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The activation of adenylate cyclase by pituitary adenylate cyclase activating polypeptide (PACAP) via helodermin-preferring VIP receptors in human SUP-T1 lymphoblastic membranes

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Competition binding curves, using [^{125}I-acetyl-His^1]PACAP-27 as radioligand and dose-effect curves of adenylate cyclase activation in human SUP-T1 lymphoblastic membranes showed that PACAP-27 and PACAP-38 stimulate the enzyme through a single class of helodermin-preferring VIP receptors with the following order of potency: helodermin = [acetyl-His^1]PACAP-27 > PACAP-38 > PACAP-27 > VIP. PACAP (6–27) (K_i 0.5–0.8 μM) and [Des-His^1, Asn^3]PACAP-27 (K_i 1–2 μM) acted as competitive antagonists. Using a series of 13 PACAP-27 analogues and fragments and three VIP analogues, we identified positions 1, 2, 3, 9 and 13 in PACAP-27 as being of importance for high-affinity binding. Thus, we added further evidence for considering that the present helodermin-preferring VIP receptors, when compared to a majority of VIP receptors and PACAP receptors, exhibit an original specificity pattern.

Introduction

The unusual VIP receptor of the human lymphoblastic cell line SUP-T1 displays similar affinity for VIP and PHI, a higher affinity for the parent peptide helodermin and no affinity for secretin and GRF [1]. It also shows remarkable high affinity for [Phe^1]VIP but rather low affinity for VIP analogues monosubstituted in position 1 to 4 by the corresponding D-amino acid [1,2]. The fragment VIP(10–28) does not recognize this receptor [2]. On a functional point of view, all VIP analogues capable to occupy this receptor activate adenylate cyclase so that, up to now, no antagonist is available. These pharmacological properties are clearly different from those of most VIP receptors and we classified the SUP-T1 lymphoblastic receptor as a 'helodermin-preferring' VIP receptor [2–4]. A similar receptor was described recently in human small cell lung carcinoma cell lines [5].

Lately, Miyata et al. isolated from ovine hypothalamus two novel peptides closely related to VIP: the amidated 38-residue Pituitary Adenylate Cyclase Activating Polypeptide (PACAP-38) and its N-terminal amidated 27-residue derivative (PACAP-27) [6,7]. Their N-terminal portion has 68% similarity with VIP. Identical peptides are present in man and rat. PACAP-27 and PACAP-38 interact with high affinity with VIP receptors in liver [8], pancreas [9] and lung membranes [10] from rat. Besides, they display also high affinity towards other selective receptors (clearly distinct from VIP receptors) in rat hepatic membranes [8], in various areas of the rat central nervous system [10], in cultured

Abbreviations: PACAP-38 and PACAP-27, pituitary adenylate cyclase activating peptides in, respectively, the long version (the amidated 38-residue peptide (1–38)) and the N-terminal amidated 27-residue short version (1–27); VIP, vasoactive intestinal peptide; K_d, concentration required for half-maximal occupancy of binding sites with tracer; IC_{50}, concentration required for half-maximal inhibition of tracer binding; EC_{50}, concentration exerting half-maximal stimulation of adenylate cyclase; K_i, concentration required for half-maximal inhibition of adenylate cyclase stimulation; I.A., intrinsic activity (efficacy), considering the maximal effect of PACAP-27 as 1.

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rat astrocytes [11], a rat cell culture derived from an adrenal pheochromocytoma [12], the rat cancerous pancreatic acinar cell line AR 4-2J [13], and the human neuroblastoma cell line NB-OK [14].

In the present work we examined the interaction of both PACAPs with human SUP-T1 lymphoblastic membranes and observed that PACAP-27 and PACAP-38 bound to the helodermin-preferring VIP receptor and activated adenylate cyclase through this receptor. The N-terminal part of PACAP was found to contribute greatly to the efficient activation of receptors. The PACAP fragments PACAP (6–27) and PACAP (7–27) and the PACAP analogue [Des-His^1,Asn^3]PACAP-27 acted as antagonists of the PACAP receptor-mediated effect.

Materials and Methods

Cell culture and crude membrane preparation

The SUP-T1 cell line was cultured at 37°C in RPMI medium supplemented with 5% (by vol.) fetal calf serum (from Gibco). Stock cultures were diluted every three days with 4 volumes of fresh medium. Cells were harvested by centrifugation at 100 × *g* for 10 min, lyzed in hypotonic 1 mM $NaHCO_3$ (pH 7.0) then quickly frozen in liquid nitrogen. After thawing and centrifugation of the lysate at 2000 × *g* for 10 min at 4°C, the resulting supernatant was centrifuged at 10000 × *g* for 15 min. The final pellet was homogenized in 1 mM $NaHCO_3$ and immediately used for binding assay and adenylate cyclase assay.

Radioiodination and purification of tracers

PACAP-27 and [acetyl-His^1]PACAP-27 were radioiodinated by the chloramine T method as previously described [13,14] then purified by HPLC. The radioiodination reaction was stopped by dilution, and the mixture immediately injected into a reverse-phase 0.39 cm × 30 cm μBondapak C_{18} column (Waters Associates, Milford, MA, U.S.A.) equilibrated in solvent A (0.1% trifluoroacetic acid, 5% CH_3CN). Elution was performed with a flow rate of 1 ml/min^{-1} with a 0–35% linear gradient of solvent B (0.1% trifluoroacetic acid, 80% CH_3CN) over 5 min, followed by a 35–75% linear gradient of solvent B over 40 min. Free ^{125}I and unlabelled peptides were separated from three or four peaks of trichloroacetic acid-precipitable ^{125}I. The major radioactive peak (in each case the second peak) showed the highest capacity to bind to SVP-T1 membranes, after CH_3CN evaporation. The residue was diluted in 10 mM sodium phosphate (pH 7.4) and 150 mM NaCl, containing 0.02% Tween 20 to avoid peptide adsorption. The tracer was stored at −20°C. The specific radioactivity of both tracers was typically 2 mCi/nmol.

Binding of [^{125}I]PACAP-27 and [^{125}I-acetyl-His^1]PACAP-27 to SUP-T1 membranes

Binding was performed in 50 mM Tris-maleate buffer (pH 8.0) containing 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 1% (mass/vol.) bovine serum albumin, 5–10 pM tracer, increasing concentrations of unlabelled peptide and approx. 20 μg membrane protein, in a total volume of 120 μl. Incubations were conducted at 37°C for 20 min to allow equilibrium of binding and terminated by dilution with 2 ml ice-cold 0.1 M sodium phosphate buffer (pH 7.4), followed by rapid filtration through glass-fiber filters (GF/C, Whatman, Maidstone, Kent, U.K.) soaked in 0.1% poly(ethyleneimine) for at least 24 h. The filters were rinsed three times with 2 ml of the same buffer, and their radioactivity was measured in a multichannel LKB Gammacounter. Non-specific binding of [^{125}I]PACAP-27 and [^{125}I-acetyl-His^1]PACAP-27 was determined in the presence of 0.1 μM PACAP-27 and accounted for approximately 8% and 4%, respectively, of the total radioactivity added.

Adenylate cyclase assay

The assay was performed according to the procedure of Salomon et al. [15]. Membrane protein, used at the same concentration as in the binding assays, was incubated in a total volume of 60 μl containing 0.5 mM [α-^{32}P]ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho*enol*pyruvate, 30 μg/ml pyruvate kinase and 30 mM Tris-HCl, at a final pH of 7.5. The reaction was initiated by adding membranes, and was terminated after a 12-min incubation at 37°C by adding 0.5 ml of a 0.5% sodium dodecyl sulfate solution containing 0.5 mM ATP, 0.5 mM cAMP and 20000 cpm [8-^{3}H]cAMP (to determine cAMP recovery). cAMP was separated from ATP by two successive chromatographies on Dowex 50 W-X8 and neutral alumina.

Peptides and other chemicals

Synthetic helodermin, VIP and PACAP-38 were from Novabiochem (Laüfelfingen, Switzerland). All PACAP analogues and fragments were synthesized with an Automated Applied Biosystem apparatus (Foster City, CA, U.S.A.) using the Fmoc strategy: a 4-(2',4'-dimethoxyphenylfluoren-9-ylmetoxycarbonylamino-methyl)phenoxy resin and fluoren-9-ylmethoxycarbonyl labelled amino acids activated with *N*-hydroxybenzotriazole and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate [16] were used. The peptides were purified by HPLC and their conformity established by both total amino acid composition and Edman degradation in a 477 A sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The different sequences are presented in Table I.

All reagents for peptide synthesis were purchased from Novabiochem. The origin of the reagents used for peptide radioiodination, adenylate cyclase assay and binding assay is detailed in Ref. 17.

Results

1. General characteristics of PACAP binding sites

PACAP binding sites were characterized by the binding of [^{125}I]PACAP-27 and [^{125}I-acetyl-His^{1}]PACAP-27. The binding of both tracers was rapid at 37°C and reached steady state after 20–30 min then persisted for at least 60 min. It was saturable with complete inhibition of tracer binding in the presence of 0.3 μM unlabelled PACAP and reversible as studied by isotopic dilution. Furthermore tracer dissociation was markedly accelerated by addition of 10 μM GTP. Data on [^{125}I-acetyl-His^{1}]PACAP-27 binding are presented in Fig. 1. This tracer was used in all further experiments as it showed higher affinity and a higher ratio of total binding over non specific binding than [^{125}I]PACAP-27. Tracer binding was inhibited by PACAP-27, PACAP-38, VIP and helodermin (Fig. 2) but not by secretin, human growth hormone releasing factor, glucagon or unrelated peptides such as substance P, neurotensin, somatostatin or cholecystokinin each at 1 μM. When testing the specificity of binding sites with synthetic analogues and PACAP fragments, [acetyl-His^{1}]PACAP-27 showed a higher affinity than PACAP-27 (Fig. 3) and [^{125}I-acetyl-His^{1}]PACAP-27

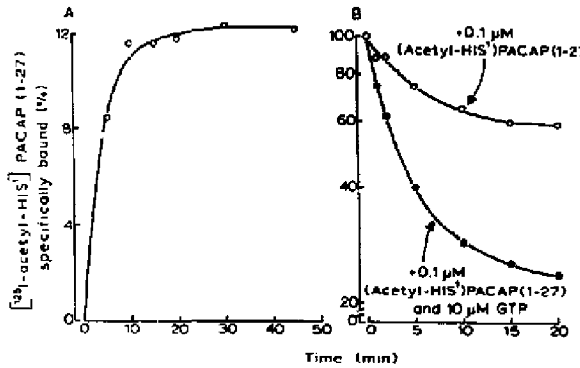

Fig. 1. Association (A) and dissociation (B) kinetics at 37°C of [^{125}I-acetyl-His^{1}]PACAP(1–27) on membranes from human SUP-T1 lymphoblasts. The results were the means of experiments performed in duplicate on three different membrane preparations. For association kinetics the tracer bound was expressed in % of the tracer offered. Dissociation was induced by adding, after a 20 min preincubation with tracer, 0.1 μM unlabelled [acetyl-His^{1}]PACAP-27 either alone (○) or with (●) 10 μM GTP. The results were expressed in % of tracer bound before addition of the unlabelled ligand.

proved to be a better probe than ^{125}I-PACAP itself as it showed higher affinity and allowed a higher ratio of total binding over non specific binding (data not shown). Therefore, further data were obtained with this tracer whose association and dissociation rates are illustrated in Fig. 1.

TABLE 1

Sequences of amino acids for PACAP(1–38), PACAP(1–27), VIP, helodermin, VIP analogues, PACAP analogues and fragments

All peptides except helodermin are amidated (*).

Peptide	1 5 10 15 20 25 30 35
PACAP(1–38)	H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-G-K-R-Y-K-Q-R-V-K-N-K-*
PACAP(1–27)	H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
VIP	H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*
Helodermin	H-S-D-A-I-F-T-E-E-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-A-S-I-L-G-S-R-T-S-P-P-P-S
[Ser^{9}]VIP	H-S-D-A-V-F-T-D-S-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*
[Tyr^{13}]VIP	H-S-D-A-V-F-T-D-N-Y-T-R-Y-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*
[Ser^{9},Tyr^{13}]VIP	H-S-D-A-V-F-T-D-S-Y-T-R-Y-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*
PACAP(2–27)	S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
PACAP(3–27)	D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
PACAP(5–27)	I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
PACAP(6–27)	F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
PACAP(7–27)	T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Acetyl-His^{1}]PACAP(1–27)	AC-H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Phe^{1}]PACAP(1–27)	F-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Ala^{2}]PACAP(1–27)	H-A-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Asn^{3}]PACAP(1–27)	H-S-N-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Glu^{3}]PACAP(1–27)	H-S-E-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Des-His^{1},Ala^{2}]PACAP(1–27)	- A-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Des-His^{1},Asn^{3}]PACAP(1–27)	- S-N-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
[Des-His^{1},Glu^{3}]PACAP(1–27)	- S-E-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-*
	1 5 10 15 20 25 30 35

2. Specificity of receptors labelled with [125I-acetyl-His1]PACAP-27 and adenylate cyclase coupling

Tracer binding was dose-dependently inhibited by PACAP and related peptides with the following decreasing order of binding affinities: helodermin > PACAP-38 > PACAP-27 > VIP (Fig. 2). The corresponding IC_{50} values are quoted in Table II. The four peptides stimulated adenylate cyclase, their order of affinity was in good agreement with binding data. Besides, helodermin and both PACAPs were equally efficient, and VIP was slightly but not significantly less active (I.A. of 0.9 as compared to 1.0 for helodermin and PACAPs: Fig. 2 and Table II). All curves for binding inhibition and adenylate cyclase activation were compatible with the existence of one homogeneous class of receptors.

We then synthesized a series of VIP analogues, PACAP fragments, and PACAP analogues in order to address four questions:

(a) Concerning amino acids that are involved in a preferential recognition of PACAP over VIP, based on

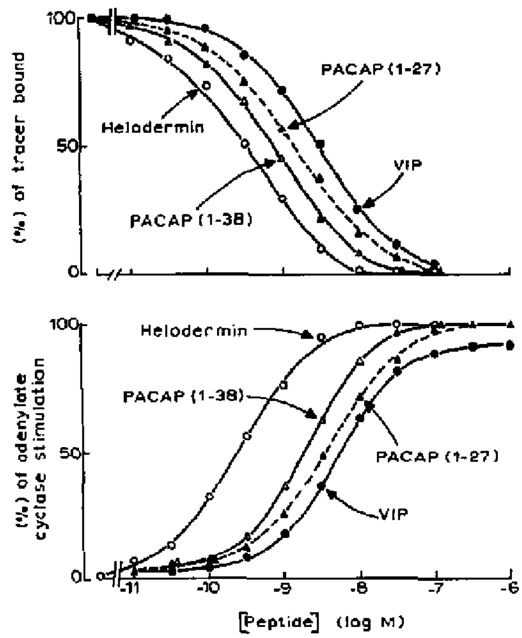

Fig. 2. Dose-effect curves of inhibition of [125I-acetyl-His1]PACAP-38 binding (A) and adenylate cyclase stimulation (B) by helodermin (○), PACAP-38 (△), PACAP-27 (▲) and VIP (●) in SUP-T1 lymphoblastic cell membranes. The results were the means of at least three experiments performed in duplicate. Binding data were expressed as a % of the [125I-acetyl-His1]PACAP-27 specifically bound in the absence of unlabelled peptide. Adenylate cyclase stimulation was expressed as a % of the increase in cAMP produced in the presence of 1 μM PACAP-27. The basal activity was 4.2±0.3 pmol cAMP produced min^{-1} (mg $protein)^{-1}$ and the activity as stimulated by 1 μM PACAP-27 was 152±8 pmol cAMP min^{-1} (mg $protein)^{-1}$ (means±S.E., $n = 5$).

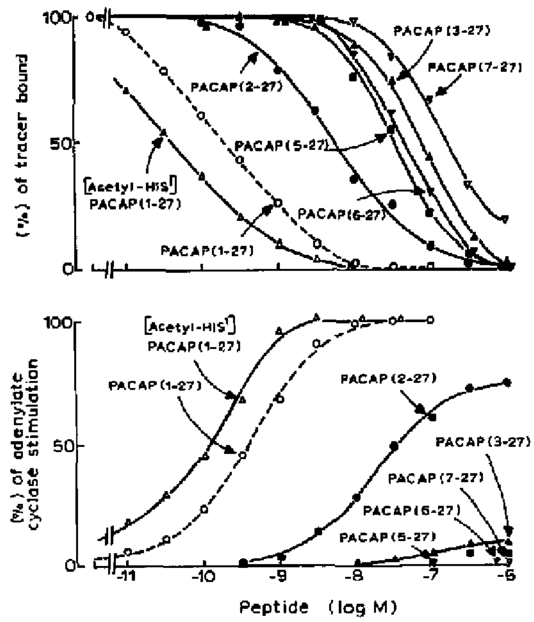

Fig. 3. Dose-effect curves of inhibition of [125I-acetyl-His1]PACAP-27 binding (A) and adenylate cyclase stimulation (B) by [acetyl-His1]PACAP-27 (△), PACAP-27 (○), PACAP(2–27) (●), PACAP(3–27) (▲), PACAP(5–27) (■), PACAP(6–27) (▼) and PACAP(7–27) (▽). Experimental conditions and the expression of results were identical to those of Fig. 2.

the ligand ability to occupy receptors and to stimulate adenylate cyclase, [Ser9-Tyr13]VIP was indistinguishable from PACAP-27, [Tyr13]VIP was slightly less potent and efficient than PACAP-27 and [Ser9]VIP was no more potent and efficient than VIP (Table II).

(b) The minimal PACAP sequence required for receptor occupancy and adenylate cyclase activation was tested systematically: PACAP (2–27) was already 30-fold less potent than PACAP-27 but retained 75% of its efficacy on adenylate cyclase. PACAP(3–27), -(5–27), -(6–27) and -(7–27) were, respectively, 400-, 200-, 300- and 1000-fold less potent than PACAP(1–27) for binding. PACAP(3–27) and -(5–27) retained a low but significant ability to stimulate adenylate cyclase (Fig. 3 and Table II). PACAP(6–27), when tested in combination with PACAP-27, helodermin or VIP, shifted the adenylate cyclase activation curve rightward without altering the maximal effect of the agonist (Fig. 4). The K_i of PACAP (6–27) was estimated as 500–800 nM for all three agonists tested (Table II).

(c) Considering the distinguished role of amino acids 1, 2 and 3 of PACAP for efficient interaction with receptors, we wondered what could be the bearing of their substitution or alteration. The acetylation of the NH_2 terminus increased the peptide potency 6-fold with no change in efficacy (Fig. 3 and Table II). The replacement of histidine-1 by phenylalanine provoked

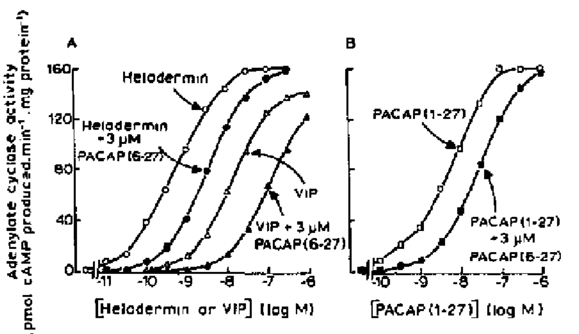

Fig. 4. Dose-effect curves of adenylate cyclase activation in SUP-T1 lymphoblastic cell membranes by helodermin (○, ●, A), VIP (△, ▲, A) and PACAP(1–27) (□, ■, B) in the absence (open symbols) or presence (closed symbols) of 3 μM PACAP(6–27). The results were the means of experiments performed in duplicate on three different membrane preparations and were expressed in pmol cAMP produced min^{-1} (mg protein)$^{-1}$.

a 10-fold decrease in potency and a slightly reduced efficacy (Fig. 5 and Table II). Substitution of serine-2 by alanine increased 4-fold the peptide potency. Substitution of aspartic acid-3 by glutamic acid was without significant effect while substitution by asparagine markedly reduced the potency as well as efficacy (Fig. 5, Table II).

When the same substitutions of serine or aspartate were operated in the absence of histidine-1 i.e. on

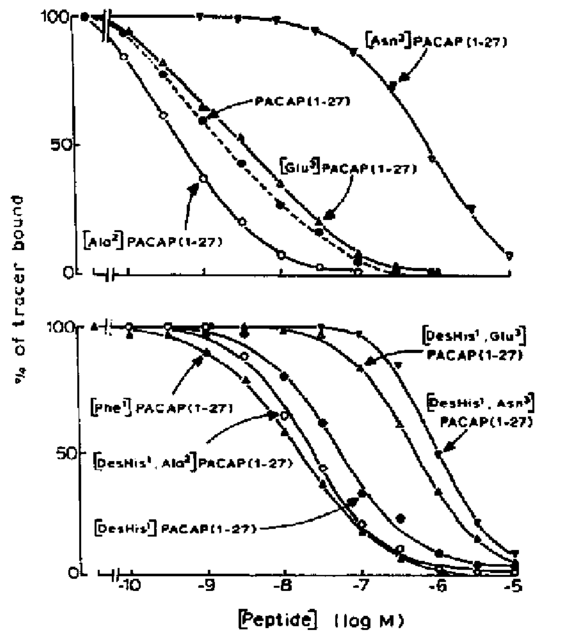

Fig. 5. Dose-effect curves of inhibition of [^{125}I-acetyl-His^1]PACAP-27 binding by: (A): PACAP-27 (●), [Ala^2]PACAP-27 (○), [Glu^3]PACAP-27 (△) and [Asn^3]PACAP-27 (▽) and (B): [Phe^1]PACAP-27 (▲), [Des-His^1]PACAP-27 (●), [Des-His^1,Ala^2]PACAP-27 (○), [Des-His^1,Asn^3]PACAP-27 (▼) and [Des-His^1,Glu^3]PACAP-27 (△). Experimental conditions and the expression of results were identical to those of Figs. 2 and 3.

TABLE II

Capacity of helodermin, VIP, VIP analogues, PACAP, PACAP analogues and PACAP fragments to inhibit [^{125}I-acetyl-His^1]PACAP-27 (IC_{50} in nM) and to stimulate adenylate cyclase (K_{act} in nM) or inhibit its PACAP-27-stimulated activity (K_i in nM) in SUP-T1 cell membranes

The intrinsic activity (I.A.) is the ratio between the maximal enzyme activity in the presence of each peptide tested and the maximal activity in the presence of 1 μM PACAP(1–27). Values were the means ± S.E. ($n \geq 3$). Experiments were performed in duplicate. n.d. = not measurable. * not significantly lower than 1.

Peptide	IC_{50} (nM)	K_{act} (nM)	K_i (nM)	I.A.
Helodermin	0.3 ± 0.1	0.3 ± 0.1		1.0
PACAP-38	0.8 ± 0.2	2.0 ± 0.5		1.0
PACAP-27	2 ± 1	5 ± 1		1.0
VIP	5 ± 2	10 ± 2		0.9 *
[Ser^9]VIP	5 ± 3	10 ± 2		0.9 *
[Tyr^{13}]VIP	4 ± 1	6 ± 2		1.0
[Ser^9,Tyr^{13}]VIP	2 ± 1	5 ± 1		1.0
PACAP(2–27)	50 ± 10	150 ± 30		0.8
PACAP(3–27)	800 ± 50	n.d.	500 ± 100	0.1
PACAP(5–27)	400 ± 100	n.d.	330 ± 50	0.03
PACAP(6–27)	600 ± 100	-	600 ± 200	0
PACAP(7–27)	2000 ± 200	-	2000 ± 300	0
[Acetyl-His^1]PACAP(1–27)	0.3 ± 0.05	1.5 ± 0.2		1.0
[Phe^1]PACAP-27	20 ± 2	40 ± 3		0.8
[Ala^2]PACAP-27	0.5 ± 0.1	1.0 ± 2.0		1.0
[Asn^3]PACAP-27	1000 ± 100	2000 ± 100		0.5
[Glu^3]PACAP(1–27)	3.0 ± 0.5	10 ± 5		1.0
[Des-His^1,Ala^2]PACAP-27	20 ± 5	80 ± 5		1.0
[Des-His^1,Asn^3]PACAP-27	1000 ± 100	-	1000 ± 100	0
[Des-His^1,Glu^3]PACAP-27	600 ± 100	1000 ± 100		0.3

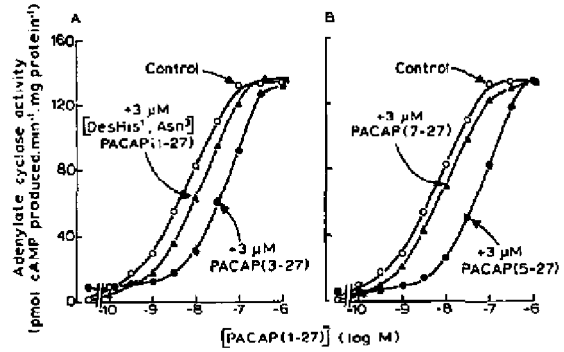

Fig. 6. Dose-effect curves of adenylate cyclase activation in SUP-T1 lymphoblastic cell membranes by PACAP-27 in the absence (open symbols) or presence of 3 μM [Des-His[1],Asn[3]]PACAP-27 (▲, A), 3 μM PACAP(3–27) (●, A), 3 μM PACAP(5–27) (●, B), or 3 μM PACAP(7–27) (▲, B). The results were the means of experiments performed in duplicate on three different membrane preparations and were expressed in pmol cAMP produced min^{-1} (mg $protein)^{-1}$.

[Des-His[1]]PACAP-27 (see Table I), the Ala[2] derivative showed higher potency and efficacy than PACAP(2–27); the Asn[3] derivative retained very low potency and lost all capacity to stimulate adenylate cyclase; the Glu[3] derivative was 30-fold less potent than PACAP (2–27) and exhibited reduced I.A.

(d) Finally, in addition to PACAP(6–27) whose effects have already been described in Figs. 3 and 4, the PACAP fragments (3–27), (5–27) and (7–27) and the analogue [Des-His[1],Asn[3]]PACAP-27 were also tested for their ability to inhibit PACAP-stimulated adenylate cyclase. The position of PACAP(3–27) and PACAP(5–27) as partial agonists with very low I.A. was confirmed, and PACAP(7–27) and [Des-His[1],Asn[3]]-PACAP-27 proved to be low affinity antagonists (Fig. 6).

Discussion

1. The present data show that PACAP-27 and PACAP-38, two newly discovered peptides of the VIP family, interacted with high affinity with SUP-T1 cell membranes and stimulated their adenylate cyclase. PACAP binding sites could be labelled by both [^{125}I]PACAP-27 and [^{125}I-acetyl-His[1]]PACAP-27. The latter tracer showed higher affinity than PACAP-27 itself and a better specific/non specific binding ratio. The binding sites labelled by the two tracers exhibited the characteristics of relevant receptors: binding was time-dependent, reversible, saturable, specific and inhibited by GTP. These receptors were probably identical to those previously reported as being 'helodermin-preferring' VIP receptors in this cell line. Two evidences support this conclusion: (a) tracer binding was completely inhibited by helodermin and VIP, and IC_{50} values were identical to those observed when these receptors were labelled with [^{125}I]helodermin [1] or [^{125}I-acetyl-His[1]]VIP [2,4]; (b) PACAP-27-, helodermin- and VIP-stimulated adenylate cyclase activities were competitively inhibited by PACAP(6–27) and the same K_i was found for the three agonists considered.

Several peptides of the VIP family were, thus, likely to interact with these functional receptors that showed an unusual decreasing order of binding affinity: helodermin > PACAP-38 > PACAP-27 > VIP, with PACAP-27 being 2-fold more potent than VIP. This order of potency differed clearly from that recently described for selective PACAP receptors [9–14]. However, the present helodermin-preferring VIP receptors in SUP-T1 cells also bare no resemblance with a majority of VIP receptors, based on their capability to discriminate VIP analogues [2,4] (see also Introduction).

2. By comparing PACAP-27 with PACAP fragments and monosubstituted PACAP analogues, we further specified the structural requirements for an optimal contribution of the N-terminus of the ligand to receptor recognition and subsequent adenylate cyclase activation.

As already noticed for VIP [18,19] and secretin receptors [20,21], the amino terminal residue of PACAP was particularly important for full biological activity at low peptide concentration: the absence of histidine-1 decreased 25- to 30-fold the affinity for receptors but adenylate cyclase activation remained possible. The replacement of histidine-1 by phenylalanine produced a compound only 3-fold more potent than [Des-His[1]]PACAP. Acetylation of the α-amino group of histidine 1 was beneficial for receptor recognition, an observation in line with that previously made on SUP-T1 receptors with [acetyl-His[1]]VIP [2,4] and perhaps, due to the alteration of the chemical properties of the imidazole moiety following the suppression of an ionized function by acetylation [22].

Concerning position 2, replacement of serine by alanine increased 3- to 4-fold the peptide affinity in, respectively, the absence and presence of histidine – 1. Although we have no explanation, it is of interest to note that the parent peptide PHI, with its N-terminal sequence H-A-D-G, recognizes SUP-T1 receptors with the same affinity as VIP [1,2], a rather unusual observation since PHI is 3- to 10-fold less potent than VIP on most VIP receptors [23]. Replacement of serine 2 by alanine in secretin (that possesses the same N-terminal sequence H-S-D-G as PACAP) exerts contrasting effects: it decreases 3-fold the affinity for secretin receptors in rat pancreas [24], rat heart [25] and rat/mouse neuroglioma cell membranes [21] but doubles the peptide affinity for human pancreatic membranes [20]. The absence of both His[1] and Ser[2] decreased 400-fold the peptide affinity and reduced the peptide efficacy to a mere 10%. Similar observations have been made for VIP [18] and secretin [21].

An acidic function in position 3 was also critical for the high affinity of PACAP for SUP-T1 receptors: substitution of Asp[3] by Asn reduced 500-fold the affinity of the ligand and 2-fold its efficacy while the replacement by Glu affected slightly only the binding to the receptor. Conceivably, in the native PACAP-27 molecule, efficient ligand–receptor interaction may follow a conformational state of the ligand whereby its amino-terminal histidine interacts with a negatively charged carboxyl group [22]. In the absence of histidine-1, an even more precise positioning of the carboxyl function of the ligand may be required for potent and efficient interaction with the receptor. [Des-His^1-Glu^3]PACAP-27 was indeed 12-fold less potent than [Des-His^1]PACAP-27 and only 2-fold more potent than [Des-His^1-Asn^3]PACAP-27; furthermore, it displayed only one third of the maximal intrinsic activity. [Des-His^1-Asn^3]PACAP-27 showed the same low potency as [Asn^3]PACAP-27 but was completely devoid of intrinsic activity and, accordingly, it antagonized the PACAP-stimulated response of adenylate cyclase.

3. VIP and PACAP-27 differ in several positions: 4, 5, 9, 13, 15, 24, 25, 26 and 28. As VIP was only 3-fold less potent than PACAP-27 and allowed 90% of maximal adenylate cyclase activation, it is unlikely that these amino acids were all markedly involved in high-affinity binding. However, the introduction into VIP of a tyrosine in position 13, and the simultaneous introduction of serine and tyrosine in, respectively, position 9 and 13 led to VIP analogues that were close to PACAP. By contrast, the same substitutions in VIP increase 3- to 10-fold the ligand affinity for specific PACAP receptors in rat brain membranes and on a human neuroblastoma cell line but then, these analogues still show 300-times less affinity than PACAP-27 itself [9].

4. Finally, in the present work, we observed that two PACAP analogues, devoid of intrinsic activity, were able to antagonize the helodermin-preferring VIP receptor of the human lymphoblastic cell line SUP-T1: PACAP(6–27) with a K_i of 500–600 nM and [Des-His^1-Asn^3]PACAP-27 with a K_i of 1–2 μM.

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