C. Geneticin (0.5 mg/ml) was kept in the culture medium of the stock culture. Subcultures prepared for membrane purification were done in a medium without geneticin.

The cell clones used were referred to as PACAP-r cl 2–10 (PACAP type I receptor, receptor density of 4.6 pmol/mg protein), VIP_1-r cl 3 (PACAP type II, VIP_1 receptor, receptor density of 0.85 pmol/mg protein) and VIP_2-r cl 11 (PACAP type II, VIP_2 receptor, receptor density of 0.2 pmol/mg protein).

2.2. Membrane preparation and receptor identification

Cells were harvested with a rubber policeman and pelleted by low-speed centrifugation; the supernatant was discarded and the cells lysed in 1 mM $NaHCO_3$ solution and immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4°C for 10 min at $400 \times g$, and the supernatant was further centrifuged at $20\,000 \times g$ for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$ was used immediately as a crude membrane preparation. Tracers were prepared by the iodogen method, purified by adsorption on a Sep-Pak Cartridge and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid. [^{125}I]VIP and [^{125}I]Ac-His^1-PACAP-27 had a specific radioactivity of 0.7 and 0.5 mCi/nmol, respectively. The affinity of [^{125}I]VIP for the PACAP type II, VIP_1 receptor was 0.5 ± 0.1 nM (Ciccarelli et al., 1994); the affinity of [^{125}I]Ac-His^1-PACAP-27 for the PACAP type I receptor was 14.2 ± 2.0 nM (Ciccarelli et al., 1995). Tracer affinity for the PACAP type II, VIP_2 receptor was not determined as we were unable to saturate the receptors by increasing the concentration of the radioligand. This suggested that the tracer concentration used was negligible when compared to the tracer K_d value.

In all cases, non-specific binding was defined as the residual binding in the presence of 1 μM of the unlabelled peptide corresponding to the tracer. Binding was performed at 37°C in a 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1% bovine serum albumin (pH 7.4) buffer. Bound radioactivity was separated from free by filtration through glass fiber filters GF/C presoaked for 24 h in 0.1% polyethyleneimine and rinsed 3 times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1% bovine serum albumin.

2.3. Peptide synthesis

All peptides were synthesized as C-terminal amides by solid phase methodology on a manual LKB-Biochrom-Biolynx apparatus (Cambridge, UK) using the Fmoc strategy (9-fluorenylmethoxy carbonyl). The apparatus worked by continuous flow synthesis: the Nova Syn KR 100 resin (for peptide amides) (Novabiochem, Läufelfingen, Switzerland) and fluoren-9-yl-methoxy carbonyl-labelled amino acids activated with *N*-hydroxy benzotriazole and *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (Ambrosius et al., 1989) were used. This system was necessary to synthesize parent peptides with different C-terminals. Five columns were introduced sequentially in the flow system to initiate the synthesis at positions 38, 32, 30, 29 and 27 of the PACAP molecule. The synthesis was continued with the five coupled columns. An aliquot of resin-bound peptide from each column was picked up after coupling of the PACAP residues 6, 3 and 2.

The peptides were cleaved for 3 h with a cleavage mixture containing trifluoroacetic acid 82%, phenol 4%, ethanedithiol 2%, thioanizole 4%, H_2O 4% and triisopropyl silane 4%. After partial trifluoroacetic acid evaporation, the peptides were precipitated with 10 volumes of cold ether. They were purified by reverse-phase chromatography on Jordi-Gel DVB 300 Å (10 × 1 cm) and by ion exchange chromatography on Mono S HR 5/5. Peptide purity was assessed (> 95%) by capil-

lary electrophoresis and sequence conformity was verified by sequencing.

3. Results

The affinity of the PACAP analogues was determined by competition curves analysis. The results were expressed as IC_{50} values, i.e. the concentration of unlabelled peptide that inhibited by half the binding of the tracer. The tracer concentrations were calculated to be 0.2 nM and 0.12 nM for, respectively, [^{125}I]Ac-His^1-PACAP-27 and [^{125}I]VIP. Considering the K_d for the tracers reported in the Materials and methods section, the IC_{50} values for the tested peptides were close to their K_d values. Eleven peptide concentrations were tested in a concentration range from 10^{-10} to 10^{-5} M. Computer analysis of the competition curves done with the LIGAND program revealed that the best fit was obtained in all cases assuming the existence of a single class – or state – of receptor.

Table 1
IC_{50} values (in nM) of binding of VIP, PACAP-(1–27), PACAP-(1–38) and fragments on PACAP type I receptor, PACAP type II, VIP_1 receptor; PACAP type II, VIP_2 receptor

Peptide tested	IC_{50} (nM)		
	PACAP type I-R	PACAP type II-R VIP_1	PACAP type II-R VIP_2
VIP	1000	3	3
PACAP-(1–27)	3	2	5
PACAP-(1–29)	2	1	1
PACAP-(1–30)	2	1.5	3
PACAP-(1–32)	2	1.5	3
PACAP-(1–38)	4	2	1
PACAP-(2–27)	100	80	50
PACAP-(2–29)	200	150	50
PACAP-(2–30)	100	100	50
PACAP-(2–32)	40	30	10
PACAP-(2–38)	20	40	20
PACAP-(3–27)	5000	2000	300
PACAP-(3–29)	2000	1000	200
PACAP-(3–30)	500	1000	40
PACAP-(3–32)	400	1000	40
PACAP-(3–38)	200	800	100
PACAP-(6–27)	1500	600	300
PACAP-(6–29)	300	600	200
PACAP-(6–30)	40	300	40
PACAP-(6–32)	30	300	40
PACAP-(6–38)	30	600	40

The IC_{50} values were established, using the LIGAND program, from inhibition curves of tracer binding by increasing concentrations of unlabelled peptides. The results were the means of 3 determinations and the standard deviation (S.D.) was less than 0.1 log unit in all cases. The tracer used for identification was [^{125}I]Ac-His^1-PACAP-27 for the PACAP type I and PACAP type II, VIP_2 receptors and [^{125}I]VIP for the PACAP type II, VIP_1 receptor.

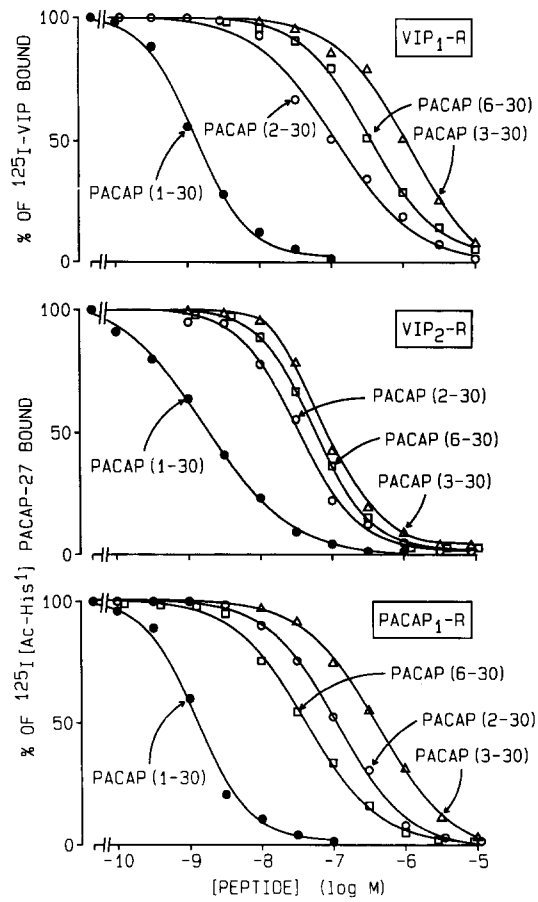

Fig. 1. Inhibition of [^{125}I]VIP binding to membranes from CHO cells expressing the PACAP type II, VIP_1 receptor (upper panel) and of [^{125}I]Ac-His^1-PACAP-27 binding to membranes from CHO cells expressing the PACAP type II, VIP_2 receptor (middle panel) and PACAP type I receptor (lower panel) by PACAP-(1–30) (●), (2–30) (○), (3–30) (△), and (6–30) (□). The results were the means of three determinations.

All the data are summarized in Table 1 and typical curves are presented in Fig. 1.

4. Discussion

The present work had two main objectives: (1) to precisely define the role of the PACAP-(28–38) C-terminal sequence in PACAP receptor occupancy; (2) to compare the ability of N- and C-terminally shortened PACAP molecules to occupy the three recombinant PACAP receptors: the selective PACAP type I receptors and two non-selective PACAP type II receptors.

Previous data obtained for membrane preparations from rat and human cell lines (Robberecht et al., 1992a,b) and tissues (Hou et al., 1994) containing a majority of PACAP-selective receptors (PACAP type I receptor) have shown that, although PACAP-27 and

PACAP-38 have a similar affinity for the receptors, the N-terminally shortened analogues of PACAP-38 have a higher affinity than the analogues derived from PACAP-27. The present work confirmed this finding (compare the IC_{50} values of PACAP-(1–27) and PACAP-(1–38), PACAP-(2–27) and PACAP-(2–38), PACAP-(3–27) and PACAP-(3–38), PACAP-(6–27) and PACAP-(6–38) in Table 1 on the PACAP type I receptor) and delineated the amino acids of the PACAP-(28–38) extension involved in this effect.

The affinities of PACAP-(1–27), PACAP-(1–29), PACAP-(1–30), PACAP-(1–32) and PACAP-(1–38) for the PACAP type I receptor were similar. After deletion of His^1, PACAP-(2–32) and PACAP-(2–38) were equally potent, but 3- to 10-fold more potent than PACAP-(2–27), PACAP-(2–29) and PACAP-(2–30). After deletion of His^1 and Ser^2, there was a sharp contrast between the potency of fragments (3–38), (3–32) and (3–30) (200–500 nM) and that of fragments (3–29) and (3–27) (2000–5000 nM). After deletion of the N-terminal sequence His^1, Ser^2, Asp^3, Gly^4, $Ileu^5$, the fragments (6–38), (6–32) and (6–30) were equally potent (30–40 nM), but 10-fold more potent than fragment (6–29) and 50-fold more potent than fragment (6–27).

Thus, the increased affinity observed with the N-terminally truncated PACAP-38 analogues was already detectable with the PACAP-32 or PACAP-30 fragments, suggesting that the addition of at least two basic residues markedly increased the affinity of these truncated peptides. PACAP-(6–38) is already known to be a PACAP receptor antagonist. We found in this work (data not shown) that PACAP-(6–27), PACAP-(6–29), PACAP-(6–30) and PACAP-(6–32) had no detectable intrinsic activity on adenylate cyclase activation.

PACAP-(1–27), PACAP-(1–29), PACAP-(1–30), PACAP-(1–32) and PACAP-(1–38) were not discriminated by the non-selective PACAP type II, VIP_1 receptor (recognizing VIP and PACAP with the same affinity). Deletion of His^1 reduced 20- to-100 fold the peptide affinities. Although the affinity of PACAP-(2–32) and PACAP-(2–38) was higher than that of PACAP-(2–27), PACAP-(2–29) and PACAP-(2–30), the differences between the two groups of peptides was less marked than on the PACAP type I receptor. The fragments (3–27) to (3–38) were about 1000-fold less potent than the complete molecules. There was no major difference between the various C-terminally shortened peptides. A similar observation was made for the (6–27) to (6–38) analogues although their potency was higher than that of the (3–27) to (3–38) molecules. Thus, at variance with what was observed on the PACAP type I receptor, the occupancy of the PACAP type II, VIP_1 receptor was only slightly affected by the C-terminal sequence 28–38.

The PACAP type II, VIP_2 receptor discriminates PACAP-27 from PACAP-38 and may be considered as a PACAP-38 preferring-receptor (IC_{50} PACAP-38: 1.0 nM; IC_{50} PACAP-27: 5 nM). This observation was also valid for the PACAP fragments since PACAP-(2–38), (3–38) and (6–38) had a lower IC_{50} than the corresponding PACAP-27 fragments. Like the occupancy of the PACAP type I receptor, but at variance with the PACAP type II, VIP_1 receptor, the fragments (3–30), (3–32), (6–30) and (6–32) were as potent or even more potent than the corresponding PACAP-38 fragments on the PACAP type II, VIP_2 receptor.

Based on the comparison of several analogues (Robberecht et al., 1992a,b; Hou et al., 1994; Clemens et al., 1992), we suggested that PACAP-(1–38) binding to the PACAP type I receptor was best explained by three anchorage areas: the first in the N-terminal triplet, the second in the middle (11–27) portion (particularly in the C-terminal part of the PACAP-27 molecule) and the third in the (28–38) extension. We also suggested that two anchorage areas are sufficient to generate high affinity binding. This interpretation may be extended to the PACAP type II, VIP_2 receptor. Furthermore, the third anchorage point may probably be more precisely positioned in the 28–32 portion of the molecule as all the fragments having the C-terminal sequence 28–32 were as potent as those having the 28–38 end. As this portion of the molecule is not supposed to influence the general configuration of the PACAP molecule (Wray et al., 1993) it is likely that the positive charges in the sequence Gly-Lys-Arg-Tyr-Lys-NH_2 interact directly with the receptor. The corresponding receptor binding site is not yet established.

PACAP type I and type II receptors may be coexpressed in the same tissues (Suda et al., 1992; Usdin et al., 1994) or in the same cells (Vertongen et al., 1994). The contribution of each receptor subtype to the overall response is difficult to establish. Molecules like PACAP-(3–30) and PACAP-(3–32) that discriminated between the three receptor types might be useful. Similarly, the fragments (6–30), (6–32) and (6–38) might be helpful for discriminating the two VIP receptors.

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