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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

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The existence of specific receptors for the two PACAPs (Pituirary Adenylate Cyclase Activating Peptides of 27 and 38 amino acids) was previously demonstrated on membranes from the pancreatic acinar cell line AR 4-2J (Buscail et al., FEBS Lett. 202, 77–81, 1990) by [^{125}I]PACAP-27 binding. Here we demonstrate, by comparing Scatchard analysis of saturation curves and competition binding curves obtained with [^{125}I]PACAP-27 and [^{125}I]PACAP-38 as radioligands, the coexistence of two classes of receptors : 1/ PACAP-A receptors that recognize PACAP-27 and PACAP-38 with the same high affinity (K_d 0.3 nM) and 2/ PACAP-B receptors that recognize PACAP-38 with a high affinity (K_d 0.3 nM) and PACAP-27 with a lower affinity (K_d 30 nM). These two receptors are coupled to adenylate cyclase but can be clearly distinguished by the ability of PACAP(6–27) to specifically inhibit PACAP-27 adenylate cyclase activation.

Pituitary adenylate cyclase activating polypeptide (PACAP) receptor; Adenylate cyclase; Rat pancreatic acinar cell line AR 4-2J

1. INTRODUCTION

Recently, Miyata et al. isolated from ovine hypothalamus two amidated peptides named, respectively, Pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP-38) [1] and the N-terminal amidated 27-residue derivative (PACAP-27) [2,3]. PACAP-27 is 68% similar to VIP. PACAP-38 and PACAP-27 show high affinity for VIP receptors in membranes from lung [4], pancreas [5] and liver [6] from rat, and for helodermin-preferring receptors in the human lymphoblastic cell line SUP-T1 ([7] and Gourlet et al., unpublished data). In addition, PACAP-38 and PACAP-27 interact also with highly selective PACAP receptors of rat central nervous system [4,8,9], cultured rat astrocytes [10], human neuroblastoma cells NB-OK [11], rat adrenochromaffin cells [12], and rat cancerous pancreatic acinar cells AR 4-2J [13]. These PACAP receptors are labelled by [^{125}I]PACAP-27, show almost equivalent affinity for PACAP-27 and PACAP-38 (K_d 0.2–0.4 nM), recognize VIP at high concentration only (K_d around 3 μM), and are positively coupled to adenylate cyclase.

In the present study, we compared the binding properties of [^{125}I]PACAP-27 and [^{125}I]PACAP-38 on AR 4-2J pancreatic cell membranes. Our data suggest that PACAP-38, besides the previously described PACAP-27 receptor [13], interact also with a second binding site more selectively for the longer PACAP form. Indeed, the fragment PACAP(6–27) inhibited the PACAP-27-stimulated but not the PACAP-38-stimulated adenylate cyclase activity, allowing a clear functional discrimination between PACAP-27 receptors and PACAP-38 receptors.

Abbreviations: PACAP-38 and PACAP-27, pituitary adenylate cyclase activating polypeptides in, respectively, the long version (the amidated 38-residue peptide(1–38)) and the N-terminal amidated 27-residue derivative version(1–27); VIP, vasoactive intestinal peptide; rat PHI and PHV, rat peptide histidine-isoleucinamide(1–27) and rat peptide histidine valinamide(1–44)

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2. MATERIALS AND METHODS

2.1. *Cell culture and crude membrane preparation*

The pancreatic acinar cell line AR 4-2J was cultured as described [13]. For membrane preparation, cells were mechanically detached, washed with fresh culture medium, pelleted at 50 × *g* for 10 min, lysed in hypotonic 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 $NaHCO_3$ in order to obtain a protein concentration of approximately 200 μg/ml and immediately tested.

2.2. *Peptide synthesis*

PACAP-27 and PACAP(6–27) were synthesized by solid phase technique on an Automated Applied Biosystems apparatus (Foster

City, CA, USA) using the Fmoc strategy [5]. Their conformity was established by both total amino acid composition and Edman degradation. PACAP-38 and VIP were obtained from Novabiochem (Läufelfingen, Switzerland).

2.3. *Peptide radioiodination*

PACAP-27 was radioiodinated by the chloramine-T method and purified by HPLC as described [13]. PACAP-38 was labelled by the iodogen technique [14], then purified by HPLC under conditions similar to those used for PACAP-27. Four radioactive peaks were separated in these conditions. The first two peaks were separated from unlabelled PACAP-38 and were equally able to specifically bind to membranes. The last two peaks bound poorly and with a high non-specific component. Although the precise nature of each peak was not established, the two first peaks were likely to be monoiodinated forms of PACAP-38. These two peaks were pooled and stored in 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 1.5% (w/v) bovine serum albumin and 0.1% (w/v) Tween 20. Their specific radioactivity was typically 2 mCi/nmol.

2.4. *Binding studies*

Binding was conducted as previously described [11,13]. Briefly, AR 4-2J cell membranes were incubated at 37°C in a total volume of 0.12 ml containing 50 mM Tris-maleate (pH 8.0), 5 mM $MgCl_2$, 1.0% (w/v) bovine serum albumin, 100 KIU/ml Trasylol, 0.5 mg/ml bacitracin, and [^{125}I]PACAP-27 or [^{125}I]PACAP-38 (0.05 nM under standard binding conditions). Non-specific binding of [^{125}I]PACAP-27 and [^{125}I]PACAP-38 was determined in the presence of, respectively, 0.1 μM PACAP-27 and 0.1 μM PACAP-38. The separation of membrane bound and free radioactivities was achieved by rapid filtration through glass-fiber filters (GF/C, Whatman, Maidstone, Kent, UK) presoaked for 24 h in 0.1% poly(ethyleneimine) followed by rinsing with a 0.1 M phosphate buffer (pH 7.4) containing 1% (w/v) bovine serum albumin.

2.5. *Adenylate cyclase assay*

The 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 [15,16].

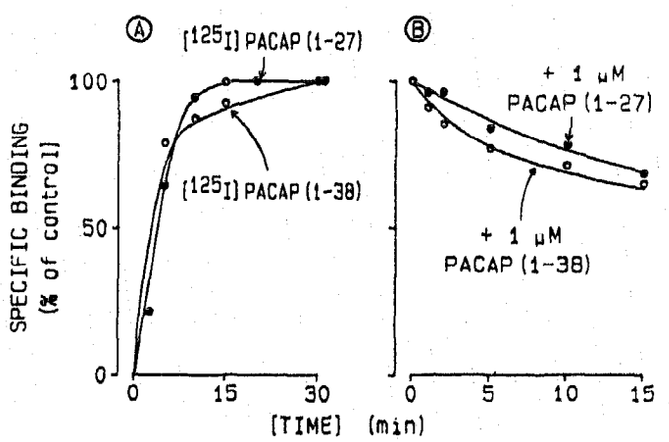

Fig. 1. Association (A) and dissociation (B) kinetics of [^{125}I]PACAP-27 and [^{125}I]PACAP-38 on rat pancreatic AR 4-2J cell membranes at 37°C. Specific binding of [^{125}I]PACAP-27 (○) and [^{125}I]PACAP-38 (●) was expressed in % of the value obtained after a 30 min incubation of each tracer. [^{125}I]PACAP-27 and [^{125}I]PACAP-38 dissociation was induced by adding, after a 30 min preincubation with tracer, 1 μM PACAP-27 and 1 μM PACAP-38, respectively. Results were the means of experiments performed in duplicate on 3 different membrane preparations.

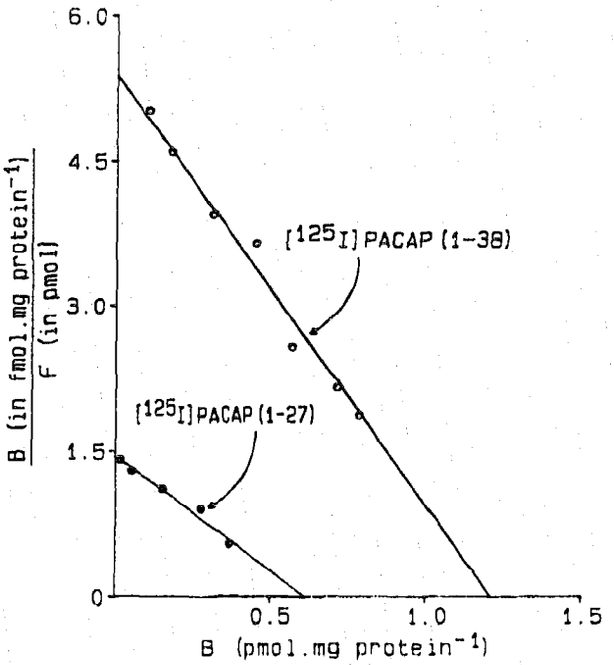

Fig. 2. Scatchard transformation of saturation curves of [^{125}I]PACAP-27 and [^{125}I]PACAP-38 binding to rat pancreatic AR 4-2J cell membranes. B_{max} was expressed in pmol of ligand bound · mg protein^{-1} and F as pM. Values were the means of experiments performed in duplicate on 3 different membrane preparations.

3. RESULTS

[^{125}I]PACAP-27 and [^{125}I]PACAP-38 bound rapidly, specifically and reversibly (by 40% after 15 min, the longest period tested), at 37°C, to AR 4-2J cell membranes (Fig. 1). Saturation curves (Fig. 2) performed with increasing concentrations of both tracers were compatible with the labelling, by each tracer, of one homogeneous class of binding sites. The total number of binding sites labelled by [^{125}I]PACAP-38 and [^{125}I]PACAP-27 was, respectively, 1.2 ± 0.2 and 0.6 ± 0.1 pmol · mg prot.$^{-1}$ (means ± SE of 3 experiments), and the corresponding K_d values were, respectively, 0.23 ± 0.03 nM and 0.40 ± 0.05 nM.

[^{125}I]PACAP-27 binding was inhibited dose-dependently by PACAP-27, PACAP-38, and related peptides with the following decreasing order of potency: PACAP-38 ⩾ PACAP-27 > PACAP(6–27) ⩾ VIP. The inhibition curve of [^{125}I]PACAP-27 binding by unlabelled PACAP-27 was flatter than with PACAP-38, and PACAP(6–27) was 300-fold less potent than PACAP(1–27) (Fig. 3).

[^{125}I]PACAP-38 binding was totally inhibited by unlabelled PACAP-38. PACAP-27 was 30-fold less potent in this respect than PACAP-38, based on the IC_{50} value. Furthermore, the slope of the competition curve of [^{125}I]PACAP-38 binding by PACAP-27 indicated that PACAP-38 recognized one class of binding sites

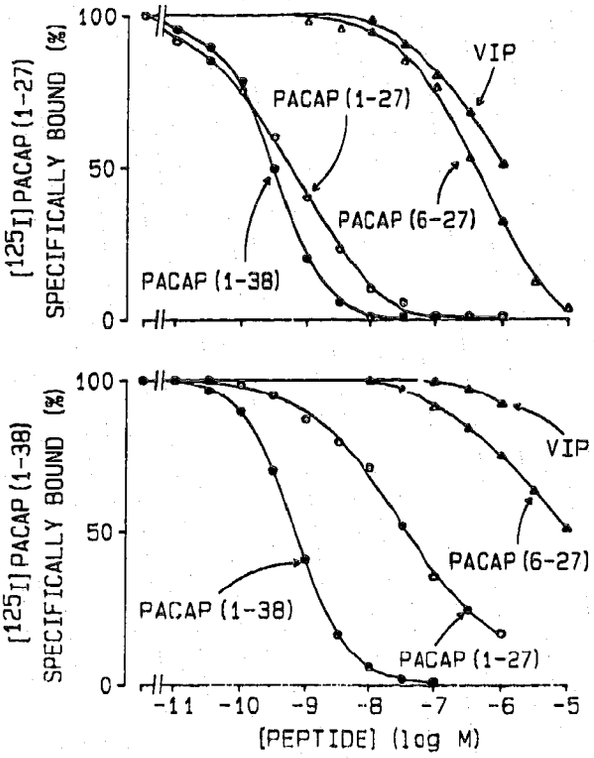

Fig. 3. Dose–effect curves of inhibition of [^{125}I]PACAP-27 (upper panel) and [^{125}I]PACAP-38 (lower panel) binding to rat pancreatic AR 4-2J cell membranes by PACAP-38 (●), PACAP-27 (○), PACAP(6–27) (△) and VIP (▲). Results were the means of experiments performed in duplicate on 3–5 different membrane preparations.

(Hill coefficient of 0.92 ± 0.06; mean ± SD; n = 3) and PACAP-27 recognized more than one class of sites (Hill coefficient of 0.52 ± 0.02). This statistical analysis was further confirmed using the fitting program of Munson and Rodbard [17]. The probability that a two-site model was not better than a one site model for PACAP-27 was only 0.0001. The best fit for a two-site model was obtained considering the coexistence of 63 ± 4% of sites with a K_d of 6.7 ± 1.5 nM and 25 ± 4% of sites with a K_d of 890 ± 410 nM (means ± SE). A statistical analysis of a three-site model could not be performed. The IC_{50} of PACAP(6–27) was 100-fold higher than that of PACAP-27 and this fragment inhibited [^{125}I]PACAP-38 binding 10-times less potently than [^{125}I]PACAP-27. VIP at 1 μM inhibited [^{125}I]PACAP-38 binding by 10% only.

Confirming our previous data [13], PACAP-27 and PACAP-38 stimulated adenylate cyclase with the same efficacy, PACAP-27 being 3-fold less potent than PACAP-38 (Fig. 4). PACAP(6–27) exerted no stimulatory effect but inhibited the stimulated enzyme competitively, when given in combination with PACAP(1–27). Coupling data in Figs. 4 and 5, the K_i for PACAP(6–27) on the PACAP-27-stimulated enzyme can be estimated as 500 ± 50 nM (mean ± SE of 5 experiments), a value similar to the IC_{50} value of this

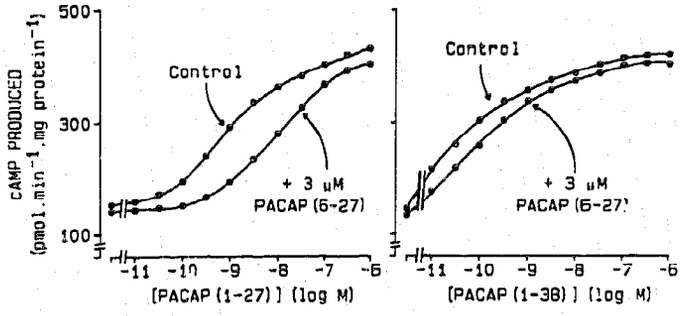

Fig. 4. Dose–effect curves of adenylate cyclase activation by PACAP-27 (left panel) and PACAP-38 (right panel), in the presence of 0.1 μM GTP and in the absence (○) or presence (●) of 3 μM PACAP(6–27). Results were expressed in pmol cyclic AMP produced · min^{-1} · mg protein^{-1} and were the means of experiments performed in duplicate on 3 different membrane preparations.

fragment on [^{125}I]PACAP-27 binding (Fig. 3). In contrast, the inhibition of the PACAP-38-stimulated enzyme, in the combined presence of 10 μM PACAP(6–27) and 0.1 nM PACAP-38, was weak and did not exceed 40%: the K_i of PACAP(6–27) on PACAP-38-stimulated adenylate cyclase could not be calculated accurately but appeared to be higher than 3 μM (Fig. 5).

4. DISCUSSION

Four arguments support the view that at least two classes of selective PACAP receptors coexist in rat pancreatic AR 4-2J membranes: (i) the total number of sites labelled by [^{125}I]PACAP-38 was two-fold higher than that labelled by [^{125}I]PACAP-27; (ii) PACAP-38, PACAP-27, PACAP(6–27) and VIP inhibited [^{125}I]PACAP-27 and [^{125}I]PACAP-38 binding with different rank of potencies; (iii) inhibition curves of tracer binding with PACAP-27 were flatter than those obtained with PACAP-38; (iv) PACAP(6–27) inhibited

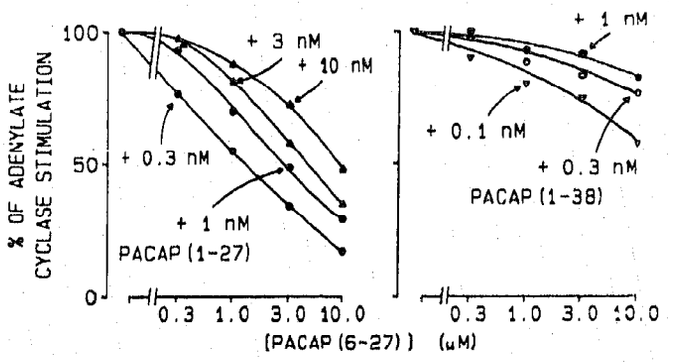

Fig. 5. Inhibition by increasing PACAP(6–27) concentrations of adenylate cyclase stimulation by different concentrations of PACAP-27 (left panel) or PACAP-38 (right panel). The results were expressed in % of adenylate cyclase activity without PACAP(6–27), after subtraction of the basal value and were the means of experiments performed in duplicate on 3 different membrane preparations.

PACAP-27-stimulated adenylate cyclase more potently than the PACAP-38-stimulated enzyme.

Thus, a first class of PACAP-A receptors seemed to recognize PACAP-27 and PACAP-38 with the same high affinity (0.3 nM) and VIP with low affinity. PACAP-27 stimulation of adenylate cyclase through these receptors was inhibited by PACAP(6–27) with a K_i of 500 nM. The second class of PACAP-B receptors recognized PACAP-38 with high affinity (0.3 nM), PACAP-27 with lower affinity (IC_{50} 30 nM), and was virtually unable to recognize VIP. PACAP-38 stimulation of adenylate cyclase occurred essentially through this class of receptors as it was only weakly inhibited by PACAP(6–27). Therefore, PACAP-38 recognized PACAP-A and PACAP-B receptors equally well but appeared to activate adenylate cyclase through PACAP-B receptors only: this could be due to a higher density of PACAP-B receptors and/or to more efficient receptor-Gs coupling.

PACAP-27 and PACAP-38 coexist in mammalian tissues [1,3] and derive from the same precursor [1–3]. Their processing remains to be established and, by analogy with the glucagon system [18], both PACAPs may conceivably result from independent, tissue-specific, cleavage rather than from successive cleavage involving PACAP-38 as a PACAP-27 precursor. The presence of distinct receptors for different molecular forms of biologically active peptides has already been documented: for instance, glucagon and oxyntomodulin (a C-terminally extended form of glucagon) act on diverse receptors in several tissues and exert different physiological functions [19,20]. This is by no means a general rule as, by way of another example, the three different molecular forms of rat PHI (PHI, PHI-glycine and PHV [21,22]) bind to the same receptors [23].

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