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The pituitary adenylate cyclase activating polypeptide (PACAP I) and VIP (PACAP II VIP_1) receptors stimulate inositol phosphate synthesis in transfected CHO cells through interaction with different G proteins

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Abstract

The PACAP receptor (PACAP I receptor, selective for PACAP) and the PACAP II VIP_1 receptor (recognizing PACAP and VIP with the same high affinity) were stably expressed in Chinese Hamster Ovary (CHO) cells. Cell lines expressing different receptor densities, as measured by binding saturation curves, were selected. Inositol phosphate production was stimulated dose dependently in all the cell lines by PACAP and VIP, and the order of potency of the agonists was identical to that of high affinity receptor occupancy. The stimulatory effect of a saturating peptide concentration was proportional to the total receptor density. At similar receptor densities, however, the PACAP receptor mediated stimulation was higher than the VIP receptor-mediated stimulation. Pretreatment of the cells with pertussis toxin for 8 h had no effect on receptor densities, did not alter the PACAP stimulated inositol phosphate synthesis by the cells expressing the PACAP I receptor but markedly inhibited the response of the cells expressing the PACAP II VIP_1 receptor. Thus, the present results indicate that the two G_s-coupled PACAP I and PACAP II VIP_1 receptors may stimulate IP production. The maximal stimulation depended on the number of receptor expressed; the PACAP I and PACAP II VIP_1 receptors probably activated the phospholipase C through G proteins of the G_q, and of the G_i/G_o families, respectively.

Keywords: VIP1 receptor; PACAP receptor; Inositol phosphate; G protein

1. Introduction

Two natural forms of PACAP: PACAP-27 and the C-terminally prolonged peptide PACAP-38 are synthesized by alternative processing of a single peptide precursor. Both PACAPs (PACAP-27 and PACAP-38) and VIP activate two receptor classes: the PACAP I receptors, which are selective for both PACAPs and have a low affinity for the parent peptide VIP, and the PACAP II receptors, which are non selective and have a similar high affinity for VIP and both PACAPs. The latter receptors were previously named VIP receptors [1,2].

Both receptor classes activate the adenylate cyclase. In several cells naturally expressing the selective PACAP I receptors [3–6], PACAPs also stimulate the IP_3 production and increase the cytoplasmic

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calcium concentration. In contrast, in rat GH_3 pituitary cells [5] that express VIP receptors, PACAP and VIP stimulate cyclic AMP production but have a negligible effect on inositol phosphate accumulation. These observations suggested that the PACAP I receptor but not the PACAP II receptors are able to stimulate both messenger pathways.

The concept that a single ligand can activate more than one second messenger pathway is not new, but proof that this may occur through interaction with a single receptor was offered only recently following the expression of recombinant receptors, in cell lines that do not constitutively express the receptor studied or any related receptor. It was thus demonstrated that several recombinant G_s-coupled receptors can stimulate not only the cyclic AMP production but also the phospholipase C activity (dopamine D_1 [7], TSH [8], LH [9], PTH [10], calcitonin [11], glucagon [12], glucagon-like peptide-I [13], vasopressin V2 [14], β_2-adrenergic receptor [14] and PACAP receptors [15,16]). In most of the systems studied, IP increases occur at higher agonist concentrations than the cyclic AMP increases and require a higher receptors density [14,17,18]. The presence of receptor reserve, common for cyclic AMP increase, has not been demonstrated for the stimulation of the IP cascade by these G_s-coupled receptors. Thus, the coupling of these receptors to G_s is much more efficient than their coupling to the G protein(s) activating phospholipase C.

Three receptors recognizing VIP and PACAP have been cloned [19–22]: it is now possible to test the hypothesis that the PACAP I (PACAP-preferring receptor) and PACAP II VIP_1 receptors are indeed coupled to different G proteins, and that PACAP II VIP_1 receptors, unlike naturally expressed and transfected PACAP I receptors [2,15,16], are really unable to activate the $Gq/_{11}$-phospholipase C second messenger pathway.

To investigate this question, we decided to compare the inositol phosphate production in cells stably transfected with either PACAP I or PACAP II VIP_1 receptors. We chose CHO cells for these experiments because they present a rather unusual property: phospholipase C can be stimulated in these cells not only by G proteins belonging to the $G_{q/11}$ family, but also by G proteins belonging to the $G_{i/o}$ family. Since activation of $G_{i/o}$ proteins is readily abolished by pertussis toxin treatment, it is easy to investigate the ability of a single receptor to interact with the three major G protein families in CHO cells: G_s activates the adenylate cyclase, while $G_{i/o}$ and $G_{q/11}$ activate the phospholipase C in a pertussis toxin-sensitive and -insensitive manner, respectively.

It has been suggested that the capacity of a receptor to couple to different G proteins is largely dependent on the number of receptors expressed at the cell surface [14]. If this is the case, the differences between the response of cells expressing PACAP I and PACAP II VIP_1 receptors might be quantitative rather than qualitative. To test this hypothesis we compared the ability of recombinant PACAP I and PACAP II VIP_1 receptors, stably expressed at different densities to stimulate phospholipase C activity.

We had previously observed that ^{125}I-PACAP-38 labelled not only the same receptor population as ^{125}I-PACAP-27 but also an additional receptor population [23,24]. In a subsequent study, we synthesized several PACAP analogues of differing lengths (not naturally expressed) and demonstrated that PACAP-29 (PACAP-27, Gly 28, Lys 29 amide) was the shortest peptide with the same pharmacological profile as PACAP-38 [25].

The comparison of the binding properties of ^{125}I-PACAP-27 and ^{125}I-PACAP-29 with adenylate cyclase stimulation by recombinant PACAP I, PACAP II VIP_1 and chimeric $PACAP/VIP_1$ receptors expressed by CHO cells led us to suggest that ^{125}I-PACAP-29 but not ^{125}I-PACAP-27 labelled all the PACAP receptors, coupled as well as uncoupled to G proteins [26]. We therefore decided to use this tracer, rather than ^{125}I-PACAP-27, to evaluate the total receptor densities in our different cell lines.

2. Materials and methods

2.1. Construction of the expression plasmids, transfection, selection and expression in CHO cells

Expression and selection in cloned CHO cells has been described previously [16,27,28] and is briefly summarized below.

The DNA coding for the rat PACAP II VIP_1 receptor and for the rat PACAP I normal (short) receptor were introduced into a mammalian expression vector derived from pRC/RSV (Invitrogen) that

contains the selectable neomycin phosphotransferase gene, as detailed previously [16,27,28]. The resulting recombinant plasmids were transfected into the CHO cell line DG 44 by electroporation [27,28]. Forty eight hours after transfection, geneticin-resistant cells were selected, and cloned by dilution. Several clones that expressed the PACAP I or the PACAP II VIP_1 receptors respectively were selected on the basis of PACAP stimulated adenylate cyclase activity, and the presence of the expected mRNA, confirmed by RT-PCR. For each recombinant receptor, up to five clones were finally selected on the basis of the receptor density expressed at the cell surface, as explained below.

2.2. Cell cultures

Cells were maintained in α minimal essential medium (αMEM+) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 400 μg/ml geneticin at 37°C in an atmosphere of 95% air-5% CO_2. The subcultures for the experiments were maintained without geneticin in the medium.

2.3. Determination of receptor density

The density of receptors in each of the PACAP I and PACAP II receptor expressing cell lines was determined by Scatchard transformation of saturation binding curves. The tracer used was ^{125}I-PACAP-29 [26] iodinated by the iodogen technique [29], and purified by adsorption on a Sep-Pak Cartridge and elution with 50% acetonitrile in 0.1% trifluoroacetic acid. As receptor saturation could not be achieved with the tracer, the saturation curves were obtained by incubating a fixed amount of tracer and increasing concentrations of unlabelled PACAP-29 [26]. Binding conditions were as follows: crude membranes prepared as described previously [27,28] were incubated in a 50 mM Tris-maleate buffer at pH 7.8 containing 0.5 mM $MgCl_2$, 0.5% BSA and 1 mg/ml bacitracin in the presence of 40 000 cpm of ^{125}I-PACAP-29 and increasing concentrations of unlabelled PACAP-29. Membrane dilution was adjusted for each membrane preparation so that tracer binding was proportional to the amount of membrane protein in the assay; after a 15 min incubation at 37°C the radioactivity bound to the membranes was separated from the free radioactivity by rapid filtration through glass-fiber filters (GF/C Whatman, Maidstone, United Kingdom) presoaked for 24 h in 0.1% polyethyleneimine. Four cell lines expressing different PACAP I receptor densities and five cell lines expressing different PACAP II VIP_1 receptor densities were selected.

2.4. Measurement of accumulation of IPs

Cells were seeded in Falcon 175 cm^2 culture dishes with 40 ml of Dulbecco's medium enriched with 10% FCS and 1 μCi *myo*-[^{3}H]inositol/ml and were grown for 72 h. When pertussis toxin was tested, 100 ng/ml of the toxin was added to the culture 8 h before cell harvesting. The cells were rinsed with Dulbecco's medium, harvested with trypsin/EDTA, washed twice by centrifugation and resuspended with Dulbecco's medium containing 15 mM LiCl then preincubated for 10 min at 37°C. Aliquots of the cell suspension were incubated for 15 min in the presence of the tested peptides. The reaction was terminated by the addition of trichloroacetic acid (TCA) 5% (w/v, final concentration), the samples were sonicated and centrifuged. The supernatant was collected and the pellet washed with 5% TCA. The supernatants were combined. TCA was eliminated by four extractions with diethyl ether. After evaporation of residual ether, 100 μl of 10 mM Tris was added to the extract and IPs produced were separated from *myo*-inositol according to a simplified Dowex chromatography procedure derived from that previously described [16]. After elution of *myo*-inositol, the IPs were eluted with 3 ml of 1.0 M formate. We verified that *myo*-inositol was discarded and that IP recovery was 95% when a mixture of standard ^{3}H-IPs (including equal amounts of labelled inositol-1-phosphate, inositol-1,4-bisphosphate and inositol-1,4,5-trisphosphate) was subjected to the same procedure. At the *myo*-[^{3}H]inositol concentration used in these studies, basal IP levels varied between 8 000 and 10 000 dpm per assay and the duplicates were within 5% of each other.

2.5. Chemicals

PACAP-(1–27), PACAP-(1–29) (= PACAP-(1–27)-Gly^{28}-Lys^{29}-amide [25]), PACAP-(1–38) and VIP were synthesized by solid phase methodology. *Myo*-

[^{3}H]inositol (specific radioactivity: 18.3 Ci/mmol) was obtained from Amersham International (Bucks, UK).

3. Results

3.1. PACAP-27, PACAP-38 and VIP stimulated IP production in selected CHO cell lines that expressed PACAP I receptors and PACAP II VIP$_1$ receptors

We first confirmed our previous finding [16] that PACAP-27, PACAP-38 and VIP stimulate dose dependently IP production in clone 2–10 expressing the 'normal' (short) PACAP I receptor (Fig. 1, left panel). A maximal 2.5-fold stimulation was achieved in the presence of 1 μM PACAP-27, PACAP-29 or PACAP-38. PACAP-27, PACAP-38 and VIP also stimulated the IP production in CHO cells, that expressed a comparable (20 pmol/mg protein) PACAP II VIP$_1$ receptor density (Fig. 1, right panel). The three peptides were equipotent; a 1.5 fold stimulation was observed at 1 μM peptide concentrations.

On the two clones studied, the EC$_{50}$ values of the peptides were in perfect agreement with the concentrations required for occupancy of the high affinity receptors which are selectively labelled by ^{125}I-PACAP-27 [26–28].

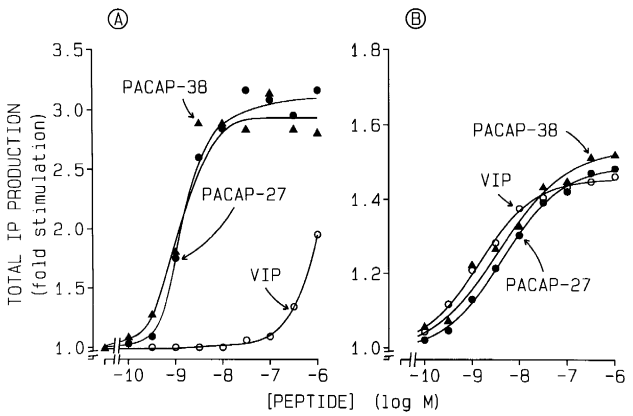

Fig. 1. Dose-response curves of PACAP-27 (●), PACAP-38 (▲) and VIP (○) on the total IP levels after 15 min incubation of CHO cells expressing equivalent concentrations of the PACAP I receptor (panel A) or the PACAP II VIP$_1$ receptor (panel B) (receptor densities ≈ 20 pmol/mg protein). The data are expressed in fold stimulation of IP production (mean of three experiments). The standard deviation was always below 15% of the average value.

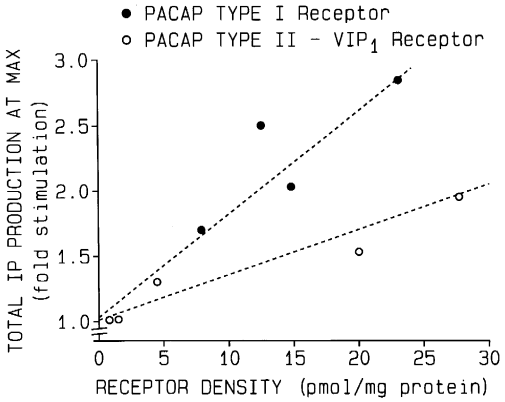

Fig. 2. Effect of receptor density on the total IP production (measured in the presence of 1 μM PACAP-27) of different clones of CHO cells expressing the PACAP I receptor (●) or the PACAP II VIP$_1$ receptor (○) (two experiments in each clone). The standard deviation was always below 15% of the average value.

3.2. Relationship between the number of receptors expressed and the maximal response to PACAP in various transfected CHO cell lines

We compared the stimulation of IP production in several other CHO cell lines, expressing different densities of PACAP I or of PACAP II VIP$_1$ receptors. The receptor density was measured by saturation curves, using ^{125}I-PACAP-29 that labelled G protein-coupled and uncoupled receptors [26]. Scatchard plots were in all cases linear. IP production was evaluated by complete dose-response curves of PACAP-27, PACAP-38 and VIP. In each responsive preparation, the EC$_{50}$ values were identical to those presented in Fig. 1. The maximum IP stimulation is plotted as a function of receptor density in Fig. 2. The maximal inositol phosphate response to PACAP increased with receptor density. At equal receptors densities, the maximal response to PACAP was higher when mediated through PACAP I receptors than through PACAP II VIP$_1$ receptors.

3.3. Effect of pertussis toxin pretreatment on the IP stimulation

The CHO cell lines expressing the highest PACAP I receptors (clone 2–10), and PACAP II VIP$_1$ recep-

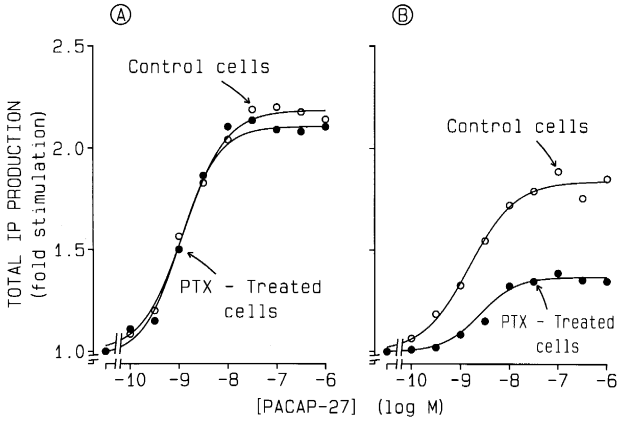

Fig. 3. Dose-response curves of PACAP-27 on the total IP levels after 15 min incubation of CHO cells expressing the PACAP I receptor (panel A) or the PACAP II VIP_1 receptor (panel B), pretreated (●) or not (○) with 100 ng/ml of pertussis toxin 8 h before cell collection. Results expressed in fold stimulation of IP production (mean of three experiments). The standard deviation was always below 15% of the average value.

tors (clone 21) densities were pretreated for 8 h with pertussis toxin before measurement of IP production. The response of the clone expressing the PACAP I receptor was unaffected by the treatment (Fig. 3, left panel). The maximum response but not the peptide sensitivity was markedly reduced in the clone expressing the highest density of PACAP II VIP_1 receptor (Fig. 3, right panel). Pertussis toxin pretreatment did not significantly modify the receptors densities in the two clones tested (data not shown).

4. Discussion

We demonstrated in this work that, like the PACAP I receptors [3–6,16], the PACAP II VIP_1 receptors are capable of stimulating the IP production in transfected CHO cells.

Four (G-protein-activated) phospholipase Cβ isoforms have been well characterized. All these isoenzymes can be activated (to various extents) by α subunits of the $G_{q/11}$ protein family [30], but only two of the four isoforms (β_2 and β_3) can also be activated by G proteins βγ subunits [30–33]. As a result, activation of the abundant $G_{i/o}$ protein family may also result in phospholipase C activation in those cells which express the β_2 and β_3 phospholipase C isoforms. The two mechanisms of phospholipase C activation are easily discriminated by pertussis toxin, which catalyzes ADP-ribosylation of the α subunits of G_i and G_o, and uncouples these G proteins from their cognate receptors.

Phospholipase C stimulation by PACAP and VIP, acting on the PACAP II VIP_1 receptor, was markedly inhibited by preincubation of the cells with the toxin. As the receptor density was not affected by the toxin, it is likely that G_i or G_o proteins are involved in this response. In contrast, the stimulatory effect of PACAP and – at much higher concentrations – of VIP on the PACAP I receptor was unaffected by the pertussis toxin pretreatment, suggesting that this receptor may be coupled to G proteins of the $G_{q/11}$ family. Thus, two receptors of the same subfamily may be coupled to different G proteins. The observation that the 'coupling efficacy' (Fig. 2) of the PACAP I receptor to phospholipase C is higher than that of the PACAP II VIP_1 receptors is also compatible with that interpretation: $G_{q/11}$ proteins also activate phospholipase C isoenzymes (β_1, β_4) which are not sensitive to $G_{i/o}$ proteins, and phospholipase C β_2 and β_3 activation by the βγ subunits released by $G_{i/o}$ is markedly inhibited if the enzyme is phosphorylated by protein kinase A (in the presence of high cAMP levels) [34]. Similar results were obtained with the muscarinic receptors: muscarinic agonists stimulate IP_3 production through interaction with M_2 or M_3 receptors; but, at similar receptor densities, the M_2 response (mediated by $G_{i/o}$ proteins) was lower than the M_3 effect (mediated by $G_{q/11}$ proteins) and the M_2 response but not the M_3 response was inhibited following pertussis toxin treatment ([35], [36] and results not shown).

The physiological relevance of the dual coupling of PACAP I and PACAP II VIP_1 receptors is unclear. As previously observed for several other G_s-coupled receptors (introduction), the efficiency of PACAP I receptors coupling with $G_{q/11}$ and of PACAP II VIP_1 receptors coupling with $G_{i/o}$ is weak, as compared with their interaction with G_s. On the other hand: (1) the PACAPs (and VIP) concentrations necessary to achieve half maximal stimulation of the phospholipase C were low (in the nM range); (2) PACAPs stimulate the IP_3 production and cytoplasmic calcium concentration not only in transfected LLC-PKI [15] and CHO cells [16], but also in normal pituitary gonadotropes [3], in rat gonadotrope-derived α-T3-1

cells [4] as well in PC 12 cells [5] and in the neuroblastoma cell line NB-OK-1 [6] (i.e. in several cells spontaneously expressing the selective PACAP I receptor). (3) In contrast with $G_{q/11}$, $G_{i/o}$ proteins interact with ion (potassium or calcium) channels, and are not usually coupled to phospholipase C. If VIP_1 receptors not only interact with G_{i3} proteins in the absence of GTP [37], but also functionally activate them, this might explain why VIP does stimulate the synthesis of NO through a pertussis toxin-sensitive mechanism in the gastric muscle cells [38].

It might be argued that the receptor concentrations obtained in our transfected cells are markedly greater than the receptor concentrations commonly observed in normal tissues. We should like to stress at this point the fact that we used an unusual tracer (^{125}I-PACAP-29) which (in contrast with ^{125}I-VIP and ^{125}I-PACAP-27) does not label only the G protein-coupled receptors but also recognizes the uncoupled receptors (in excess over the G proteins). In the CHO cell lines used in this study, ^{125}I-PACAP-27 labelled only 11 and 27% of the PACAP II VIP_1 and PACAP I receptors recognized by ^{125}I-PACAP-29, respectively [26].

In conclusion, the present results suggest that the G_s-coupled PACAP I and PACAP II VIP_1 receptors stimulate IP production in transfected CHO cells. The maximal stimulation was dependent on the number of receptor expressed. The PACAP I receptors probably activated the phospholipase C through a G protein of the G_q family, whereas the PACAP II VIP_1 receptors acted through G proteins belonging to the G_i or G_o family.

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