Presence of highly selective receptors for PACAP (pituitary adenylate cyclase activating peptide) in membranes from the rat pancreatic acinar cell line AR 4-2J

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We characterized highly selective receptors for PACAP, the pituitary adenylate cyclase activating peptide, in the tumoral acinar cell line AR 4-2J derived from the rat pancreas. PACAP, a novel hypothalamic peptide related to vasoactive intestinal peptide (VIP), was tested as the full natural 38-residue peptide (PACAP-38) and as an N-terminal amidated 27-residue derivative (PACAP-27). The binding sites showed considerable affinity for [^{125}I]PACAP-27 (K_d = 0.4 nM) and PACAP-38, while their affiity for VIP and the parent peptide helodermin was 1000-fold lower. These receptors were coupled to adenylate cyclase, the potency of PACAP-38 and PACAP-27 (K_{act} = 0.2 nM) being much higher than that of VIP (K_{act} = 100 nM) and helodermin (K_{act} = 30 nM). Chemical cross-linking of [^{125}I]PACAP-27 followed by SDS-PAGE and autoradiography revealed a specifically cross-linked peptide with an M_r of 68000 (including 3000 for one PACAP-27 molecule).

Pituitary adenylate cyclase activating peptide receptor; Vasoactive intestinal peptide; Helodermin; Adenylate cyclase; Pancreatic acinar cell line AR 4-2J; (Rat)

1. INTRODUCTION

The AR 4-2J cell line derives from azaserine-induced hyperplastic nodules of the rat exocrine pancreas [1]. CCK_A [2], CCK_B [3], bombesin [4], somatostatin [5], insulin [6], substance P [7], EGF [8], FGF [9], and VIP [10] receptors have already been characterized on the cell surface. In contrast, secretin receptors were detected neither in binding studies nor by adenylate cyclase activation, contrasting with the presence of such receptors in non-transformed rat pancreatic acinar cells. In the present study, we tested the possible interaction of PACAP-38 and a shorter derivative (PACAP-27) on AR 4-2J cell membranes. PACAP-38 is a newly discovered peptide of ovine hypothalamic origin [11] that presents 68% homology with VIP in its N-terminal (1-28) moiety. Our assumption was that PACAP would interact with the VIP receptors of AR 4-2J cells. We found, to our surprise, that PACAP interacted with considerable affinity with highly specific PACAP receptors, coupled to adenylate cyclase, and distinct from VIP-preferring receptors. The functional and structural characteristics of this new type of receptor are described in the present paper.

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Abbreviations: K_d, concentration required for half-maximal inhibition of tracer binding to a given class of receptors; PACAP-38 and PACAP-27, pituitary adenylate cyclase activating peptides in full natural version (38 residues) and as an amidated N-terminal peptide (27 residues); VIP, vasoactive intestinal peptide; rat PHI and PHV, peptide histidine isoleucinamide (1-28) and peptide histidine valine (1-42) from rat; GRF, growth hormone releasing factor; CCK, cholecystokinin; EGF, epidermal growth factor; FGF, fibroblast growth factor; KIU, kallikrein inhibitor unit

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

The full 38 residue peptide (PACAP-38) and its N-terminal amidated 27 residue derivative (PACAP-27-NH_2, quoted below as PACAP-27) were synthesized by solid phase techniques as previously described [11,12]. All other peptides and chemicals were of the highest analytical grade available.

2.2. *Peptide radioiodination*

PACAP-27 was radioiodinated by the chloramine T method and purified as described in [13]. Tracer specific radioactivity was 1 mCi/0.1 nmol.

2.3. *Binding studies*

Binding studies with [^{125}I]PACAP-27 were conducted as described for VIP receptor identification [14]. Briefly, membranes were incubated at 37°C in a total vol. of 0.12 ml containing 50 mM

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Tris/maleate, 5 mM $MgCl_2$, 1.0% (w/v) bovine serum albumin, 100 KIU/ml Trasylol, 0.5 mg/ml bacitracin (pH 7.4) and [^{125}I]PACAP-27 (10000 to 160000 cpm/assay in saturation experiments, and 20000 cpm/assay in standard assay conditions, corresponding to a final tracer concentration of 0.2 nM). Non-specific binding was determined in the presence of 0.1 μM PACAP-27 in competitive studies and 0.3 μM PACAP-27 for Scatchard plots. The separation of membrane bound and free radioactivities was achieved by rapid filtration through glass-fiber filters (GF/C; Whatman, Maidstone, Kent, England) and presoaked for 24 h in 0.1% poly(ethyleneimine).

2.4. *Adenylate cyclase assay*

An adenylate cyclase assay was performed as previously described, at 37°C, in the presence of 0.1 μM GTP, and in a final volume of 0.06 ml [15].

2.5. *Cell culture and crude membrane preparation*

The pancreatic acinar cell line AR 4-2J was maintained as a subconfluent culture in Dulbecco's modified Eagle's medium enriched with penicillin, streptomycin, amphotericin B, and 10% (v/v) fetal calf serum. Cells were grown in 175 cm^2 flasks at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity. For membrane preparation, cells were mechanically detached, washed with fresh culture medium without added fetal calf serum, pelleted at 50 × g for 10 min, lysed with 1 mM $NaHCO_3$ and quickly frozen in liquid N_2. The lysate was then defrosted, centrifuged at 4°C for 10 min at 2000 × g and the supernatant was centrifuged for 10 min at 15000 × g. The pellet was resuspended in 1 mM $NaHCO_3$ and immediately tested.

2.6. *Cross-linking of [^{125}I]PACAP-27 to AR 4-2J membranes*

Membranes were incubated for 20 min at 37°C with 0.5 nM [^{125}I]PACAP-27 in the medium used for the binding assay (see above). After incubation, membranes were washed twice by centrifugation/resuspension in cold 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and resuspended in the same buffer. Disuccinimidyl suberate (1 mM final concentration) was dissolved immediately before use in dry dimethylsulfoxide and added to 99 vol. of the membrane suspension. After a 30-min incubation at 4°C, membranes were washed twice in cold phosphate buffer (see above) then solubilized in an electrophoresis sample buffer made of 125 mM Tris/HCl (pH 6.8) enriched with 5% (w/v) SDS, 1% (w/v) dithiothreitol, 4% (w/v) 2-mercaptoethanol, 10% (w/v) sucrose and 0.02% (w/v) Bromophenol blue. SDS-PAGE was performed under reducing conditions as in [16] with a 12% homogeneous polyacrylamide separating gel (180 × 200 × 1.5 mm). Autoradiographies were conducted for two weeks at −80°C.

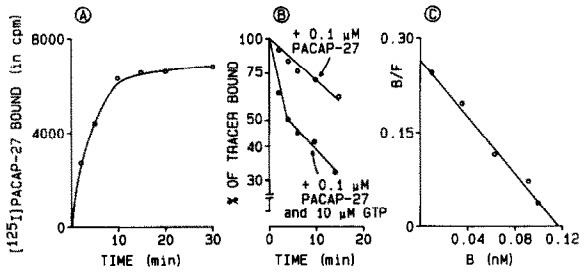

Fig.1. (A) Kinetics of specific [^{125}I]PACAP-27 binding to AR 4-2J membranes. The results are representative of experiments performed in duplicate on 3 different preparations. (B) Kinetics of dissociation of prebound [^{125}I]PACAP-27. After a 20-min incubation of membranes in the presence of [^{125}I]PACAP-27, 0.1 μM unlabelled PACAP-27 was added in the absence (○) or presence (●) of 10 μM GTP, to induce dissociation. The results are the means of experiments performed in duplicate on 3 different preparations. (C) Scatchard representation of a saturation curve of [^{125}I]PACAP-27 binding to AR 4-2J membranes. The results are representative of 3 experiments.

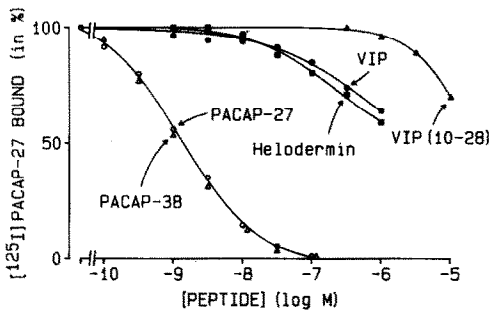

Fig.2. Inhibition of [^{125}I]PACAP-27 binding to AR 4-2J membranes by increasing concentrations of unlabelled PACAP-27 (○), PACAP-38 (△), VIP (●), helodermin (■), and VIP (10-28) (▲). The results are expressed in % of tracer specifically bound in the absence of unlabelled peptide and are the means of experiments performed in duplicate on at least 3 different preparations.

3. RESULTS

Specific [^{125}I]PACAP-27 binding to AR 4-2J membranes was rapid (fig.1A), reversible (fig.1B) and saturable (fig.1C). At 37°C, apparent binding equilibrium was attained after 15 min. Tracer bound after 20 min dissociated with a t_{1-2} of 20 min, as studied by isotopic dilution. This t_{1-2} was transiently reduced to 4 min in the added presence of 10 μM GTP. The Scatchard plot of the saturation curve of tracer binding revealed one class of binding sites with a K_d of 0.4 nM.

PACAP-27 and PACAP-38 were equally potent (IC_{50} of 1.0 nM) to inhibit the binding of 0.2 nM [^{125}I]PACAP-27 while VIP and helodermin were both 1000-fold less potent. Ten μM VIP (10–28) inhibited

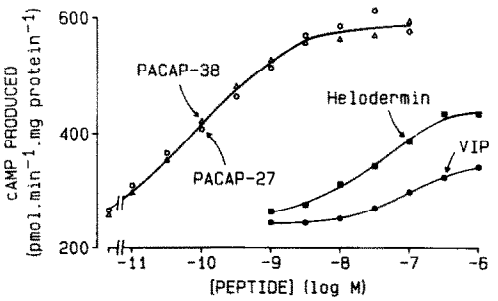

Fig.3. Adenylate cyclase stimulation of AR 4-2J membranes by increasing concentrations of PACAP-27 (○), PACAP-38 (△), VIP (●), and helodermin (■) in the presence of 10 μM GTP. The results, expressed in pmol cyclic AMP produced · min^{-1} · mg $protein^{-1}$ are the means of experiments performed in duplicate on 3 different preparations.

2.7. *Protein concentration*

The protein concentration was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.

tracer binding by 30% (fig.2). PHI, PHI-Gly and PHV from rat [18], and porcine secretin, porcine glucagon and human GRF (1–29)-NH_2 were unable, at a 1 μM concentration, to significantly affect tracer binding (not shown).

PACAP-27 and PACAP-38 were equally potent and efficient in stimulating adenylate cyclase with a K_{act} of 0.2 nM (fig.3). VIP and helodermin showed lower potency (K_{act} of 100 and 30 nM, respectively) and reduced efficacy (30% and 50%, respectively, of the maximum effects of PACAP-27 and PACAP-38). We were unable to consistently inhibit the stimulatory effects of PACAP-27 and PACAP-38 with high concentrations of VIP or helodermin (data not shown).

After [^{125}I]PACAP-27 cross-linking to AR 4-2J membranes, SDS-PAGE followed by autoradiography revealed a unique M_r 68 000 band, under reducing conditions (fig.4). The labelling of this band was markedly reduced when membranes were incubated in the presence of 0.1 μM PACAP-27, but was unaffected by the presence of 0.1 μM VIP in the incubation medium.

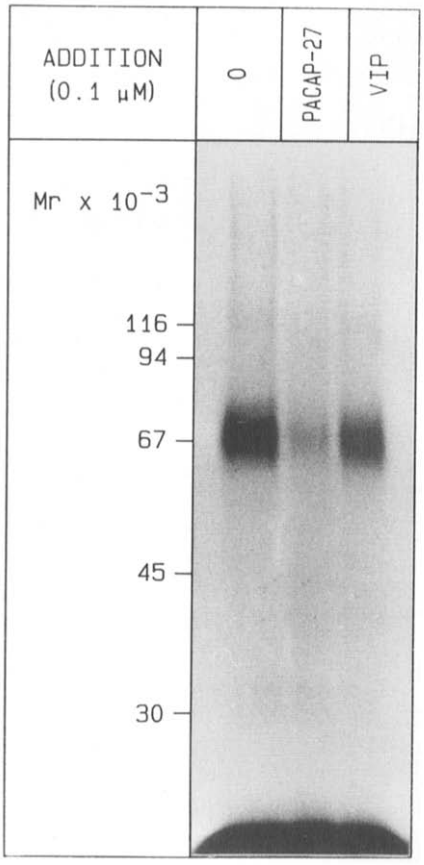

Fig.4. Cross-linking of [^{125}I]PACAP-27 to AR 4-2J membranes. Membranes were incubated with [^{125}I]PACAP-27 in the absence or presence of 0.1 μM unlabelled PACAP-27 or unlabelled VIP. Cross-linking was performed by disuccinimidyl suberate and, after washing, membranes were solubilized and submitted to SDS-PAGE under reducing conditions. Autoradiographies were conducted for two weeks at −80°C (see section 2). Similar results were obtained when the incubation buffer contained ovalbumin instead of bovine serum albumin.

4. DISCUSSION

The present data demonstrate unambiguously the presence, on membranes from the rat pancreatic acinar cell line AR 4-2J, of new specific binding sites for the novel hypothalamic peptide PACAP. These binding sites, when tested with the radioligand [^{125}I]PACAP-27, showed high selectivity as they recognized only PACAP-38 and PACAP-27 with high affinity (IC_{50} of 1 nM). Parent peptides such as VIP, PHI, secretin, helodermin, GRF and glucagon (sequences illustrated in table 1) were at least 1000-fold less potent than PACAP. This high degree of selectivity was comparable to that observed for secretin receptors and VIP receptors when tested for, respectively, VIP and secretin interactions [19]. PACAP binding sites had an M_r of 68 000 (including 3000 for one PACAP-27 molecule), a value somewhat higher than that of 61 000 for VIP binding sites in the same cell line, under reducing conditions [10].

The present PACAP binding sites were coupled to adenylate cyclase and the K_{act} for both PACAP-38 and PACAP-27 was lower (0.2 nM) than their K_d in binding studies, suggesting that only a fraction of receptors needed to be occupied for maximal enzyme activation. Our data on VIP- and helodermin-mediated adenylate cyclase activation did not establish whether these peptides interacted with PACAP receptors or with specific low-affinity VIP/helodermin receptors. We favor the second hypothesis for two reasons: (i) the discrepancy between the VIP and helodermin concentrations required for PACAP receptor occupancy and adenylate cyclase activation was not due to receptor spareness, as the first two peptides showed lower intrinsic activity than PACAP; (ii) high- and low-affinity VIP receptors that also recognize helodermin, have both been identified in AR 4-2J cells [10].

PACAP-38 was isolated from ovine hypothalamus on the basis of its ability to stimulate rat pituitary adenylate cyclase [11] but little is known on other biological effects. The existence of PACAP-27 was deduced, on theoretical ground only, when considering that the consensus sequence Gly-Lys-Arg in PACAP-38 could conceivably facilitate the generation of an amidated peptide. A much higher accumulation of cyclic AMP is observed in pituitary cells submitted to PACAP in place of any other peptide [11]. The in vivo vasodepressor activity of PACAP suggests that it could interact with peripheral VIP receptors but extrahypothalamic locations of PACAP have not yet been established. Our discovery of specific PACAP receptors in an acinar cell line derived from the exocrine pancreas suggests, however, that PACAP could be a new brain-gut peptide.

Table 1

Comparison of the amino acid sequence of PACAP-38 [11] with the related peptides helodermin, VIP and PHI [18]

Species	Peptide	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-*
	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-*
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
b/p	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	PHV (1-42)	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
b/p	Secretin	H-S-D-G-T-F-T-S-E-L-S-R-L-R-D-S-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-*
b/p	Glucagon	H-S-Q-G-T-F-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T

Differences with PACAP-38 are underlined; * = NH_2. b, bovine; hs, *Heloderma suspectum* (gila monster); o, ovine; p, porcine; r, rat

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