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Differential Signaling and Immediate-Early Gene Activation by Four Splice Variants of the Human Pituitary Adenylate Cyclase-Activating Polypeptide Receptor (hPACAP-R)

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an important, newly isolated neuroenteric hormone in the vasoactive intestinal polypeptide (VIP), secretin, and glucagon family, having been isolated in 1989 from ovine hypothalamus by Arimura and shown to have potent adenylate cyclase-stimulating activity. Two biologically amidated forms of the hormone, PACAP-38 (P-38) and PACAP-27 (P-27), are processed from the same gene, share identical 27 N-terminal amino acids, and show high interspecies conservation of their amino acid sequences.^{1,2}

Radioligand binding and cloning studies have identified three major types of PACAP receptors (types 1, 2, and 3) differing in their affinity for PACAP, VIP, and helodermin.^{2–6} The type 1 PACAP receptor has high affinity for only PACAP-38 and PACAP-27, the type 2 PACAP receptor (classic VIP receptor) shows high affinity for PACAP-38, PACAP-27, and VIP, and the type 3 PACAP receptor shows high affinity for PACAP, VIP, and helodermin. The three PACAP receptor subtypes show different as well as overlapping tissue distributions. The PACAP receptor splice variants described in this study show the highest affinity for P-38 and P-27 compared to VIP or helodermin consistent with the type 1 PACAP receptor pharmacology.⁵

PACAP hormone expression has been identified in numerous tissues including the brain, gastrointestinal tract, adrenal gland, and testis by immunohistochemistry and radioimmunoassay.^{7,8} Although its exact role has not been clearly determined, the broad distribution of PACAP suggests that it may act as a neurotransmitter or neuromodulatory peptide. Preliminary studies have shown that PACAP acts as a growth factor in some cultured cells such as in hippocampal cells where the hormone appears to exert a protective effect against the lethal actions of the gp 120 envelope protein of the human immunodeficiency virus (HIV).⁹ Another important effect of PACAP is its ability to stimulate cellular growth and differentiation in both cultured cells and in transplanted tumors

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in whole animals. In AR-42J cells, PACAP stimulates an increase in ornithine carboxylase activity and cell proliferation.⁸ In the rat pheochromocytoma cell line, PC-12, PACAP increases neurite outgrowth.¹⁰ PACAP-27 has been recently shown to stimulate colony formation in the nonsmall cell lung cancer cell line, NCI-H838, an effect that is antagonized by the PACAP antagonist PACAP(6–38) in the absence or presence of PACAP-27.¹¹ In nude mice transplanted with NCI-H838 xenografts, the PACAP antagonist PACAP(6–38) significantly slows tumor growth. Taken together, these findings suggest that PACAP may exhibit profound effects for modulating the growth of tumoral cells.

In native cell systems, PACAP-Rs have been shown to exhibit coupling to both adenylate cyclase and phospholipase C signal transduction pathways such as in the rat anterior pituitary gonadotrophs, and the rat pheochromocytoma cell line, PC-12.^{10,12} In PC-12 cells, PACAP-Rs also show differential affinities for the ligands P-38 and P-27 such that P-38 is 1000-fold more potent than P-27 at stimulating cAMP. Thus, in the rat, the variable expression of a single gene for a hormone and a single gene for its receptor may result in diverse cellular activities.

We previously cloned the rat type 1 PACAP receptor (rPACAP-R) cDNA encoding a unique 495 amino acid protein belonging to the seven transmembrane VIP/secretin/glucagon family of G-protein coupled receptor.⁵ The rat type 1 PACAP-R was later shown to be expressed as four major splice variants due to alternative splicing of two exons and shown to exhibit variable tissue expression and coupling to adenylate cyclase (AC) and phospholipase C (PLC).¹³ To determine whether the human PACAP-R gene is also alternatively spliced to generate physiologically relevant splice variants and to characterize their pharmacology and signal transduction, the cDNAs of four major hPACAP-R splice variants and their gene were cloned.¹⁴ The hPACAP-R gene contains two variably spliced exons that express four splice variant homologs similar to the rPACAP-R splice variants (null, SV-1, SV-2, SV-3). Unlike the rPACAP-R splice variants, all of the hPACAP-R splice variants demonstrate coupling to both AC and PLC. No differences in the potencies or efficacies were detected for either P-38- or P-27-stimulated increases in AC or PLC. However, the efficacy of PACAP-stimulated coupling to PLC differed among the hPACAP-R splice variants. Ligand-induced expression of the protooncogenes, c-fos, and c-myc paralleled the magnitude of PLC stimulation for the hPACAP-R splice variants. These differences in receptor-effector coupling may represent a novel mechanism in humans by which the specificity of receptor splice variants leads to greater diversity in cellular physiology by affecting the magnitude of ligand-stimulated phospholipase C response and expression of immediate-early gene transcription.

MATERIALS AND METHODS

Establishment of Stably Transfected NIH/3T3 cells. Expressing hPACAP-R Splice Variants

The coding regions for each hPACAP-R splice variant cDNAs were subcloned into pCDL-SRα/NEO at EcoRI restriction sites.¹⁴ Each splice variant was stably transfected into NIH/3T3 cells using electroporation (2×10^7 cells, 500 mFd, 0.25 kV) and the cells plated at various dilutions in Dulbecco's Modified Eagle Medium (DMEM) containing calf serum (10%) using Geneticin® selection. To eliminate the possibility of clonal variation, five

different clones within each group of splice variants were characterized. In addition, to evaluate the effects of receptor density on efficacy for cAMP and total IP stimulation, clones of NIH/3T3 cells expressing similar numbers of receptors (10,000–20,000 receptors/cell) were used.

Binding Assays

Suspended NIH/3T3 cells (500 μ L) were incubated for 60 minutes at 37°C with 50 pM of [125 I]-PACAP-27 either with or without the indicated concentrations of unlabeled peptides P-27, and P-38. Cells were subsequently pelleted (10,000 $\times$ g) and washed three times at 4°C with 200 mL PBS containing BSA 4 mg/mL and centrifuged at 10,000 $\times$ g. The cell fraction was subsequently assayed for gamma radioactivity.

cAMP Assays

Intracellular cAMP levels were assayed using a modification of the procedure described by Salomon *et al.*¹⁵ Briefly, stably transfected NIH/3T3 cells were plated on 24-well culture dishes overnight with DMEM containing 10% calf serum in the presence of [3 H]-adenine at a concentration of 2 mCi/mL. The cells were washed with DMEM alone and incubated with or without the indicated concentrations of P-38 and P-27 in DMEM containing BSA (1%) and 3-isobutyl 1–1-methyl-xanthine (IBMX) for 30 minutes and then aspirated. One hundred microliters of 5% SDS/1 mM cAMP solution was used to lyse the cells followed column chromatography with a Dowex AG-50W-X4 resin (BioRad, Richmond, CA) and an aluminum oxide column (Sigma, St. Louis, MO). Eluates were collected in scintillation vials and counted using a Beckman liquid scintillation counter.

Inositol Phosphate Assay

Total [3 H]-inositol phosphates were measured by strong anion exchange chromatography (Dowex AG 1-X8), using a modification of the method described by Berridge *et al.*¹⁶ Briefly, transfected cells were plated on 24-well culture plates with DMEM/10% calf serum in the presence of 100 mCi/mL myo-2-[3 H] inositol and incubated overnight. The following day, the medium was aspirated and the cells were incubated with PI buffer (20 mM HEPES, 2 mM CaCl_2 , 1.2 mM MgSO_4 , 10 mM LiCl, 11.1 mM, glucose, 0.5% BSA) and exposed to the indicated concentrations of peptide. Eluates were assayed using a Beckman liquid scintillation counter.

Northern Blot Analysis

Two micrograms of poly (A)+ RNA isolated by oligo d(T) chromatography were loaded in each lane, separated by electrophoresis on a 1.5% agarose/formaldehyde denaturing gel and blotted overnight onto S&S Nytran® (Schleicher and Schuell, Keene, NH), as described previously.⁵ The blot was hybridized with a [32 P]-random prime labeled probe overnight and washed at high stringency (3 $\times$ 20' washes with 0.1 $\times$ SSC, 0.1% SDS @ 42°C). The blot was exposed for 48 hours and processed using a Phosphorimager® (Molecular Dynamics, Sunnyvale, CA).

The determination of c-fos and c-myc were performed on NIH/3T3 cells stably transfected with each of the hPACAP-R splice variants. The cells were exposed to the indicated

concentrations of peptide and total RNA was isolated and separated by electrophoresis and blotted on Nytran® as described previously. Specific ³²P dCTP cDNA probes for c-fos and c-myc were allowed to hybridize to the blots overnight and washed at high stringency conditions the following day. Blots were exposed to Kodak XAR-2 film for 24–48 hours and the quantity of hybridizing probe quantitated using a densitometer.

RESULTS AND DISCUSSION

Cloning of the Human PACAP Receptor Gene and Four Splice-Variant cDNAs

As described previously, the hPACAP-R gene was cloned from a human placental genomic library using a full-length hPACAP-R cDNA probe.¹⁴ DNA sequence analysis of a region of the gene encoding the third intracellular loop from the longest clone identified two exons using criteria of splice donor/acceptor consensus sequences (Exons SV-1 and SV-2). This organization in gene structure is similar to the rat giving rise to four major human splice variants SV-1, SV-2, SV-3, or null by alternative splicing. Four hPACAP-R splice-variant cDNAs were isolated and cloned using both low stringency library screening with a radiolabeled rPACAP-R cDNA probe and the polymerase chain reaction as described.¹⁴ FIGURE 1 shows the deduced amino acid structure of the hPACAP-R.¹⁴ The hPACAP-R-null splice variant represented the predominant clone in the cDNA library encoding a unique 468 amino acid protein sharing a 93% homology to the rPACAP-R.⁵ A second clone identified by library screening contained an additional 84 nucleotides encoding 28 amino acids in the third intracellular loop (hPACAP-SV2) (Exon SV2) that is identical to the rPACAP-R-hop splice variant.¹³ A third splice variant, hPACAP-R-SV-I (Exon SV-2) included 84 nucleotides encoding 28 amino acids in the third intracellular loop that showed the greatest similarity to the published rPACAP-R-hip but differed by two amino acids (A415T, and L430P).¹⁴ The fourth major splice-variant cDNA cloned, hPACAP-R-SV3 (Exons SV-1 and SV-2), contained an additional 168 bases encoding 56 amino acids in the third intracellular loop and showed the greatest homology to the previously published rPACAP-R hip-hop splice variants.⁵

Functional Expression of the Human PACAP Receptor Splice Variants

Pharmacological Characterization—The displacement curves for P-38 and P-27 are nearly identical for each of the hPACAP-R splice variants stably expressed in NIH/3T3 cells.¹⁴ For both P-38 and P-27, half-maximal inhibition (IC₅₀) was observed at 22 nM and 32 nM, respectively (TABLE 1). Cells transfected with the SV-1 splice-variant receptor cDNA showed approximately twofold less affinity for either P-38 or P-27. Neither VIP nor secretin showed high affinity for the cloned hPACAP-R splice variants at concentrations up to 1 μM. These ligand binding affinities are similar to the previously published rat type 1 PACAP-R (IC₅₀'s P-38 = 8 nM; P-27 = 20 nM) and are similar to studies reported for native tissues such as human brain and rat hypothalamic, anterior pituitary and brain membranes,¹⁸ AR-42J cells, and human NB-OK1 cells.^{5,17,19,20}

Stimulation of Adenylate Cyclase—Each of the four splice variants showed nearly identical dose response curves for stimulation of adenylate cyclase with detectable stimulation for both P-38 and P-27 at 0.1 nM, half-maximal (EC₅₀) stimulation at

approximately 0.7–1 nM for P-38 which was approximately twofold greater than P-27, and maximal stimulation at 50–100 nM for both P-38 and P-27. P-38 and P-27 increased intracellular cAMP with nearly identical potencies ($EC_{50} \approx 1$ nM) for each of the four hPACAP-R splice variants (TABLE 1). Stimulation of adenylate cyclase was not observed for VIP and secretin at concentrations up to 1 μ M similar to rat type 1 PACAP receptors⁵ These results are in contrast to results obtained for the rPACAP-R splice variants, wherein the rat P-38 and P-27 are up to 60-fold less potent in stimulating cAMP for the “hip” and “hip-hop” splice variants compared to the “null” and “hop” splice variants.¹³ Differences in receptor density ($\approx 10,000$ to 200,000 receptors/cell) did not influence either the potency or efficacy for adenylate cyclase activation similar to results observed for transfected 5-hydroxytryptamine 1A receptors.²¹ To determine the efficacy for stimulating adenylate cyclase, five separate clones of NIH/3T3 cells stably transfected with each splice variant were used to minimize the effects of clonal variation. As shown in TABLE 1 each of the hPACAP-R splice variants showed similar efficacies for stimulating cAMP.

Stimulation of Total Inositol Phosphates for hPACAP-R Splice Variants—

PACAP-38 and PACAP-27 stimulated total inositol phosphates with a similar dose response relationship. In addition, all of the splice variants showed nearly the same dose response to P-38 and P-27, with detectable stimulation at 10 nM, half-maximal stimulation (EC_{50}) at 35–50 nM, and maximal stimulation at 1 mM (TABLE 1). This dose response for IP stimulation paralleled the ligand binding affinity, suggesting that, unlike for adenylate cyclase stimulation, there is minimal receptor “spareness” for phospholipase C coupling. These findings for the hPACAP-R splice variants are different from the results reported for the rPACAP-R splice variants in which P-38 and P-27 failed to stimulate IP for the rat “hip” splice variant and stimulated the “hip-hop” splice variant with a potency that was threefold lower than the “null” and “hop” splice variant.¹³ These differences between the rat and human splice variants may reflect differences in the complement of G-proteins and downstream effectors for the different transfected cell lines (LLC-PK1 versus NIH/3T3), expression of receptors in transient versus stable transfections, or differences in receptor structure between rats and humans.

Although the potencies for stimulation of total inositol phosphates for each human splice variant were similar, their efficacies for stimulating total inositol phosphates were different, with the SV-2 isoform having nearly a 7-fold greater efficacy compared to the null variant and almost an 11-fold greater efficacy compared to the SV-1 and SV-3 splice variants (TABLE 1). To explain our findings and reduce the potential for clonal variation, five distinct clones of NIH/3T3 cells transfected with each splice variant were used to determine efficacy. To further exclude the potential impact of variable receptor densities on studying the potencies and efficacies of each splice variant, clones expressing similar receptor densities (50,000–80,000 receptors/cell) for each splice variant were used. Each of the hPACAP-R splice variants increased intracellular cAMP to nearly the same maximal level. P-38 stimulated total inositol phosphates in a dose-dependent manner with stimulation at 1 nM, half-maximal at 10 nM and maximal at 1 mM. The null and SV-1 splice variants were nearly identical in their ability to stimulate IP. The stimulation of inositol phosphates by the SV-2 splice variant was nearly threefold greater than the null and SV-1 splice variants, and the

SV-3 was intermediate in its ability to maximally stimulate IP. These results suggest that each human PACAP-R splice variant couples with different affinities to heterotrimeric G-proteins similar to $\alpha 2A$ -adrenergic and muscarinic receptor sub-type.^{22,23} In humans, the ability of PACAP-R splice variants to activate G proteins with different efficacies leading to differences in maximal stimulation of PLC may result in a greater diversity in signal transduction.

PACAP-Induction of Immediate Early-Gene Expression—As discussed earlier, PACAP shows a significant effect on native and tumoral cells containing PACAP receptors. To determine whether this effect is mediated by the induction of immediate-early gene transcription, we investigated the effects of P-38 and P-27 on the induction of c-fos and c-myc mRNA in the stably transfected NIH/3T3 cells. Northern blot analysis using a specific radiolabeled probe for c-fos and c-myc were performed on the mRNA isolated from ligand-stimulated NIH/3T3 cells stably expressing the respective hPACAP-R splice variant. Densitometry was used to quantitate the relative abundance of c-fos or c-myc mRNA. PACAP-27 and PACAP-38 nearly equally stimulated the expression of c-fos and c-myc. As shown in FIGURE 2, the hPACAP-R-SV2 showed the greatest stimulation of c-fos mRNA with a half-maximal stimulation at approximately 10 nM and maximal response at 100 nM. The maximal response observed for cells stably expressing the hPACAP-R-SV-2 was nearly threefold greater than the hPACAP-R-null and nearly fivefold greater than the hPACAP-R-SV1 and hPACAP-R-SV3. Similar, but less dramatic, to the results obtained for c-fos, both the hPACAP-R-SV2 and the null splice variants showed nearly identical responses for stimulating c-myc expression that were approximately twofold greater than the stimulation measured for the hPACAP-R-SV1 (Fig. 3) These results closely parallel the rank order increases observed for PLC stimulation, suggesting a similar mechanism for activation of PLC and immediate-early gene expression, which may offer another possible mechanism for regulating cellular growth and differentiation by the alternative expression of specific hPACAP-R splice variants.

This study demonstrates that the gene organization of PACAP-R splice variants is highly conserved between rats and humans and that humans also express at least four of the five possible splice variants. However, unlike the rat, all of the human splice variants are capable of stimulating both cAMP and total inositol phosphates with nearly identical potencies. The efficacy of PACAP-stimulated activation of PLC varies for the particular human splice variant, with the hPACAP-R-SV2 being the most efficacious. Similar to the efficacy for stimulating PLC, the hPACAP-R-SV2 shows the greatest coupling to the expression of the immediate-early gene expression. The physiologic significance of this differential signaling may in part be dependent on the cell-specific expression of hPACAP-R splice variants similar to that observed in the rat. These structural and functional differences in the PACAP-R splice variants may help explain the differences in ligand affinity and potency for stimulation of different intracellular signals observed in a variety of native cells, and therefore may represent a novel signaling pathway in which ligand-activation of a specific receptor splice variant may induce varying degrees of cellular responses.

SUMMARY

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide belonging to the VIP/secretin/glucagon family, is present in the hypothalamus, anterior pituitary, and adrenal gland where it regulates hormone release, in the GI tract where it modulates motility, and in human tumoral cell lines where it shows a growth-promoting effect. It is now appreciated that alternative splicing of two exons of the rat PACAP-R gene generate four major rPACAP-R splice variants that are differentially expressed in tissues and variably coupled to intracellular second messengers. Because of the potential implications of these findings in human physiology, we cloned the hPACAP-R gene. Similar to the rat, two exons (SV-1 and SV-2) are alternatively spliced to account for four major hPACAP-R receptor splice variants. These splice variants (hPACAP-R-null, hPACAP-R-SV 1, hPACAP-R-SV2, hPACAP-R-SV-3) were cloned from a human frontal cortex cDNA library, stably transfected in NIH/3T3 cells and each characterized for ligand affinity, stimulation of adenylate cyclase (AC) and phospholipase C (PLC), and ligand-induced expression of the proto-oncogenes, c-fos, and c-myc. Stably transfected NIH3T3 cells expressing similar numbers of receptors of the four splice variants showed nearly identical responses for ligand affinity and potency for P-38- and P-27-stimulated increases in cAMP and total inositol phosphates. However, each receptor splice variant differed in their ligand-stimulated efficacy for total inositol phosphate stimulation. The hPACAP-R-SV2 showed the greatest efficacy for stimulating phospholipase C that was approximately seven-fold greater than the hPACAP-R-SV1, twofold greater than the hPACAP-R-Null, and 1.5-fold greater than the hPACAP-R-SV-3 splice variants. To determine whether the splice variants also differ in their ability to stimulate immediate early gene expression, c-fos and c-myc transcripts were assayed by Northern blot and quantified by densitometry. PACAP-38 increased c-fos and c-myc expression for all four of the receptor splice variants that paralleled the efficacy for PLC stimulation, with the the SV-2 splice variant showing the greatest stimulation. These results show that the hPACAP-R-SV2 exhibits enhanced efficacy for coupling to both PLC and activation of the protooncogenes, c-fos and c-myc suggesting a novel and potentially important mechanism for differentially activating signal transduction pathways that influence cellular growth and differentiation.

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FIGURE 1.

Model of the deduced amino acid sequence and partial gene structure for the hPACAP-R. The seven transmembrane regions and the intracellular and extracellular loops are based on the criteria of Kyte and Doolittle. The letters outside the circles show the corresponding amino acid for the rPACAP-R.⁵ Amino acids are numbered based on the first in frame ATG of the cloned cDNA. The *arrowhead* shows the site of a potential signal peptide cleavage site between a.a. 19 and 20 that are conserved among members of the PACAP/VIP/secretin receptors. Potential N-linked glycosylation sites are shown by the figure "4". Seven cysteines, five in the amino terminus and two in the second and third extracellular loops that are highly conserved in the VIP/secretin family of receptors, are shown by the solid triangles. Shown below is a model of the two exons (SV-1, SV-2) that encode the splice variants of the hPACAP-R. Transcription of the 84 bp SV-1 exon gives rise to the hPACAP-R-SV-1; transcription of the 84 bp SV-2 exon gives rise to the hPACAP-R-SV-2;

transcription of both exons to the hPACAP-R-SV-3; and transcription of neither exon to the hPACAP-R-null splice variants (shown by the *arrow*).

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FIGURE 2.

Stimulation of c-fos mRNA expression for each of the hPACAP-R splice variants. NIH/3T3 cells were stably transfected with each splice variant cDNA and evaluated for ligand-induced expression of c-fos mRNA at the indicated concentrations of peptide. Relative expression levels of c-fos mRNA were determined from Northern blot analysis using densitometry. All four splice variants showed maximal increases in c-fos mRNA levels at 100 nM after 4 h of incubation. The hPACAP-R-SV-2 (*open circles*) showed the greatest increase in c-fos expression that was approximately threefold greater than the null, and fivefold greater than the SV-1 and SV-3 splice variants. These results parallel the results obtained for PACAP-induced maximal stimulation of PLC by the hPACAP-R-SV-2 splice variant (TABLE 1).

FIGURE 3.

Stimulation of c-myc mRNA expression for each of the hPACAP-R splice variants. NIW3T3 cells were stably transfected with each splice variant cDNA and evaluated for ligand-induced expression of c-myc mRNA at the indicated concentrations of peptide. Relative expression levels of c-myc mRNA were determined from Northern blot analysis using densitometry. All four splice variants showed maximal increases in c-myc mRNA levels with incubations of 100 nM of PACAP-27 after 4 h. The hPACAP-R-SV-2 showed the greatest increase in c-myc expression compared to the other splice variants. The SV-3 splice variant showed an intermediate response. These results parallel the results obtained for PACAP-induced maximal stimulation of PLC and c-fos expression by the hPACAP-R-SV-2 splice variant (TABLE 1).

TABLE 1.Comparison of the Relative Potencies and Efficacies for Each of the hPACAP-R Splice Variants^a

	EC ₅₀ (nM)						
	<i>Kⁱ</i> (nM)		cAMP		Total IP		Total IP (cpm)/receptor
	P-38	P-27	P-38	P-27	P-38	P-27	P-38
Hu-PACAP-Null	20	30	0.6	0.8	35	50	0.11
Hu-PACAP-SV-1	40	60	0.8	1.0	35	50	0.07
Hu-PACAP-SV-2	20	30	1.0	1.0	35	50	0.54

^aThese results show the IC₅₀'s values for radioligand binding, the EC₅₀'s for stimulation of cAMP and IP, and the relative efficacies for each of the hPACAP-R splice variants. In order to normalize for total receptor number, efficacy was expressed as total stimulation of inositol phosphates (CPM) per receptor. P-38 and P-27 were nearly equipotent for ligand binding affinity, cAMP stimulation, and total IP turnover in each of the variants. All of the splice variants showed similar potencies for ligand binding, cAMP stimulation, and total IP turnover. The SV-2 variant had a ≈ 6-fold greater efficacy than the null variant and a ≈ 9-fold greater efficacy than the SV-1 variant for stimulating phospholipase C. These results suggest that although each of the human splice variants showed a similar profile for potency, each variant differed by the efficacy for coupling to phospholipase C.