0026-895X/92/020347-09$03.00/0

MOLECULAR PHARMACOLOGY, 42:347-355

Receptor Occupancy and Adenylate Cyclase Activation in AR 4–2J Rat Pancreatic Acinar Cell Membranes by Analogs of Pituitary Adenylate Cyclase-Activating Peptides Amino-Terminally Shortened or Modified at Position 1, 2, 3, 20, or 21

PATRICK ROBBERECHT, PHILIPPE GOURLET, PHILIPPE DE NEEF, MARIE-CLAIRE WOUSSEN-COLLE, MARIE-CLAIRE VANDERMEERS-PIRET, ANDRÉ VANDERMEERS, and JEAN CHRISTOPHE

Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, B-1070 Brussels, Belgium

Received December 31, 1991; Accepted May 7, 1992

SUMMARY

In AR 4–2J rat pancreatic acinar cell membranes, receptors for the two pituitary adenylate cyclase-activating peptides (PACAP) PACAP-27 (the short version of PACAP) and PACAP-38 [the long version, with a carboxyl-terminal (residues 28–38) extension] can be subdivided into (a) type A receptors, with high affinity (K_d, 0.3–0.5 nM) for both PACAP-27 and PACAP-38, and (b) type B receptors, with high affinity for PACAP-38 (K_d, 0.3 nM) but low affinity for PACAP-27 (K_d, 20 nM). Determinants of agonist/antagonist activity in 47 PACAP-27 and PACAP-38 analogs (mono- or disubstituted in positions 1, 2, 3, 20, and 21) or amino-terminally shortened were tested by (a) the occupancy of PACAP-A receptors, preferentially labeled with [^{125}I-*N*-acetyl-His^1]PACAP-27, and that of PACAP-A and -B receptors, both labeled with ^{125}I-PACAP-38, and (b) the resulting activation or inhibition of adenylate cyclase. For PACAP-A receptor recognition, deprotonated His^1 was a major determinant for PACAP-27 but not PACAP-38; the K_d of ^{125}I-PACAP-27 decreased 2.4-fold at 37° between pH 6.0 and 7.5 and 3.6-fold at 15°, whereas the IC_{50} of [*N*-acetyl-His^1]PACAP-27 was less affected and that of PACAP(2–27), PACAP(2–38), and PACAP(1–38) was pH independent. In addition, PACAP-A receptors coupled to adenylate cyclase were much more sensitive to PACAP-38 derivatives than to PACAP-27 derivatives; for instance, [D-Phe^2]PACAP-38 was a more potent antagonist (K_i, 5 nM) than [D-Phe^2]PACAP-27 (K_i, 350 nM), and PACAP(6–38) was a more potent antagonist (K_i, 7 nM) than PACAP(6–27) (K_i, 300 nM). PACAP-B receptors, apart from showing high affinity for PACAP-38, displayed relatively high affinity for amino-terminally shortened PACAP-38 fragments and poor affinity for PACAP-27 and PACAP-27 fragments.

PACAP-27 exhibits 68% homology to VIP, with this homology being especially evident in the amino-terminal areas (residues 1–3 and residues 6–8) and in the central region (residues 14–23). In contrast, the extended carboxyl-terminal end (residues 29–38) of PACAP-38, which contains six cationic amino acid residues, shows no homology with any known parent peptide of similar length (Table 1). PACAP-27 and PACAP-38 are present in the central nervous system (most notably in the hypothalamus) (1–3) and in the digestive tract innervation (4). Their physiological role is not yet established, but pharmacological studies demonstrate a stimulatory effect on pancreatic enzyme secretion (5, 6), as well as on insulin release (7).

Specific PACAP receptors linked to adenylate cyclase are present in the rat pancreatic cancer acinar cell line AR 4–2J (8), as well as in liver (9) and lung (10) from rat. Their poor affinity for VIP allows a clear distinction from the VIP receptors present in normal pancreas (11), liver (9), and lung (10) from rat, which interact with VIP and PACAP with similarly high affinity. We recently demonstrated that PACAP receptors in rat pancreatic AR 4–2J membranes can be subdivided into two subclasses (12) by comparison of Scatchard plots of saturation curves and competition binding curves, using ^{125}I-PACAP-27 and ^{125}I-PACAP-38 as radioligands. PACAP-A receptors (0.6 pmol/mg of protein from crude membranes) recognize

This work was supported by Grant 3.4504.85 from the Fund for Medical Scientific Research (Belgium) and "Concerted Research Action" 87/92408 from the Ministry of Scientific Policy (Belgium).

ABBREVIATIONS: PACAP-38 and PACAP-27, pituitary adenylate cyclase-activating peptides in, respectively, the long version (the amidated peptide consisting of residues 1–38) and the short version (the amidated peptide consisting of residues 1–27); [AC-His^1]-, *N*-acetyl-His^1; VIP, vasoactive intestinal peptide; PHI, peptide histidine-isoleucinamide; GRF, growth hormone-releasing factor; IC_{50}, concentration of unlabeled analog inhibiting 50% of [^{125}I-AC-His^1]PACAP-27 or [^{125}I]PACAP-38 binding; K_d, equilibrium dissociation constant of the radioligand; K_{act}, concentration producing half-maximal stimulation of adenylate cyclase (EC_{50}); K_i, concentration required for half-maximal inhibition of adenylate cyclase stimulation; IA, intrinsic activity (efficacy), considering the maximal effect of PACAP-27 as 1; PACAP, pituitary adenylate cyclase-activating peptide; HPLC, high performance liquid chromatography.

TABLE 1

Comparison of the amino acid sequence of PACAP-38 and PACAP-27 with seven related peptides

Differences from PACAP-38 are underlined (*, NH_2).

Species[a]	Peptide	Amino acids 1 5 10 15 20 25 30 35 40 45
o	PACAP-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-*
o	PACAP-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-*
b/do/h/p/r	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-*
b	PHI	H-A-D-G-V-F-T-S-D-Y-S-R-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-I-*
r	PHI	H-A-D-G-V-F-T-S-D-Y-S-R-L-L-G-Q-I-S-A-K-K-Y-L-E-S-L-I-*
r	PHV (1–42)[b]	H-A-D-G-V-F-T-S-D-Y-S-R-L-L-G-Q-I-S-A-K-K-Y-L-E-S-L-I-G-K-R-I-S-S-S-I-S-E-D-P-V-P-V
hs	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
r	Secretin	H-S-D-G-T-F-T-S-E-L-S-R-L-Q-E-G-A-R-L-Q-R-L-L-Q-G-L-V-*
p	GRF (1–44)	Y-A-D-A-I-F-T-N-S-Y-R-K-V-L-G-Q-L-S-A-R-K-L-L-Q-D-I-M-S-R-Q-Q-G-E-R-N-Q-E-Q-G-A-R-V-R-L-*

[a] b, bovine; do, dog; h, human; hs, *Heloderma suspectum* (Gila monster); o, ovine; p, porcine; r, rat.
[b] PVH (1–42), peptide histidine-valine-(1–42).

PACAP-27 and PACAP-38 with equally high affinity and VIP with low affinity. PACAP-B receptors are present in equivalent concentrations and recognize PACAP-38 with high affinity but PACAP-27 with a 100-fold lower affinity; they virtually do not recognize VIP.

In the present *in vitro* study, we hoped to tailor efficient, potent, and selective antagonists of the PACAP-27 and PACAP-38 families by appropriate modifications in the amino-terminal region of the molecule. In general, major changes in intrinsic activity are observed in VIP-related peptides, with replacement of amino acid residues in position 1, 2, or 3 or use of amino-terminally shortened fragments (for reviews, see Refs. 13 and 14). Therefore, utilizing PACAP-27 and PACAP-38 as reference peptides, we compared the ability of mono- or disubstituted PACAP-27 and -38 analogs to occupy PACAP-A and PACAP-B receptors and to stimulate (or inhibit) adenylate cyclase activity. To explore, among other factors, the steric and stereoelectronic requirements at position 2, we compared phenylalanine and arginine, two amino acids with bulky side chain groups, with serine and alanine. We also substituted D-amino acids, because they facilitate a reverse turn (15). In addition, we tested the effects of a progressive amino-terminal deletion of PACAP-27 and PACAP-38.

Experimental Procedures

Cell culture and crude membrane preparation. The rat pancreatic acinar cell line AR 4–2J was grown as described (8, 12), at 37°, in Dulbecco's modified Eagle's medium supplemented with 10% (by volume) fetal calf serum (GIBCO), 20 mM sodium pyruvate, 20 mM glutamine, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B). Stock cultures were diluted every 3 days with 4 volumes of fresh medium.

For crude membrane preparation, the cells at confluence were detached with a rubber policeman, centrifuged at 100 × *g* for 10 min, rinsed with fresh culture medium, pelleted again, lyzed in hypotonic 1 mM $NaHCO_3$ (pH 7.0), and then quickly frozen in liquid nitrogen. The lysate was defrosted and centrifuged at 2000 × *g* for 10 min at 4°. The resulting supernatant was centrifuged at 20,000 × *g* for 10 min. The final pellet was rehomogenized in 1 mM $NaHCO_3$ and immediately tested in binding and adenylate cyclase assays.

Radioiodination of PACAP(1–27), [AC-His^1]PACAP(1–27), and PACAP(1–38) and purification of the tracers. PACAP(1–27) and [AC-His^1]PACAP-27 were radioiodinated by the Iodogen technique (16) and purified by HPLC, as described in Refs. 12 and 17. After purification and CH_3CN evaporation, the major radioactive peak showing the highest capacity to bind to AR 4–2J cell membranes was diluted in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.2% (w/v) bovine serum albumin, 0.01% (w/v) sodium azide, and 0.05% (v/v) Tween 20 (to avoid peptide adsorption to the tubes). The tracer was stored at −20° and retained high affinity and low nonspecific binding properties for 2 months. Specific radioactivity was typically 2 mCi/nmol, based on iodine incorporation.

PACAP-38 was also labeled by the Iodogen technique (17) and then purified by HPLC, under conditions similar to those used for ^{125}I-PACAP-27 and [^{125}I-AC-His^1]PACAP-27. The single radioactive peak was separated from unlabeled PACAP-38 and stored as for [^{125}I-AC-His^1]PACAP-27; its specific radioactivity was 1.0 mCi/nmol.

Binding of [^{125}I-AC-His^1]PACAP-27 to rat pancreatic acinar AR 4–2J cell membranes. Competition curves were performed in a 50 mM Tris-maleate buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 100 kallikrein inhibitory units/ml Trasylol, 1% (w/v) bovine serum albumin, 20,000 cpm/assay [^{125}I-AC-His^1]PACAP-27 (corresponding to 0.10 nM tracer), increasing concentrations of unlabeled peptide, and 5 μg of AR 4–2J membrane protein, in a total volume of 120 μl. Incubations were conducted at 37° for 20 min, to allow binding equilibrium, and were terminated by dilution with 2 ml of ice-cold 0.1 M sodium phosphate buffer (pH 7.4), followed by rapid filtration through glass fiber filters (G/FC; Whatman, Maidstone, Kent, UK) that had been presoaked in 0.1% poly(ethyleneimine) for at least 24 hr. The filters were rinsed three times with 2 ml of the same phosphate buffer, and their radioactivity was measured in a multichannel LKB γ counter. Nonspecific binding of [^{125}I-AC-His^1]PACAP-27 and ^{125}I-PACAP-38 were determined in the presence of 0.1 μM PACAP-27 and 0.1 μM PACAP-38, respectively, and accounted for <15% and 40% of total binding, respectively. Total binding was always <15% of the total radioactivity added.

IC_{50} values were graphically calculated from dose-effect curves established up to a 10 μM peptide concentration. We verified that the tracer concentration did not affect the concentration of unlabeled peptide necessary for half-maximal inhibition of tracer binding, so that IC_{50} values of unlabeled peptides were close to K_d values.

Adenylate cyclase assay. Adenylate cyclase activity was determined by the procedure of Salomon *et al.* (18). GTP (10 μM) was systematically added to the incubation medium. The reaction was terminated after a 15-min incubation at 37°. The EC_{50} value was calculated from the complete dose-effect curve or by extrapolation (assuming that all peptides acted through interaction with a single class of receptors). To determine the K_i, complete dose-effect curves of PACAP-27 or PACAP-38 with adenylate cyclase were obtained in the

absence or presence of at least two concentrations of antagonist. The K_i value was derived from

$$\frac{[A']}{[A]} - 1 = \frac{[I]}{K_i}$$

where $[A']$ and $[A]$ are the two PACAP-27 (or PACAP-38) concentrations giving the same stimulation in, respectively, the presence and the absence of concentration $[I]$ of antagonist. We made no attempt to determine the K_i of a partial agonist when its IA was higher than 0.20. Each IA was the ratio between the maximal enzyme activity observed in the presence of a given agonist and the maximal effect of PACAP-27, after subtraction of the basal unstimulated value.

Protein concentration. The protein concentration was determined by the method of Lowry *et al.* (19), using bovine serum albumin as standard.

Synthesis and purification of PACAP-27 and PACAP-38 analogs. Synthetic PACAP-38 was a gift from Dr. J. P. Durieux (Novabiochem, Laüfelfingen, Switzerland). All PACAP analogs and fragments were synthesized by solid phase methodology, with an Applied Biosystems automated 431 A apparatus (Foster City, CA), using the 9-fluorenylmethoxycarbonyl strategy; a 4-(2′,4′-dimethoxyphenyl-fluoren-9-yl-methoxycarbonyl-aminomethyl)phenoxy resin and fluoren-9-yl-methoxycarbonyl-labeled amino acids activated with *N*-hydroxybenzotriazole and *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (20) were used.

The peptides were purified by preparative reverse phase chromatography, using a Nucleosil 300–5 C18 column (25 × 1 cm). Peptides were eluted from the column with a 0.1% trifluoroacetic acid buffer and a 20–60% acetonitrile gradient over 40 min. The flow rate was 2.0 ml/min, and the typical load was 10–20 mg of peptide. The eluant was monitored by UV detection at 226 nm. The eluant was fractionated on the basis of UV absorbance, and peptide-containing fractions were collected, lyophilized, and further purified by analytical HPLC, using a Vydac 218 TP 104 column (25 × 0.46 cm) with a 0.1% trifluoroacetic acid buffer and a 25–50% acetonitrile gradient. The identity of the peptides was established by at least 10 cycles of Edman degradation (in an Applied Biosystems 477 A sequencer coupled to a 120 A phenylthiohydantoin amino acid analyzer; Foster City, CA).

Other chemicals. All reagents for peptide synthesis were purchased from Novabiochem. The sources of reagents used for peptide radioiodination, adenylate cyclase assay, and binding assay are detailed in Refs. 9, 11, and 17. Carrier-free $Na^{125}I$ (300 mCi/ml), [α-^{32}P]ATP (10 Ci/mmol), and [8-^{3}H]cAMP (20–30 Ci/mmol) were purchased from Amersham International (Amersham, Bucks, Great Britain). All other reagents were of the highest grade available.

Results

General characteristics of PACAP-A and PACAP-B receptors. The binding of [^{125}I-AC-His^1]PACAP-27 and [^{125}I]PACAP-38 to PACAP receptors was rapid (attaining equilibrium after 10–15 min at 37°, reversible, and saturable (data not shown), in line with our previous data on the same membrane preparation (12). Except where indicated, we used [^{125}I-AC-His^1]PACAP-27 instead of ^{125}I-PACAP-27, taking advantage of a higher ratio of total binding to nonspecific binding (ratio of 4 versus 3). The dose-effect curves for inhibition of binding of [^{125}I-AC-His^1]PACAP-27 and ^{125}I-PACAP-38 at equilibrium (20 min) were shallower with PACAP-27 than with PACAP-38 (Fig. 1). Inhibition by unlabeled PACAP-27 was compatible with the coexistence of two classes of PACAP-27 receptors, with IC_{50} values of 0.5 nM and 20 nM (Table 2). On the other hand, competition curves with the two radioligands and unlabeled PACAP-38 indicated the presence of only one class of receptors, with an IC_{50} of 0.3 nM (Table 3), suggesting that PACAP-38 was unable to discriminate between the two receptor classes. Saturation curves with increasing concentrations of both radioligands indicated that, in line with Ref. 12, the receptor density for ^{125}I-PACAP-38 (2.0 ± 100 pmol/mg of protein) was 2-fold higher than that for ^{125}I-PACAP-27 (1.0 ± 100 pmol/mg of protein) (data not shown).

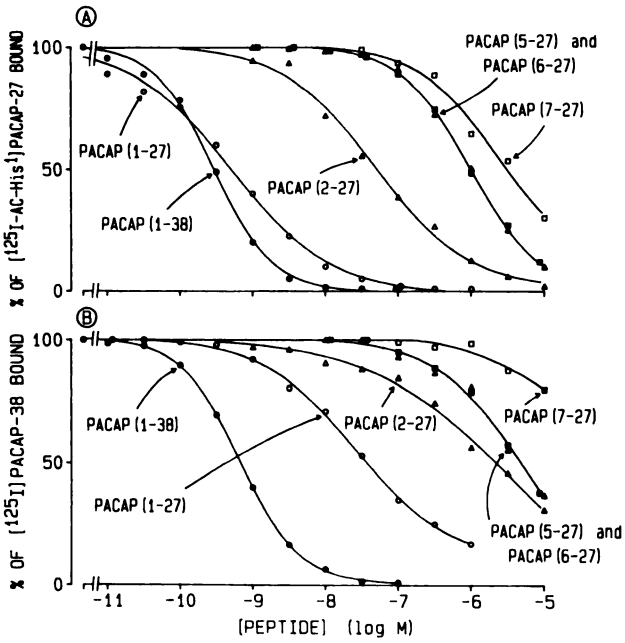

Fig. 1. Dose-effect curves for inhibition of [^{125}I-AC-His^1]PACAP(1–27) (A) and ^{125}I-PACAP-38 (B) binding, in rat pancreatic AR 4–2J cell membranes, by PACAP-27, PACAP-38, and four amino-terminally shortened PACAP-27 analogs. Binding data were obtained after 20 min at 37° and are expressed as a percentage of radioligand specifically bound in the absence of unlabeled peptide. The results are the means of at least three experiments performed in duplicate.

PACAP-27 and PACAP-38 stimulated adenylate cyclase dose-dependently, with similar potency (K_{act}, 1.0 and 0.2 nM, respectively) and efficacy (IA, 1), in the presence of 10 μM GTP (Fig. 2; Tables 2 and 3). The enzyme activity under maximal stimulation was 2.5-fold higher than under resting conditions.

These data confirmed (12) the coexistence of PACAP-A receptors without significant preference for PACAP-27 over PACAP-38 (IC_{50}, 0.5 nM versus 0.3 nM) and of PACAP-B receptors that recognized PACAP-38 with high affinity (IC_{50}, 0.3 nM) and PACAP-27 with low affinity (IC_{50}, 20 nM).

pH dependence of PACAP-A receptor occupancy by PACAP-27, PACAP-38, and PACAP analogs. ^{125}I-PACAP-27 binding, tested at equilibrium (after 20 min), increased from pH 6.0 to pH 7.5, due to a 2.4-fold rise in affinity; at 37°, the K_d decreased from 1.03 ± 0.10 nM to 0.65 ± 0.10 nM and 0.43 ± 0.08 nM (mean values ± standard errors; three experiments) at pH 6.0, 6.5, and 7.5, respectively, with no change in the number of receptors. The sharpest K_d fall was between pH 6.0 and 6.5, and radioligand binding was optimal at pH 7.5–8.0 (Fig. 3, *left*). This pH effect was amplified 2.4-fold when the temperature was reduced from 37° to 15° (Fig. 3, *right*). We next analyzed this pH dependence in competition studies conducted with unlabeled analogs (Figs. 4 and 5). The IC_{50} of [AC-His^1]PACAP-27 showed a lesser beneficial effect when the pH was raised from pH 6.0 to 8.0, at both 37° and 15°. The low potency of [Phe^1]PACAP-27 increased only 2-fold between pH 6.0 (IC_{50}, 80 nM) and pH 8.0 (IC_{50}, 40 nM). The reduced potency of PACAP(2–27) (IC_{50}, 40 nM) was unaffected by pH variation

TABLE 2

Binding of PACAP-27 fragments and analogs and adenylate cyclase activation in rat pancreatic AR 4-2J cell membranes

Values are means of at least three experiments. The maximal range of IC_{50}, K_{act}, or K_i values was 2.

Peptide tested	Binding (IC_{50})		Adenylate cyclase			
	[^{125}I-AC-His^1]PACAP-27	^{125}IPACAP-38	K_{act}	IA	K_i	
					PACAP-27	PACAP-38
	nM		*nM*		*nM*	
PACAP (1–27)	0.5	20	1.0	1.00		
PACAP (2–27)	40	1,000	100	0.85		
PACAP (3–27)	3,000	ND[a]	2,000	0.30	ND	ND
PACAP (5–27)	1,000	ND	500	0.20	ND	ND
PACAP (6–27)	800	8,000		0	300	2,000
PACAP (7–27)	3,000	ND		0	4,000	>10,000
PACAP (9–27)	2,000	>30,000		0	ND	ND
[AC-His^1]PACAP-27	0.25	30	1.0	0.85		
[Phe^1]PACAP-27	30	ND	50	0.85		
[Ala^2]PACAP-27	0.25	10	0.3	1.00		
[Des-His^1,Ala^2]PACAP-27	600	10,000	500	0.55		
[AC-His^1,Ala^2]PACAP-27	0.80	ND	1.5	0.95		
[D-Ala^2]PACAP-27	0.80	100	3.0	1.00		
[AC-His^1,D-Ala^2]PACAP-27	1.5	200	8.0	1.00		
[Arg^2]PACAP-27	500	ND	200	0.30		
[AC-His^1,Arg^2]PACAP-27	400	ND	200	0.35		
[D-Arg^2]PACAP-27	300	>10,000	300	0.50		
[AC-His^1,D-Arg^2]PACAP-27	600	>30,000	300	0.50		
[Phe^2]PACAP-27	200	ND	200	0.33	ND	ND
[AC-His^1,Phe^2]PACAP-27	30	ND	40	0.31	ND	ND
[D-Phe^2]PACAP-27	300	4,000		0.20	350	1,000
[AC-His^1,D-Phe^2]PACAP-27	2,000	>30,000		0.20	ND	ND
[Glu^3]PACAP-27	20	>10,000	30	0.85		
[Des-His^1,Glu^3]PACAP-27	4,000	>10,000		0	ND	ND
[Asn^3]PACAP-27	600	6,000	500	0.80		
[Des-His^1,Asn^3]PACAP-27	2,000	>10,000		0	1,500	5,000
[Gly^{20}]PACAP-27	4	ND	5	1.00		
[Gly^{20},Gly^{21}]PACAP-27	100	ND	80	1.00		

[a] ND, not determined.

TABLE 3

Binding of PACAP-38 fragments and analogs and adenylate cyclase activation in rat pancreatic AR 4-2J cell membranes

Values are means of at least three experiments. The maximal range of IC_{50}, K_{act}, and K_i values was 2.

Peptide tested	Binding (IC_{50})		Adenylate cyclase			
	[^{125}I-AC-His^1]PACAP-27	^{125}IPACAP-38	K_{act}	IA	K_i	
					PACAP-27	PACAP-38
	nM		*nM*		*nM*	
PACAP (1–38)	0.3	0.3	0.2	1.00		
PACAP (2–38)	4.0	ND[a]	6.0	0.80		
PACAP (4–38)	30	30		0	11	300
PACAP (5–38)	20	20		0	25	1000
PACAP (6–38)	3	10		0	7	150
PACAP (7–38)	20	50		0	25	150
[Ala^2]PACAP-38	1.5	2	0.8	0.95		
[AC-His^1,Ala^2]PACAP-38	0.4	8	8	0.90		
[D-Ala^2]PACAP-38	0.10	0.4	0.3	1.00		
[AC-His^1,D-Ala^2]PACAP-38	0.10	1.5	0.3	0.90		
[Arg^2]PACAP-38	30	50	100	0.50		
[AC-His^1,Arg^2]PACAP-38	8	150	200	0.50		
[D-Arg^2]PACAP-38	2	15	10	0.40	ND	ND
[AC-His^1,D-Arg^2]PACAP-38	2	20	30	0.40	ND	ND
[Phe^2]PACAP-38	5	8	5	0.47	ND	ND
[AC-His^1,Phe^2]PACAP-38	2	10	3	0.38	ND	ND
[D-Phe^2]PACAP-38	2	30		0	5	40
[AC-His^1,D-Phe^2]PACAP-38	2	30		0	ND	ND
[Des-His^1,Glu^3]PACAP-38	40	100		0	30	1000
[Ala^3]PACAP-38	3	20	30	0.30	ND	ND

[a] ND, not determined.

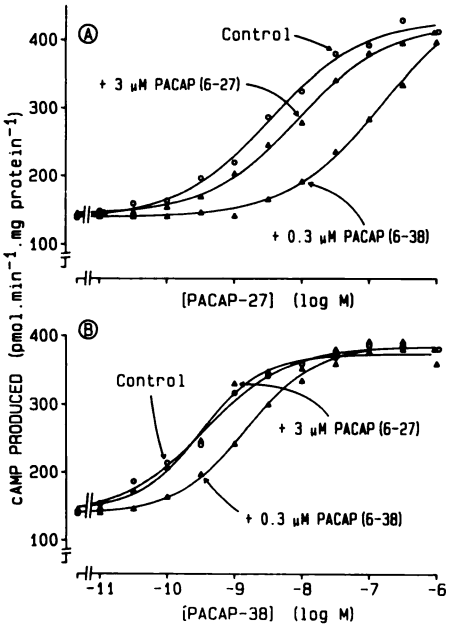

Fig. 2. Dose-effect curves for adenylate cyclase stimulation, in rat pancreatic AR 4–2J cell membranes, by PACAP(1–27) (A) and PACAP(1–38) (B). The enzyme activity was tested in the presence of 10 μM GTP for 10 min, in the absence or presence of 3 μM PACAP(6–27) or 0.3 μM PACAP(6–38). Enzyme activity (in pmol of cAMP produced/min/mg of protein) when unstimulated was 140 ± 10 and when stimulated by 1 μM PACAP-27 and 1 μM PACAP-38 was 400 ± 20 and 380 ± 30, respectively (means ± standard errors of experiments performed in duplicate on six different membrane preparations.

at 37° and was affected very modestly at 15°. In contrast, the very low potency of PACAP(7–27) depended strongly on the pH, with IC_{50} values of 20,000, 10,000, and 2,000 nM at, respectively, pH 6.0, 7.0, and 8.0 at 37°, with similar values at 15°. Interestingly, the IC_{50} of PACAP-38, compared with PACAP-27, was pH and temperature independent, i.e., remained practically constant (0.2 nM) at both 37° and 15°.

Interaction of analogs and fragments of PACAP-27 and PACAP-38 with PACAP-A receptors and adenylate cyclase. K_{act} (or K_i) values of PACAP-27 analogs were, in general, comparable to their IC_{50} values when [^{125}I-AC-His^1]PACAP-27 was used as tracer (Fig. 6; Tables 2 and 3). To test the role of amino acids 1, 2, and 3, we examined the effects of their alteration or substitution, namely, acetylation of the free α-NH_2 function of His^1 in PACAP-27, replacement of His^1 in PACAP-27 by Phe^1, and replacement of Ser^2 by Ala^2, Phe^2, or Arg^2 as the L- or D-isomer and also in the [AC-His^1] format. [Ala^2]PACAP-27 was the only agonist showing IC_{50} and K_{act} values slightly lower than those of PACAP-27. The K_{act} (K_i) value increased with (L- or D-) Phe^2 and Arg^2 replacements. The IA decreased as follows in the PACAP-27 series: Ala^2 ≥ Ser^2 = D-Ala^2 > D-Arg^2 > Arg^2 = Phe^2 > D-Phe^2; it decreased as follows for the PACAP-38 series: Ser^2 = Ala^2 = D-Ala^2 > Arg^2 = Phe^2 ≥ D-Arg^2 ≫ D-Phe^2 (the latter analog, with an IA of 0, acted as a weak antagonist, with a K_i of 350 nM). In the PACAP-27 series, *N*-acetylation of His^1 increased the potency (but not the IA) in [AC-His^1,Phe^2]PACAP-27 but decreased the potency in [AC-His^1,D-Phe^2]PACAP-27; it exerted no effect on the IC_{50} and K_{act} of the Arg^2 and D-Arg^2 PACAP-27 analogs (Table 2). In the PACAP-38 series of analogs, *N*-acetylation was rather detrimental, increasing the K_{act} of the [AC-His^1,Ala^2]-, [AC-His^1,Arg^2]-, and [AC-His^1,D-Arg^2]- derivatives of PACAP-38 (Table 3).

Substitution of Asp^3 by glutamate decreased 40-fold the potency of [Glu^3]PACAP-27, and substitution by asparagine was even more deleterious, reducing the potency 1200-fold and the efficacy moderately (by 20%) (Table 2).

Replacement of His^1 in PACAP-27 by the more polar amino acid Phe^1 decreased the potency markedly (60-fold). The favorable role of His^1 was also evident when the effects of Ser^2 or Asp^3 substitutions in the absence of His^1, i.e., in [Des-His^1] analogs, were considered; [Des-His^1,Ala^2]PACAP-27 showed 15-fold lower potency and reduced efficacy, as compared with PACAP(2–27), and [Des-His^1,Asn^3]PACAP-27 and [Des-His^1,Glu^3]PACAP-27 showed very low potency and could not stimulate adenylate cyclase ([Des-His^1,Asn^3]PACAP-27 was a weak antagonist, with a K_i of 1500 nM) (Table 2). The longer [Des-His^1,Glu^3]PACAP-38 analog kept a relatively good potency (IC_{50}, 40 nM) (Fig. 7) but acted as an antagonist (K_i, 30 nM) (Table 3).

We next tested the minimal amino-terminal sequence required for receptor binding and adenylate cyclase activation, by a progressive deletion of amino acids in the residue 1–8 amino-terminal part of PACAP-27 and PACAP-38 (Figs. 1, 2, 7, and 8; Tables 2 and 3). IC_{50} values for amino-terminally shortened PACAP derivatives increased as follows: PACAP-38 ≥ PACAP-27 > PACAP(2–38) = PACAP(6–38) > PACAP(5–38) = PACAP(7–38) ≥ PACAP(4–38) ≥ PACAP(2–27) ≫

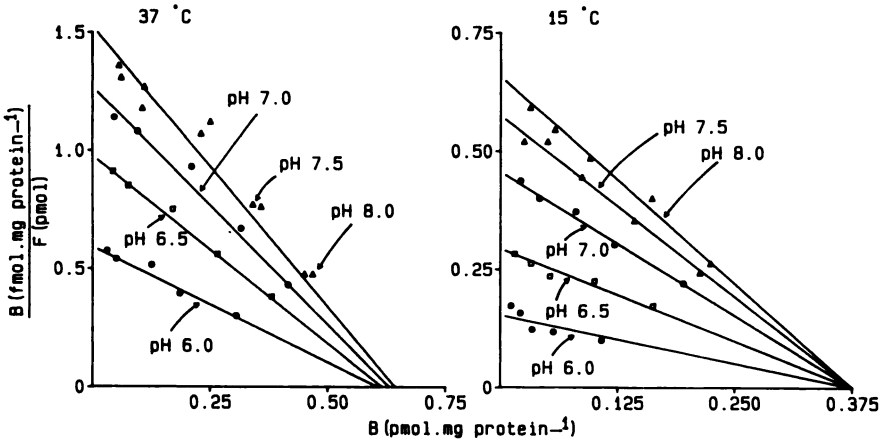

Fig. 3. Scatchard representation of saturation curves obtained after incubation of increasing concentrations of ^{125}I-PACAP-27 with rat pancreatic AR 4–2J cell membranes for 20 min at 37° (*left*) and 15° (*right*) and at pH 6.0, 6.5, 7.0, 7.5, and 8.0. The results are the means of three experiments performed in duplicate, and the membrane preparations in *left* and *right* were different.

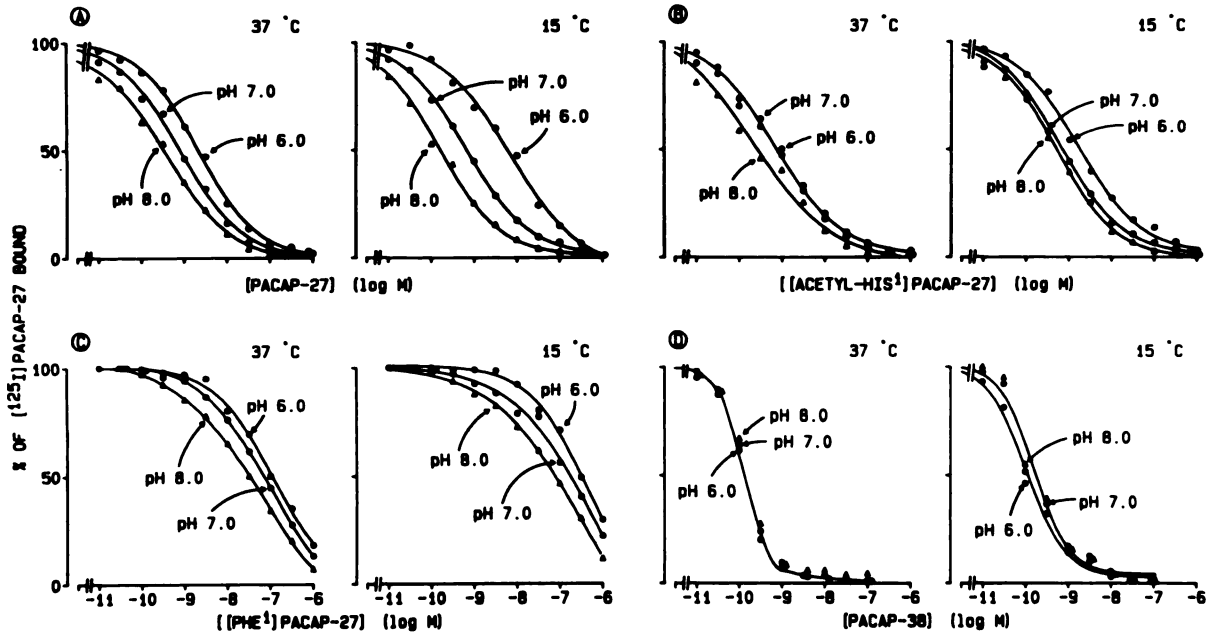

Fig. 4. Dose-effect curves for inhibition of ^{125}I-PACAP(1–27) binding at 37° or 15° by PACAP-27 (A), [AC-His^1]PACAP-27 (B), [Phe^1]PACAP-27 (C), and PACAP-38 (D), in rat pancreatic AR 4–2J cell membranes, at pH 6.0, 7.0, and 8.0. The results are the means of at least three experiments performed in duplicate. Binding data, obtained after 20 min at 37°, are expressed as a percentage of ^{125}I-PACAP(1–27) specifically bound in the absence of unlabeled peptide.

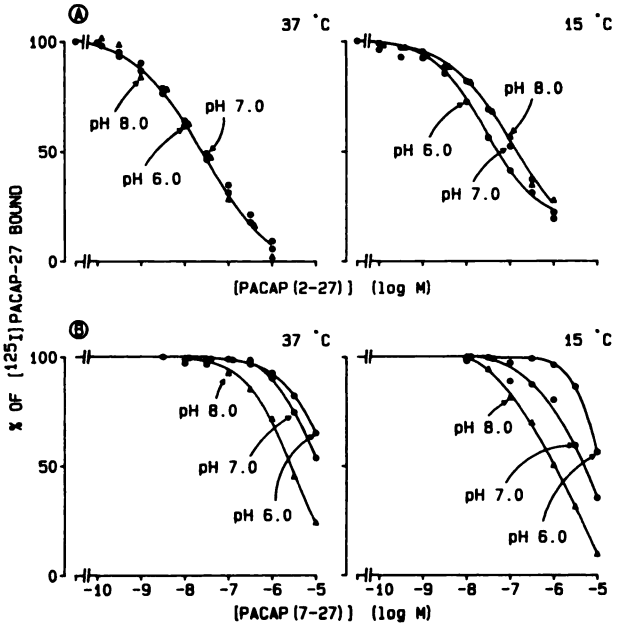

Fig. 5. Dose-effect curves for inhibition of [^{125}I-AC-His^1]PACAP(1–27) binding at 37° or 15° by PACAP(2–27) (A) or PACAP(7–27) (B), in rat pancreatic AR 4–2J cell membranes, at pH 6.0, 7.0, and 8.0. The results are the means of at least three experiments performed in duplicate. Binding data, obtained after 20 min at 37°, are expressed as a percentage of [^{125}I-AC-His^1]PACAP specifically bound in the absence of unlabeled peptide.

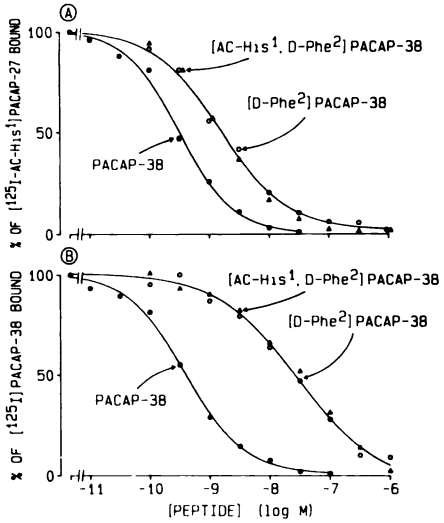

Fig. 6. Dose-effect curves for inhibition of [^{125}I-AC-His^1]PACAP(1–27) (A) and ^{125}I-PACAP-38 (B) binding, in rat pancreatic AR 4–2J cell membranes, by PACAP-38, [D-Phe^2]PACAP-38, and [AC-His^1,D-Phe^2]PACAP-38. Binding data were obtained after 20 min at 37° and are expressed as a percentage of radioligand specifically bound in the absence of unlabeled peptide. The results are the means of at least three experiments performed in duplicate.

PACAP(6–27) > PACAP(7–27). PACAP(2–38) was only 13 times less potent than PACAP(1–38), whereas PACAP(2–27) was 80 times less potent than PACAP-27. PACAP(2–27) was 50–80-fold less potent than PACAP-27 in binding but retained 85% of its efficacy for adenylate cyclase. The IC_{50} of PACAP(3–27), PACAP(5–27), PACAP(6–27), PACAP(7–27), and PACAP(9–27) at PACAP-A receptors was, respectively, 6000-, 2000-, 1600-, 6000-, and 4000-fold higher than that of PACAP(1–27). PACAP(6–27), having lost the ability to stimulate adenylate cyclase, behaved as an antagonist (Table 2), i.e., shifted the adenylate cyclase activation curve of PACAP-27 rightward, with a K_i of 300 nM (Fig. 2, A). PACAP(7–27) behaved as a weaker antagonist.

The K_i of PACAP(6–38) for PACAP-38 enzyme stimulation was 15-fold higher than its IC_{50} for ^{125}I-PACAP-38-labeled receptors, whereas its K_i for PACAP-27 enzyme stimulation was only 2-fold higher than its IC_{50} for [^{125}I-AC-His^1]PACAP-27-labeled receptors.

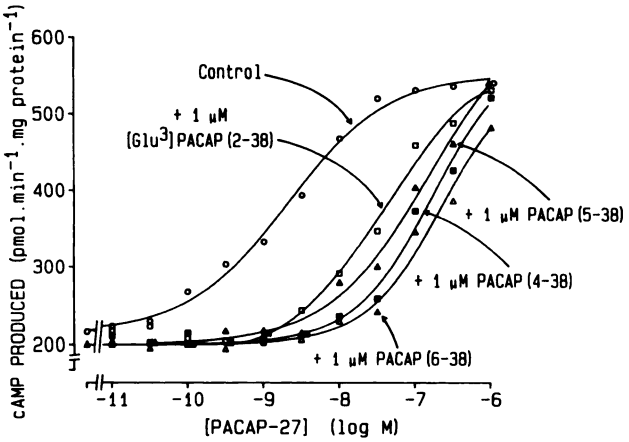

Fig. 7. Dose-effect curves for adenylate cyclase stimulation, in rat pancreatic AR 4–2J cell membranes, by PACAP-27. The enzyme activity was tested as in Fig. 2, in the presence of 10 μM GTP for 10 min, in the absence or presence of 1 μM [Glu³]PACAP(2–38), PACAP(4–38), PACAP(5–38), or PACAP(6–38). The results are the means of experiments performed in duplicate on three different membrane preparations and are expressed in pmol of cAMP produced/min/mg of protein.

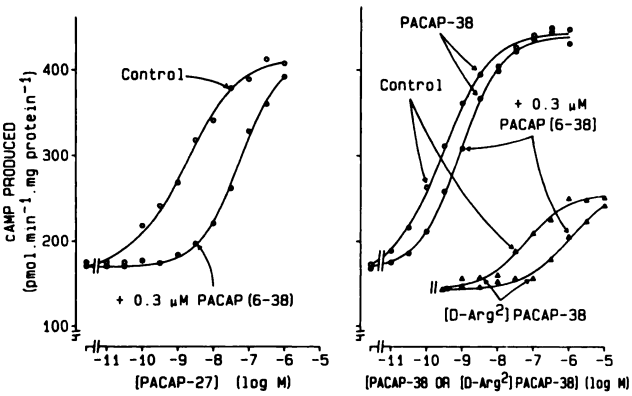

Fig. 8. Dose-effect curves for adenylate cyclase stimulation in rat pancreatic AR 4–2J cell membranes by PACAP(1–27) (*left*) and PACAP(1–38) or [D-Arg²]PACAP(1–38) (*right*). The enzyme activity was tested as in Fig. 2, in the presence of 10 μM GTP, in the absence (*open symbols*) or presence (*closed symbols*) of 0.3 μM PACAP(6–38). The results are the means of experiments performed in duplicate on three to six different membrane preparations and are expressed in pmol of cAMP produced/min/mg of protein.

Interaction of PACAP analogs and PACAP fragments with PACAP-B receptors. PACAP-B receptors, as well as PACAP-A receptors, were labeled with ^{125}I-PACAP-38. PACAP-B receptors exhibited high affinity for PACAP-38 but poor affinity for PACAP-27, amino-terminally shortened PACAP-27 fragments, and all substituted PACAP(1–27) and PACAP(1–38) analogs. Their affinity for amino-terminally shortened PACAP-38 fragments was, however, only slightly lower than (or similar to) that of PACAP-A receptors labeled with [^{125}I-AC-His¹]PACAP-27. In particular, the IC_{50} of PACAP(6–38) for ^{125}I-PACAP-38-labeled receptors (all receptors) was only 3-fold higher than that for [^{125}I-AC-His¹]PACAP-27-labeled receptors (the PACAP-A receptors).

Discussion

PACAP-A and -B receptors and their coupling to adenylate cyclase in rat pancreatic AR 4–2J cell membranes. Competition binding curves in the presence of 0.1 nM [^{125}I-AC-His¹]PACAP-27 and 0.1 nM ^{125}I-PACAP-38 suggested the coexistence of two classes of binding sites, one of the A type, with an IC_{50} of 0.5 nM and 0.3 nM, respectively, for unlabeled PACAP-27 and PACAP-38, and another class, of the B type, with an IC_{50} of 0.3 nM for unlabeled PACAP-38 and 20 nM for unlabeled PACAP-27. [^{125}I-AC-His¹]PACAP occupied PACAP-A receptors preferentially, considering the 120-fold lower IC_{50} of [AC-His¹]PACAP-27 in competition with [^{125}I-AC-His¹]PACAP-27, compared with ^{125}I-PACAP-38 (Table 2); thus, [^{125}I-AC-His¹]PACAP was largely A specific. PACAP-38-preferring receptors (type B) were, like type A receptors, recognized by ^{125}I-PACAP-38. Furthermore, the slope of competition curves of ^{125}I-PACAP-38 binding indicated that PACAP-38 did not discriminate among the receptors, whereas PACAP-27 did.

Were both receptor subtypes coupled to adenylate cyclase? With most PACAP-27 analogs, there was a good correlation between the IC_{50} for binding and the K_{act} for adenylate cyclase activation, suggesting that the effects of PACAP-27-derived agonists could be entirely accounted for by their interaction with PACAP-A receptors. The same held true for inactive fragments; the inhibitory effects of both PACAP(6–27) and PACAP(6–38) (taken as examples) correlated perfectly with their ability to occupy PACAP-A receptors. The data concerning the agonist effects of PACAP-38 derivatives and the inhibitory effects of PACAP-38 fragments on PACAP-38 stimulation are more difficult to explain, due to the fact that none of these molecules was highly selective for the PACAP-A receptor subtype. On the basis of the K_{act} of adenylate cyclase activation, it is tempting to consider that agonists of the PACAP-38 series, such as [AC-His¹,Ala²]PACAP-38, [Arg²]PACAP-38, [AC-His¹,Arg²]PACAP-38, and [D-Arg²]PACAP-38, acted through receptors labeled by ^{125}I-PACAP-38 (Table 3) and to extend this conclusion to all PACAP-38 analogs. However, the poor inhibitory effects of PACAP-38 fragments PACAP(4–38), PACAP(5–38), PACAP(6–38), and PACAP(7–38) on PACAP-38-stimulated adenylate cyclase do not support this hypothesis; these fragments were 3–50-fold less potent for inhibition of PACAP-38-stimulated adenylate cyclase than for PACAP-B receptor occupancy and there was, therefore, no correlation between the K_i of PACAP-38 fragments for the stimulatory effect of PACAP-38 and their affinity for PACAP-A or PACAP-B receptors.

We have no explanation for this discrepancy, but we have formulated three hypotheses. (a) Full adenylate cyclase activation by PACAP-38 could require only a small proportion of the total number of receptors, so that antagonism would be observed only at a high degree of receptor occupancy. The fact that the partial agonist [D-Arg²]PACAP-38 was inhibited, with high potency, by PACAP-38 fragments supports this hypothesis, but the lack of dissimilarity between IC_{50} and K_{act} values is not in favor of spareness. (b) The discrepancy could result from an artifactual selectivity if binding equilibrium was attained more rapidly for PACAP-38 than for PACAP-27. To evaluate the possibility of nonequilibrium data, we repeated our dose-effect curves for enzyme activation (with or without inhibitor) but, instead of using the usual 15-min incubation period, we used three shorter periods (2, 5, and 10 min) and one longer period (30 min); the K_i for PACAP(6–38) was unaffected by these changes. (c) PACAP-38 (but not necessarily its derivatives) could bind nonspecifically to test tubes, shifting the dose-effect curve of adenylate cyclase activation to the right. Our

data were, however, not modified when 1% bovine serum albumin was added to the enzyme assay medium (data not shown).

Structural requirements of PACAP analogs for receptor occupancy and adenylate cyclase coupling. PACAP, VIP, PHI, GRF, and glucagon are structurally related peptides (Table 1), and Asp^3, Phe^6, and Thr^7 are common to all amino acid sequences. The specificity of PACAP-27, compared with VIP, in the residue 1–9 amino-terminal part of the molecule relies on the presence of Gly^4, Ile^5, and Ser^9, instead of Ala^4, Val^5, and Asn^9 in VIP. Whatever the specific tertiary structure that might result in this amino-terminal region, receptor recognition and the ensuing adenylate cyclase activation depended clearly on the residue 1–3 amino-terminal sequence of PACAP-27, with a contribution of each amino acid in this area. We tested whether amino acid substitutions among these first amino acids could alter the ability of the resulting analog to couple the PACAP receptor with adenylate cyclase more than it affected the binding affinity (Table 2).

The IC_{50} of PACAP-27 analogs with the 1-position modified or deleted increased in the following order: [AC-His^1]PACAP-27 ≤ PACAP-27 < [Phe^1]PACAP-27 ≤ PACAP(2–27). Their K_{act} increased as follows: PACAP-27 = [AC-His^1]PACAP-27 < [Phe^1]PACAP-27 < PACAP(2–27); the IA decreased as follows: PACAP-27 ≥ PACAP(2–27) = [AC-His^1]PACAP-27 = [Phe^1]PACAP-27. As indicated by the good potency and efficacy of [AC-His^1]PACAP-27, a free α-amino group was relatively unimportant. Acetylation of the free amino group was even slightly beneficial for receptor recognition and K_{act} in the case of [AC-His^1,Phe^2]PACAP-27. This observation is at variance with the optimal receptor recognition requirements of VIP, secretin, and glucagon, which entail a free α-NH_2 for His^1 (13, 14, 21, 22).

These data probably reflected the influence exerted by the α-amino group of His^1 on the ionization of the imidazole ring. From the pH dependence (sharpest K_d decrease between pH 6.0 and 6.5) and temperature dependence (amplification of the pH effect at 15°, compared with 37°) of PACAP receptor occupancy (see Results), we conclude that the deprotonated imidazole ring of His^1 was a major determinant for optimal affinity recognition of PACAP-27 by the receptors. In strong contrast to PACAP-27, the interaction of PACAP-38 was pH independent, suggesting that (a) both ligands bind to the same receptor through different domains or (b) an intramolecular interaction of the cationic carboxyl-terminal extension of PACAP-38 with His^1 alters the pK_a of the imidazole function while the ligand binds to receptors. It must be noted that the pH dependence of PACAP(7–27) was at much higher K_d levels and could be concerned, for example, with the ionization of a histidine residue in the receptor binding domain itself.

The integrity of the imidazole ring of His^1 was obviously important, because replacing His^1 by aromatic phenylalanine in PACAP-27 decreased the potency 60-fold, i.e., made this compound as potent and efficient as [Des-His^1]PACAP-27.

Changes in position 2 of PACAP-27 directly altered receptor-effector coupling. [L- and D-Ala^2] derivatives were full agonists, whereas [L- and D-Arg^2]- and [L- and D-Phe^2]- derivatives were partial agonists. More precisely, replacement of Ser^2 in PACAP-27 by alanine doubled the potency, but the simultaneous acetylation of His^1 neutralized this modest advantage. A similar replacement in secretin (which possesses the same amino-terminal sequence, H-S-D-G, as PACAP) exerts contrasting effects; it doubles the peptide affinity for human pancreatic membranes (22) but decreases 3-fold the affinity for secretin receptors in rat pancreas (21). The replacement of Ser^2 by D-Ala^2 (in PACAP-27 as well as in the *N*-acetylated His^1 derivative) decreased slightly the affinity but provoked no change in IA. The beneficial effect of Ser^2, compared with Ala^2, was obvious when working in the [Des-His^1] format; the IC_{50} of [Des-His^1,Ala^2]PACAP-27 was 15-fold higher than that of PACAP(2–27), and its K_{act} was 5 times higher (Table 2). Increasing the bulkiness in position 2 played a highly unfavorable role, with the hydrophobicity of Phe^2 and D-Phe^2 being as deleterious as the basicity of Arg^2 and D-Arg^2 (Fig. 1). Substitution of Ser^2 by Phe^2 decreased the potency and efficacy (−67%) of the PACAP derivative. [D-Phe^2]PACAP-27 was a partial agonist (IA, 0.20). Thus, position 2 could only tolerate isosteric substitutions.

An acidic aspartic function in position 3 was also critical for the high affinity of PACAP-27; substitution of Asp^3 by asparagine reduced the affinity of the ligand by as much as 1200-fold and its efficacy by −20%, whereas replacement by glutamate affected the binding affinity much less dramatically (40-fold) and the efficacy by only 13%. In the absence of His^1, [Des-His^1,Asn^3]PACAP-27 showed lower potency than [Asn^3]PACAP-27 and, being devoid of IA, it antagonized the PACAP-27-stimulated response of adenylate cyclase (K_i, 1500 nM). [Des-His^1,Glu^3]PACAP-27 was 100-fold less potent than [Des-His^1]PACAP-27.

The two highly hydrophilic residues (Lys^{20} and Lys^{21}) located in the central portion of a hydrophobic domain in PACAP-27 were of functional importance for receptor interaction; their replacement by helix-breaking Gly^{20} and Gly^{21} reduced the affinity of the resulting peptide 200-fold.

From investigations on sequence deletions, it appears that the amino-terminal part of PACAP-27 was variously implicated in receptor binding and receptor-effector coupling. With deletions increasing further away from the amino terminal in the PACAP-27 series, the IC_{50} of carboxyl-terminal PACAP fragments increased, decreased, and then increased again, giving an irregular trend, PACAP(2–27) < PACAP(6–27) < PACAP(5–27) < PACAP(3–27) = PACAP(7–27) = PACAP(9–27). PACAP(2–27), PACAP(3–27), and PACAP(5–27), with reduced binding potency, were still biologically active. For example, PACAP(3–27), with its 6000-fold reduced affinity, maintained an IA of 0.30, whereas PACAP(6–27), which was still able to recognize PACAP-A receptors, lacked the key elements required to mediate signal transduction. PACAP(7–27) and PACAP(9–27) could still bind with low potency (IC_{50}, 3000 and 2000 nM, respectively), indicating that the residue 9–27 region contained enough of the PACAP sequence to bind to the receptor. To conclude, our data demonstrated that the amino-terminal part of PACAP was absolutely required for initiating the biological response and was an important determinant of receptor affinity.

The carboxyl-terminal hydrophilic extension present in fragments and derivatives of PACAP-38 facilitated high affinity interactions with receptors, indicating a profound change in the requirements for optimal receptor activation. Among amino-terminally shortened peptides, PACAP(4–38), PACAP(5–38), and PACAP(7–38) showed similar affinity (IC_{50}, 20–30 nM). Their lack of efficacy for stimulating adenylate

cyclase (IA, 0) allowed all three fragments to inhibit the PACAP-27-stimulated adenylate cyclase (K_i in the 11–25 nM range) (Table 3). PACAP(2–38) and PACAP(6–38) showed even higher binding potency (IC_{50}, 3–4 nM) but PACAP(2–38) stimulated adenylate cyclase, whereas PACAP(6–38) inhibited PACAP-27 stimulation of the enzyme (K_i, 7 nM). The irregular trend in IC_{50} and K_{act}/K_i values after progressive amino-terminal deletion suggests that the [Gly^4-Ile^5-Phe^6] tripeptidic portion of PACAP-38 was not an essential binding element.

The occupancy of receptors preferentially labeled by [^{125}I-AC-His^1]PACAP-27 and adenylate cyclase activation (or inhibition) were, in general, better achieved with PACAP-38 analogs than with PACAP-27 analogs. The reason for these differences may lie in the distinct secondary structure of the two reference peptides. Indeed, with their first amino acid residues normally present, PACAP-27 and PACAP-38 showed similar potency and efficiency for adenylate cyclase stimulation. If the residue 28–38 carboxyl-terminal extension in PACAP-38 was, then, relatively unimportant for enzyme activation, this was no longer true after minimal amino-terminal deletion; PACAP(2–27) and PACAP(2–38) were still relatively good agonists (on the basis of their IA, 0.85 and 0.80, respectively), but PACAP(2–27) was 100-fold less potent than PACAP(1–27) in terms of K_{act}, whereas PACAP(2–38) was only 30-fold less potent than PACAP(1–38). PACAP(6–38) and PACAP(6–27) were both unable to provoke receptor-enzyme coupling, but PACAP(6–38) was much more potent than PACAP(6–27), with K_i values of 7 and 150 nM, compared with K_i values of 300 nM and 2000 nM for PACAP(6–27), with, respectively, the PACAP-27- and -38-stimulated enzyme. In addition, PACAP-38, PACAP-27, PACAP(6–38), and PACAP(6–27) inhibited [^{125}I-AC-His^1]PACAP-27 and ^{125}I-PACAP-38 binding with different rank orders of potencies.

If the amino-terminal histidine, in its deprotonated form, was a major determinant for high affinity PACAP-27 recognition by PACAP-A receptors, it was less critical for PACAP-38 binding. Concerning position 2, [D-Ala^2]PACAP-38 was a potent full agonist, whereas [D-Arg^2]PACAP-38 was a poor activator and [D-Phe^2]PACAP-38 a potent inhibitor of the PACAP-27 receptor-mediated effect (K_i, 5 nM). Replacement of the acidic aspartate residue in position 3 by alanine in PACAP-38 markedly reduced adenylate cyclase activation through PACAP receptors. At variance with three PACAP-27 analogs D-amino acid-substituted in position 2, which showed no better affinity than the L-amino acid-substituted analogs, the equivalent D-amino acid-substituted PACAP-38 analogs showed higher affinity and a lower K_{act} (or K_i) value. For instance, [D-Arg^2]PACAP-38 was 10-fold more potent (IC_{50} and K_{act}) but no more active than [Arg^2]PACAP-38. [D-Phe^2]PACAP-38 and [*N*-AC-His^1,D-Phe^2]PACAP-38 were without IA, and the first was shown to inhibit competitively the PACAP-27-stimulated enzyme, with a K_i of 5 nM. [Des-His^1,Glu^3]PACAP-38 was a relatively potent antagonist of PACAP-27 (K_i, 30 nM; 50-fold lower than the PACAP-27 equivalent).

Acknowledgments

We thank N. Peuchot and M. Stiévenart for their secretarial help.

References

1. Vigh, S., A. Arimura, K. Köves, A. Somogyvári-Vigh, J. Sitton, and C. D. Fermin. Immunohistochemical localisation of the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in human and primate hypothalamus. *Peptides* **12**:313–318 (1991).
2. Köves, K., A. Arimura, T. G. Görcs, and A. Somogyvári-Vigh. Comparative distribution of immunoreactive pituitary adenylate cyclase activating polypeptide and vasoactive intestinal polypeptide in rat forebrain. *Neuroendocrinology* **54**:159–169 (1991).
3. Arimura, A., A. Somogyvári-Vigh, A. Miyata, K. Mizuno, D. H. Coy, and C. Kitada. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* **129**:2787–2789 (1991).
4. Sundler, F., E. Ekblad, A. Absood, R. Håkanson, K. Köves, and A. Arimura. Pituitary adenylate cyclase activating peptide: a novel vasoactive intestinal peptide-like neuropeptide in the gut. *Neuroscience* **46**:439–454 (1992).
5. Mungan, Z., A. Ertan, R. A. Hammer, and A. Arimura. Effect of pituitary adenylate cyclase activating polypeptide on rat exocrine secretion. *Peptides* **12**:559–562 (1991).
6. Raufman, J. P., R. Malhotra, and L. Singh. PACAP-38, a novel peptide from ovine hypothalamus, is a potent modulator of amylase release from dispersed acini from rat pancreas. *Regul. Peptides* **36**:121–129 (1991).
7. Kawai, K., C. Ohse, Y. Watanabe, S. Suzuki, K. Yamashita, and S. Ohashi. Pituitary adenylate cyclase activating polypeptide stimulates insulin release. *Life Sci.* **50**:257–261 (1992).
8. Buscail, L., P. Gourlet, A. Cauvin, P. De Neef, D. Gossen, A. Arimura, A. Miyata, D. H. Coy, P. Robberecht, and J. Christophe. Presence of highly selective receptors for PACAP (pituitary adenylate cyclase activating peptide) in membranes from the rat pancreatic acinar cell line AR 4–2J. *FEBS Lett.* **262**:77–81 (1990).
9. Robberecht, P., P. Gourlet, A. Cauvin, L. Buscail, P. De Neef, A. Arimura, and J. Christophe. PACAP and VIP receptors in rat liver membranes. *Am. J. Physiol.* **260**:G97–G102 (1991).
10. Lam, H.-C., K. Takahashi, M. A. Ghatei, S. M. Kanse, J. M. Polak, and S. R. Bloom. Binding sites of a novel neuropeptide pituitary-adenylate-cyclase-activating polypeptide in the rat brain and lung. *Eur. J. Biochem.* **193**:725–729 (1990).
11. Gourlet, P., M.-C. Woussen-Colle, P. Robberecht, P. De Neef, A. Cauvin, M.-C. Vandermeers-Piret, A. Vandermeers, and J. Christophe. Structural requirements for the occupancy of specific receptors for the pituitary adenylate cyclase activating peptide (PACAP) and adenylate cyclase activation in pancreatic and neuronal membranes. *Eur. J. Biochem.* **195**:535–541 (1991).
12. Robberecht, P., M.-C. Woussen-Colle, P. De Neef, P. Gourlet, L. Buscail, A. Vandermeers, M.-C. Vandermeers-Piret, and J. Christophe. The two forms of the pituitary adenylate cyclase activating polypeptide (PACAP(1–27) and PACAP(1–38)) interact with distinct receptors on rat pancreatic AR 4–2J cell membranes. *FEBS Lett.* **286**:133–136 (1991).
13. Robberecht, P., A. Cauvin, P. Gourlet, and J. Christophe. Heterogeneity of VIP receptors. *Arch. Int. Pharmacodyn.* **303**:51–66 (1990).
14. Christophe, J., M. Svoboda, J.-P. Dehaye, J. Winand, M. C. Vandermeers-Piret, A. Vandermeers, A. Cauvin, P. Gourlet, and P. Robberecht. The VIP/PHI/secretin/helodermin/helospectin/GRF family: structure-function relationship of the natural peptides, their precursors and synthetic analogues as tested *in vitro* on receptors and adenylate cyclase in a panel of tissue membranes, in *Peptide Hormones as Prohormones: Processing, Biological Activity, Pharmacology* (J. Martinez, ed.). Ellis Horwood Ltd., Chichester, UK, 211–243 (1989).
15. Hruby, V. J., F. Al-Obeidi, and W. Kazmierski. Emerging approaches in the molecular design of receptor-selective peptide ligands: conformational, topographical and dynamic considerations. *Biochem. J.* **268**:249–262 (1990).
16. Fraker, P. J., and J. C. Speck, Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80**:849–857 (1978).
17. Cauvin, A., P. Robberecht, P. De Neef, P. Gourlet, A. Vandermeers, M.-C. Vandermeers-Piret, and J. Christophe. Properties and distribution of receptors for pituitary adenylate cyclase activating peptide (PACAP) in rat brain and spinal cord. *Regul. Peptides* **35**:161–173 (1991).
18. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Ambrosius, D., M. Casaretto, R. Gerardy-Schahn, D. Saunders, D. Brandenburg, and H. Zahn. Peptide analogues of the anaphylatoxin C3a: syntheses and properties. *Biol. Chem.* **370**:217–272 (1989).
21. Robberecht, P., M. Waelbroeck, M. Noyer, P. Chatelain, P. De Neef, W. König, and J. Christophe. Characterization of secretin and vasoactive intestinal peptide receptors in rat pancreatic plasma membranes using the native peptides, secretin-(7–27) and five secretin analogues. *Digestion* **23**:201–210 (1982).
22. Robberecht, P., P. De Neef, M. Waelbroeck, J. C. Camus, J.-L. Scemama, D. Fourmy, L. Pradayrol, N. Vaysse, and J. Christophe. Secretin receptors in human pancreatic membranes. *Pancreas* **3**:529–535 (1988).

Send reprint requests to: Jean Christophe, Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, Building G/E-CP 611, Route de Lennik 808, B-1070 Brussels, Belgium.