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Cloning and Characterization of the Signal Transduction of Four Splice Variants of the Human Pituitary Adenylate Cyclase Activating Polypeptide Receptor:

EVIDENCE FOR DUAL COUPLING TO ADENYLATE CYCLASE AND PHOSPHOLIPASE C*

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Abstract

Alternative splicing of two exons of the rat pituitary adenylate cyclase activating polypeptide (PACAP) receptor gene generates four major splice variants that are differentially expressed in specific tissues and variably coupled to intracellular second messengers. To evaluate the potential implications of these findings in human physiology, the human PACAP receptor gene was cloned. Alternative splicing about two exons of the gene allowed for four major splice variants that were subsequently identified on cDNA cloning. Each of the four splice variant cDNAs (null, SV-1, SV-2, and SV-3) was stably expressed in NIH/3T3 cells at similar receptor densities. For each splice variant, PACAP (both PACAP-38 and PACAP-27) had similar affinity and potency for stimulating either adenylate cyclase or phospholipase C. However, each receptor splice variant differed in their ligand-stimulated maximal response (efficacy) for total inositol phosphate accumulation with the SV-2 showing the greatest efficacy, followed by the null, SV-1, and SV-3 splice variants. Therefore, unlike the rat, PACAP binds and stimulates signal transduction with nearly equal affinity and potency for each of the receptor splice variants although with varying efficacy for the stimulation of phospholipase C. These results suggest a novel and potentially important mechanism for a single hormone to not only couple to dual signal transduction cascades but also elicit tissue-specific differential activation of phospholipase C in humans.

Pituitary adenylate cyclase activating polypeptide (PACAP),[1] isolated in 1989 from ovine hypothalamus, occurs as two carboxylamidated peptides, PACAP-38 (P-38) and PACAP-27 (P-27), each possessing identical NH_2-terminal 27 amino acid residues (1). Their primary structure suggest their inclusion in the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family (1, 2). Radioligand binding and cloning studies have identified three major

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[1]The abbreviations used are: PACAP, pituitary adenylate cyclase activating polypeptide; PACAP-R, PACAP receptor; VIP, vasoactive intestinal peptide; rPACAP-R, rat PACAP-R; hPACAP-R, human PACAP-R; AC, adenylate cyclase; PLC, phospholipase C; PCR, polymerase chain reaction; BSA, bovine serum albumin; PCS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

types of PACAP receptors (PACAP-Rs) differing in their affinity for PACAP, VIP, and helodermin and having different as well as overlapping tissue distributions (2–6). The type I PACAP receptor described in this study shows the highest affinity for P-38 and P-27 compared to VIP or helodermin (5).

There have been a paucity of studies investigating the pharmacology and signal transduction of PACAP receptors in human tissues because of the difficulty in obtaining specimens. In the postmortem human brain, specific PACAP binding sites have been identified in the hypothalamus, brain stem, cerebellum, and cortex (7); however, their functional significance is unknown. PACAP has been shown to exhibit a protective role against the neuronal toxicity of the gp120 envelope protein of the human immunodeficiency virus suggesting its role as a neurotrophic factor (8). In the human gastrointestinal tract, PACAP may modulate smooth muscle motility as evidenced by a reduction in immunoreactive PACAP nerve fibers in Hirshsprung's disease (9). Type 1 PACAP receptors have also been described on the human lung carcinoma cell lines NCI-H345 and NCI-N417 and the human neuroblastoma cell line NBOK-1, where they stimulate clonal growth (10, 11).

We previously cloned the rat Type 1 PACAP receptor (rPACAP-R) cDNA encoding a unique 495-amino acid protein belonging to the VIP/secretin/glucagon family of G protein-coupled receptors (5). The type 1 rat PACAP-R was shown subsequently to be expressed as four additional splice variants as a result of alternative splicing of two exons encoding a region of the third intracellular loop (12). These splice variants were shown to exhibit variable tissue expression and coupling to signal transduction pathways leading to differences in the levels of activation of adenylate cyclase (AC) and phospholipase C (PLC). In native cell systems, PACAP-Rs have also been shown to exhibit coupling to both adenylate cyclase and phospholipase C signal transduction pathways in the rat anterior pituitary gonadotrophs (13) and the rat pheochromocytoma cell line, PC-12 (14). 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.

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, we cloned the cDNA and gene for the hPACAP-R. The present study demonstrates that the hPACAP-R gene contains two variably spliced exons that express four splice variant homologs of 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 between 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. These differences in the efficacy of receptor-effector coupling may represent a novel mechanism in humans in which multiple PACAP-R splice variants leads to greater diversity in cellular function by affecting the magnitude of the ligand-stimulated phospholipase C response.

EXPERIMENTAL PROCEDURES

Tissue Procurement, RNA Isolation, and cDNA Synthesis—

Human tissues were obtained as surgical specimens and immediately frozen in liquid nitrogen. Total RNA was isolated using a low temperature guanidine isothiocyanate method and poly(A)$^+$ RNA was enriched using oligo(dT)-cellulose chromatography (Stratagene, La Jolla, CA). Oligo(dT)-primed cDNA was synthesized using Superscript reverse transcriptase (Life Technologies, Inc.).

Isolation of cDNA Clones—

A human frontal cortex cDNA library in the λ ZAP II phagemid vector (Stratagene, La Jolla, CA) was screened using a 32P-random-primed labeled probe derived from the full-length coding region of the rPACAP-R cDNA (5). Approximately 7.5×10^5 clones were screened at low stringency (three washes with 2 × SSC, 0.1% SDS at 37 °C (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate)). The longest cDNA clone was subsequently sequenced and subcloned into the expression vector pCDL-SRα/Neo (15). All positively hybridizing clones were rescreened with two 32P-end-labeled 84-base pair oligonucleotide probes corresponding to either the rat “hip” or “hop” splice variant exon sequences (12) to identify potential human splice variant homologs.

Isolation of Genomic Clones—

A human fibroblast genomic DNA library in the λFIX II vector (Stratagene, La Jolla, CA) was screened under high stringency conditions (three washes with 0.1 × SSC, 0.1% SDS at 42 °C) using a ^{32}P-random-primed radiolabeled cDNA probe derived from the full-length coding region of the hPACAP-R cDNA.Positively hybridizing clones were evaluated for the presence of human homologs of the rat hip or hop splice variant exon sequences SV-1 and SV-2 (Fig. 2) using the polymerase chain reaction. Plaque-purified genomic clones were amplified using the sense (AGGCAATGAGTCCAGCATCTAC; nucleotides 1190–1211, Fig. 1) and antisense (TGCGACTGGCCCGGTCCACC; nucleotides 1381–1400, Fig. 1) primers with *Taq:Pfu* polymerase (15:1) during 35 cycles of 94 °C denaturation for 15 s, 65 °C annealing for 2 min, and 72 °C extension for 4 min with a final extension time of 30 min (16). PCR products were sequenced to identify exons SV-1 and SV-2 as well as consensus intron/exon splice donor and acceptor sites.

PCR Cloning of the SV-1 and SV-2 Splice Variants—

The hPACAPR-SV-1 was cloned from the human frontal cortex cDNA library in a three-step PCR using overlapping sense (nucleotides 1246–1297, Fig. 1) and antisense (nucleotides 1214–1265, Fig. 1) primers derived from the SV-1 exon paired with the respective 3’-untranslated antisense (nucleotides 1780–1800, Fig. 1) and 5’-untranslated sense (nucleotides 50–70, Fig. 1) primers. The resulting PCR products were annealed and extended to generate a full-length product that was amplified using the sense (GAATTCTGCTGGCCAAGTGTCATG, nucleotides 50–70, Fig. 1) and antisense (GAATTCCTGGGACCGCAGGCAGGTGC, nucleotides 1780–1800, Fig. 1) primers

containing an *Eco*RI restriction site (underlined). This PCR product was subsequently ligated into the pCDL-SRα/Neo expression vector at the *Eco*RI site (15).

The hPACAP-R-SV-2 was similarly cloned from the human frontal cortex cDNA library in a three-step PCR using overlapping sense (1246–1318, Fig. 1) and antisense (1245–1265, Fig. 1) primers derived from the SV-1 exon and amplified as described above for the SV-1 splice variant.

Sequence Analysis—

All genomic, cDNA, and PCR products were sequenced using the dsDNA Cycle sequencing system (Life Technologies, Inc.). DNA and amino acid sequence analysis was performed using the Geneworks® software package.

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

Following linearization of the vector at the AatII restriction site in the ampicillin resistance gene of the vector, pCDL-SRα/Neo, 20 μg of recombinant plasmid were transfected into NIH/3T3 cells using electroporation (2×10^7 cells, 500 microfarads, 0.25 kV). Clones of stably transfected cells were selected using Geneticin® (250 mg/ml, Life Technologies, Inc.).

Radioligand Binding—

^{125}I-PACAP-27 (2200 Ci/mmol) was obtained from DuPont NEN. Stably transfected NIH/3T3 cells were washed once in phosphate-buffered saline (PBS: 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.4) containing 1 mg/ml bovine serum albumin (BSA) at 4 °C, scraped from the tissue culture plate in DMEM containing 1 mg/ml BSA, centrifuged at 400 × *g*, and resuspended in the same medium at 300,000 cells/ml. Suspended cells (500 μl) were incubated for 60 min at 37 °C with 50 pM ^{125}I-PACAP-27 (2200 Ci/mmol) (DuPont NEN) either with or without the indicated concentrations of unlabeled peptides, P-27 and P-38 (Bachem, Torrance, CA). Cells were subsequently pelleted (10,000 × *g*) through a bed of 250 μl of PBS/4% BSA at 4 °C, and washed three times at 4 °C with 200 μl in PBS/4% BSA. The cell pellet was assayed for γ radioactivity (Packard, Auto-Gamma). The density of cell surface receptors expressed in each stably transfected cell line was determined by the saturable binding (total binding in the presence of ^{125}I-PACAP-27 alone less nonspecific binding in the presence of 1 μM P-38). Binding data were analyzed using the nonlinear least squares curve-fitting program, LIGAND (17). To normalize signal transduction data for receptor density, an estimate of receptor density was determined by ligand binding using a concentration of free radioligand, [L], greater than $3 \times K_d$, a concentration of the receptor, [R_{total}], less than 0.1 K_d and the relationship [R_{total}] = [RL][$K_d + L$]/[L] as described previously (18).

cAMP Assay—

Stably transfected NIH/3T3 cells were plated on 24-well culture dishes overnight with DMEM/10% calf serum in the presence of [^{3}H]adenine (Amersham Corp.) at a concentration of 2 mCi/ml. The cells were washed with DMEM, incubated with or without the indicated concentrations of P-38 and P-27 in DMEM containing 1 mg/ml BSA and 2.5

mM 3-isobutyl-1,1-methylxanthine for 30 min and then aspirated. One hundred microliters of 2% SDS, 1 mM cAMP solution was used to lyse the cells. cAMP was assayed by consecutive Dowex AG-50W-X4 resin (Bio-Rad) and aluminum oxide (Sigma) column chromatography according to a modification of the procedure described by Salomon *et al.* (19). [^{3}H]cAMP was measured using a liquid scintillation counter (Packard, Downers Grove, IL).

Inositol Phosphate Assay—

Stably transfected NIH/3T3 cells were plated on 24-well culture plates with DMEM/10% calf serum in the presence of 100 μCi/ml myo-2-[^{3}H]inositol (DuPont NEN) and incubated overnight. Following aspiration of the *myo-2*-[^{3}H]inositol, 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) in the presence of the indicated concentrations of peptide. Total [^{3}H]inositol phosphates were measured using strong anion exchange chromatography (Dowex AG 1-X8) according to a modification of the method of Berridge *et al.* (20). Total [^{3}H]inositol was measured using a liquid scintillation counter (Packard).

RESULTS

cDNA Library Hybridization Screening and Cloning of the hPACAP-R—

Relying on the conservation of the nucleotide sequence for a particular G protein-coupled receptor between species (21), a rat PACAP-R cDNA probe was used to screen a human frontal cortex cDNA library (5). Thirty-six positive cDNA clones were plaque-purified using low stringency hybridization conditions. DNA sequence analysis of the longest plaque-purified cDNA clone revealed a 98% similarity to the rPACAP-R (5). ^{32}P-End-labeled probes, derived from the published rat hip and hop sequences, were used to further screen the human cDNA library (12). The rat hop probe hybridized to three of the 36 isolated cDNA clones (hPACAP-R-SV-2), whereas the hip probe did not hybridize to any of the isolated clones. Therefore, the majority (33 out of 36) of the cDNA clones did not contain sequence homologous to the rat hip and hop splice variants and is referred to as hPACAP-R-null splice variant.

Genomic Library Hybridization Screening and Cloning of the hPACAP-R Gene—

The hPACAP-R gene was cloned from a human placental genomic library to determine whether the human PACAP-R gene structure contained exons that could be alternatively spliced to produce multiple receptor isoforms with the corresponding amino acid sequence within the third intracellular loop of the receptor similar to the rat PACAP-R gene. Twenty-seven positively hybridizing genomic clones were identified and plaque-purified by library screening. DNA sequence analysis of a region of the gene encoding the third intracellular loop identified two exons (exons SV-1 and SV-2, Fig. 1*A*) using criteria of splice donor/acceptor consensus sequences. Therefore, similar to the rat, the organization of the hPACAP-R gene allows for the alternative splicing of the two exons SV-1 and SV-2 within transcripts that results in the human PACAP-R splice variants null, SV-1, SV-2, and SV-3 (Fig. 1*B*). Additionally, as observed in the rat, two contiguous splice acceptor sites are observed for exon SV-2 giving rise to two potential transcription products (SV-2A and SV-2B, Fig. 1*B*).

Analysis of the Nucleotide Sequence of the hPACAP-R Splice Variants—

The clones isolated by cDNA library hybridization screening were subjected to DNA sequence analysis and their homology compared to the published rPACAP-R splice variants (12). The hPACAP-R-null splice variant represented the predominant clone in the cDNA library and encoded a unique 468 amino acid protein (Fig. 1*A*, omit exons SV-1 and SV-2) that had a 93% homology to the rPACAP-R (5). A second clone identified by library screening was identical to the hPACAP-R-null splice variant, except that it contained an additional 84 nucleotides encoding 28 amino acids in the third intracellular loop (hPACAP-R-SV2) (Fig. 1*A*, include exon SV-2) that were identical to the rPACAP-R-hop splice variant (12). Because hybridization screening of the human frontal cortex cDNA library failed to detect the presence of additional splice variants of the hPACAP-R despite a gene organization suggesting the potential for two additional splice variants, we used the increased sensitivity of PCR to isolate these suspected splice variants from the same human frontal cortex cDNA library. PCR using primers derived from exon SV-1 (described under "Experimental Procedures") identified a product that included 84 nucleotides encoding 28 amino acids in the third intracellular loop that showed the greatest similarity to the rPACAP-R-hip splice variant (Fig. 1*A*). This product (hPACAP-R-SV-1) differed from its rat homolog by two amino acids (A415T and L430P, Fig. 1*A*). PCR also identified a cDNA clone containing sequence transcribed from both exons (described under "Experimental Procedures") that comprised 168 base pairs encoding 56 amino acids in the third intracellular loop and showing the greatest homology to the rPACAP-R-hip-hop splice variant (Fig. 1*B*, include exons SV-1 and SV-2).

Analysis of the Deduced Amino Acid Sequence of the hPACAP-R—

An hydropathy plot of the of the deduced amino acid sequence of the hPACAP-R-null cDNA using the criteria of Kyte and Doolittle (22) identifies 8 regions of hydrophobic residues, seven corresponding to putative transmembrane domains consistent with its membership in the G protein-coupled superfamily of receptors (Fig. 1*A*). Alignment of the deduced amino acid sequence with all known deduced protein sequences showed the highest homology (93%) with the rPACAP-R sequence (Fig. 1*A*). The eighth hydrophobic region includes a potential signal peptide sequence encompassing the NH_2-terminal 20 amino acids (Fig. 1*A*). The deduced amino acid sequence has five potential *N*-linked glycosylation sites, three located in the amino terminus with the fourth and fifth in the second and third extracellular loops, respectively. Additionally, there are 7 cysteines, 5 in the amino terminus, and 1 each in the second and third extracellular loops.

Ligand Binding to hPACAP-R Splice Variants—

The displacement curves for ^{125}I-PACAP-27 binding by P-38 and P-27 were nearly identical for each of the hPACAP-R splice variants stably expressed in NIH/3T3 cells (Fig. 2). For both P-38 and P-27, detectable inhibition was observed at 50 nM and half-maximal inhibition (IC_{50}) at 22 nM and 32 nM, respectively. Cells transfected with the SV-1 splice variant receptor cDNA showed approximately 2-fold less affinity for either P-38 or P-27 (Fig. 2, *second panel from top*).

Stimulation of Adenylate Cyclase by hPACAP-R Splice Variants—

Each of the four splice variants showed nearly identical dose-response curves for PACAP stimulation of adenylate cyclase in NIH/3T3 cells stably expressing each of the four PACAP-R splice variants (Fig. 3). Stimulation was detectable for both P-38 and P-27 at 0.1 nM, half-maximal (EC 50) at 0.7 nM for P-38 that was approximately 2-fold lower than P-27, and maximal at 50–100 nM for both P-38 and P-27.

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

Each of the four splice variants showed nearly identical dose-response curves for PACAP stimulation of total inositol phosphates in NIH/3T3 cells stably expressing each of the four PACAP-R splice variants (Fig. 4). Each of the splice variants showed nearly the same dose response to P-38 and P-27, with detectable stimulation at 10nM, half-maximal stimulation (EC_{50}) at 35–50 nM, and maximal stimulation at 1 mM.

Efficacy for Stimulation of Adenylate Cyclase and Phospholipase C by hPACAP-R Splice Variants—

To determine the relative coupling of each of the four PACAP-R splice variants to mediators of intracellular signal transduction, five separate clones of each splice variant stably expressed in NIH/3T3 cells were studied to minimize the effects of clonal variation. Table I shows the average K_i values for radioligand binding inhibition, the EC_{50} values for cAMP and total IP stimulation, and the efficacy for total IP stimulation representing the average of five different clones for each of the hPACAP-R null, SV-1, and SV-2 splice variants. The K_i values and the EC_{50} values for cAMP and total IP stimulation are nearly identical for each splice variant; however, the efficacy for coupling to phospholipase C as measured by the maximal stimulation in total IP per receptor is 5–8-fold greater for the SV-2 splice variant compared to the null and SV-1 splice variants, respectively (Table I).

To exclude the potential influence that differences in receptor density may have on efficacy for coupling to second messenger pathways, clones expressing similar receptor densities (50,000–80,000 receptors/cell) for each splice variant were chosen to study their efficacy for stimulating cAMP and IP. P-38 stimulation of cAMP was dose-dependent and saturable with an observable stimulation at 0.1 nM, half-maximal stimulation at 1 nM, and maximal at 10 nM for each of the splice variants (Fig. 5). Each of the hPACAP-R splice variants increased intracellular cAMP to nearly the same maximal level (Fig. 5). P-38 stimulated total inositol phosphates in a dose-dependent manner with observable stimulation at 1 nM, half-maximal at 10 nM, and maximal at 1 mM (Fig. 6). 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 3-fold greater than the null and SV-1 splice variants, while the SV-3 was intermediate in its ability to maximally stimulate IP (Fig. 6).

DISCUSSION

The discovery of four major splice variants of the rPACAP-R that exhibit a differential response to P-38 and P-27, variable coupling to second messengers, as well as variable tissue-specific expression led to the attractive speculation that multiple PACAP-R splice

variants arising from a single gene can effect a more complex regulation of cellular second messenger signaling pathways (12). In the current study, we cloned the human PACAP-R gene and four splice variant cDNAs establishing their conservation in humans. However, in humans, unlike in rats, both P-38 and P-27 stimulate each splice variant equally well. Also, unlike the rat, each splice variant has nearly the same dose response to PACAP for activation of both adenylate cyclase and phospholipase C. However, the hPACAP-R splice variants differ in their degree of efficacy for stimulating PLC.

The hPACAP-R splice variants were cloned by hybridization screening and PCR of a human frontal cortex cDNA library. The hPACAP-R cDNA exhibits a nucleotide sequence identity of 93% with the rPACAP-R consistent with the interspecies variation observed for other G protein-coupled receptors (21). The hPACAP-R protein has a calculated molecular mass of 51 kDa, which is in close agreement with the cloned rPACAP-R (55 kDa) and with cross-linking studies of native human brain (48 kDa) (7). The deduced amino acid sequence of the hPACAP-R includes a hydrophobic signal peptide based on criteria by von Heijne (23) comprising the first 20 NH_2-terminal amino acids with a potential cleavage site identical to rPACAP-R and other members of the VIP/secretin/glucagon receptor family, suggesting that it may be important for targeting the translated receptor protein to the cell membrane (4, 6, 23–25).

Similar to other members of the VIP/secretin/glucagon receptor family, the hPACAP-R shares a long amino terminus (>120 amino acids) that may be important for ligand recognition as demonstrated by secretin/VIP chimeras (26), for naturally occurring missense mutations demonstrated for the mouse gonadotropin hormone-releasing hormone receptor resulting in a Dwarf *little* (*lit*) mouse phenotype and for the human glucagon receptor resulting in adult onset diabetes mellitus (27, 28). The hPACAP-R possesses three potential glycosylation sites in the amino terminus similar to the VIP_1, VIP_2, glucagon, GLP-1, and secretin receptors, suggesting that these receptors may be similarly processed and targeted to the cell membrane (4, 6, 23–25, 29). These conserved glycosylation sites may also be important for high affinity ligand binding, as has been shown previously for the VIP_1 receptor (30) and the gastrin-releasing peptide receptor (31). The amino terminus and second and third extracellular loops possess five cysteine residues that are highly conserved among PACAP, VIP_1, VIP_2, secretin, GLP-1, and glucagon receptors that may form disulfide bonds necessary for agonist binding conformation as shown for rhodopsin (32).

Hybridization screening of a human frontal cortex cDNA library identified the null splice variant as the predominant form and is consistent with the fact that this form is identical to the only PACAP-R splice variant previously cloned from a human pituitary cDNA library (34). A less frequent, second splice variant containing an additional 28 amino acids within the third intracellular loop (SV-2) that was identical to the 28 amino acids within the rat homolog, rPACAP-R-hop, splice variant was identified using an 84-base oligonucleotide probe specific to the rat hop sequence (5). To determine whether there could be additional splice variants, we cloned the hPACAP-R gene. DNA sequence analysis of the hPACAP-R gene revealed an intron-exon organization similar to the rPACAP-R gene in which two exons encode a portion of the third cytoplasmic loop of the receptor as predicted by consensus exon-intron sequences (33). The SV-1 and SV-2 exons are similar to the rat exons, hip and

hop, each encoding highly homologous 28-amino acid cassettes (12). Similar to the rPACAP-R hop exon, there are two acceptor sites at the 5' end of the hPACAP-R-SV-2 exon (Fig. 1*B*, *underlined*) allowing for the variable presence of splice variants SV-2a and SV-2b. Because the human gene structure could allow the expression of two additional splice variants encoded by the SV-1 exon alone and in combination with the SV-2 exon, we used the increased sensitivity of PCR to rescreen the human frontal cortex cDNA library for the expression of additional splice variants. Primers derived from the SV-1 exon were used to clone the two remaining receptor splice variant cDNAs, SV-1 and SV-3. The relative expression of each of the splice variants in the frontal cortex was null> SV-2 > SV-1 = SV-3. These results indicate that the hPACAP-R, similar to the rPACAP-R, can be expressed as four major splice variants as a result of the variable expression of two cassettes of 28 amino acids within the third cytoplasmic loop encoded by two exons that are alternatively spliced. The conservation of alternative splicing of the PACAP-R gene from rats to humans suggests that it may represent a highly conserved and efficient mechanism for creating biological diversity.

The cloning of the hPACAP-R splice variants provides a unique opportunity to characterize their pharmacology because of the difficulty in obtaining native human tissues. Ligand binding affinities (Fig. 2) for the cloned hPACAP-R null, SV-1, SV-2, and SV-3 splice variants (IC_{50} values: P-38 = 20–40 n M, P-27 = 35–70 nM) are similar to the previously published rat type 1 PACAP-R (IC_{50} values: P-38 = 8 nM, P-27 = 20 nM) (5). Clonal variation among the stably transfected cells was minimized by examining radioligand binding affinity and stimulation of adenylate cyclase and inositol phosphates for five separate clones from each splice variant. Although P-38 and P-27 binding affinities have not been reported for the remaining rPACAP-R splice variants, their pharmacology would be expected to be similar to the hPACAP-R splice variants given the high degree of nucleotide sequence homology. All of the hPACAP-R splice variants showed similar affinities for P-38 and P-27 and minimal affinity for VIP and secretin (data not shown). These results are similar to studies reported for native tissues such as human brain (7) and rat hypothalamic, anterior pituitary and brain membranes (35), AR-42 J cells, and human NB-OK1 cells (36, 37).

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 stably transfected in NIH/3T3 cells (Fig. 3). The ability for each of the hPACAP-R splice variants to stimulate adenylate cyclase with similar potencies is in contrast to results obtained for the rPACAP-R splice variants, where 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 (12). Stimulation of adenylate cyclase was not observed for VIP and secretin (data not shown) at concentrations up to 1 μM similar to rat type 1 PACAP receptors (5). Maximal stimulation of cAMP per receptor (efficacy) was similar for each splice variant (Fig. 5). Maximal stimulation (1 nM) occurred at half-maximal receptor occupancy and suggests the presence of "spare" receptors. Furthermore, differences in receptor density (≈ 10,000–200,000 receptors/cell) did not influence either the potency or efficacy for adenylate cyclase activation (data not shown) similar to results observed for transfected 5-hydroxytryptamine-1A receptors (18). These results differ from classical pharmacological models and studies evaluating the dose

responses for varying receptor densities in stably transfected cells (38). To exclude whether our non-classical results could be explained on the basis of cell receptor densities resulting in spare receptors, an analysis of potency and efficacy for each splice variant expressing lower receptor numbers (*i.e.* <10,000 receptors/cell used in this study) may be required.

Similar to PACAP stimulation of cAMP, PACAP stimulated total inositol phosphates with nearly equal potencies for each splice variant. The dose response for stimulation of inositol phosphates for each hPACAP-R splice variant receptor closely paralleled the ligand binding curves, suggesting that, unlike adenylate cyclase, there is minimal receptor "spareness" for coupling to phospholipase C. This is in contrast to results reported for the rPACAP-R splice variants where P-38 and P-27 failed to stimulate IP in the hip splice variant and stimulated the hip-hop splice variant with a potency that was 3-fold lower than the null and hop splice variants (12). These differences between the rat and human splice variants may reflect differences in the complement of G proteins and downstream effectors for different transfected cell lines (LLC-PK1 (12) *versus* NIH/3T3 (current study)), transient *versus* stable transfection and consequent differences in receptor density, or differences in receptor structure due to small differences in primary sequence.

Although the potencies for stimulation of total inositol phosphates for each human splice variant (Fig. 4) were similar, their efficacies for stimulation of total inositol phosphates for each splice variant were different, with the SV-2 isoform having nearly a 5-fold greater efficacy compared to the null variant and almost an 8-fold greater efficacy compared to the SV-1 and SV-3 splice variants (Fig. 6 and Table I). To reduce the potential for clonal variation, five distinct clones of each splice variant stably expressed in NIH/3T3 cells were used to determine efficacy. Although this study did not specifically address the stoichiometry of receptor-G protein coupling, the results suggest that each human PACAP-R splice variant couples with different affinities to heterotrimeric G proteins similar to α2A-adrenergic and muscarinic receptor subtypes (39, 40). 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 provide another mechanism for differential regulation of receptor-mediated signal transduction.

PACAP receptors, unlike the majority of G protein-coupled receptors, are capable of activating the dual signal transduction pathways involving adenylate cyclase and phospholipase C in native cells. Their ability to couple to these two pathways suggests that these receptors are capable of coupling to both $G\alpha_s$ and $G\alpha_{q/11}$ to stimulate AC and PLC, as demonstrated for the human thyrotropin, α_{2A}-adrenergic receptors, dopamine (D2), bombesin, and vasopressin receptors (41–44). Alternatively, these receptors may couple to a single G protein such as $G\alpha_s$ with subsequent stimulation of AC and PLC by disassociated $\beta\gamma$-subunits as shown by overexpression of β_2-adrenergic, luteinizing hormone, and vasopressin 2 (V2) receptors (38) in stably transfected cells. Our study did not specifically address whether overexpression of PACAP receptors enhances inositol phosphate turnover; however, we utilized stably transfected NIH/3T3 cells expressing similar receptor densities for each splice variant to minimize the effects of receptor density on signal transduction. Furthermore, receptor numbers in the range tested (10,000–200,000 receptors/cell) did not influence efficacy for phospholipase C activation (data not shown), indicating that

receptor/G protein coupling is maximal at each of these receptor densities. These observations are similar to those reported for the transfected human 5-hydroxy-tryptamine-1A receptor in which the efficacy for cAMP inhibition was not significantly increased by increasing receptor densities (18).

Although several recent studies have suggested that PACAP-R splice variants are capable of differential coupling to intracellular second messengers in a cell-specific manner, these results should be viewed with circumspection in specific cell types to exclude the expression of other receptors with high affinity for PACAP as shown recently in anterior pituitary somatotrophs and gonadotrophs (45). Therefore, in addition to the variable action of PACAP via type 1 PACAP receptor splice variants, PACAP has the further potential for inducing a variety of responses through its ability to interact with type 2 (classical VIP receptor) and type 3 PACAP (VIP_2) receptors.

Other potential mechanisms that lead to finer regulation of signal transduction need to be considered such as the differential potencies for P-38 and P-27 to couple to intracellular second messengers. Differences in receptor affinity for P-38 and P-27 have been observed for both the recombinant rPACAP-R splice variants (12) and for native receptors on PC-12 cells where P-38 is approximately 100–1000-fold more potent than P-27 for stimulating inositol phosphate turnover (14). This high affinity for P-38 *versus* P-27 for some PACAP-R splice variants may be explained on the basis of multiple affinity states of the receptors as shown for cholecystokinin receptor (46). These observations indicate that two genes, one gene encoding the hormone and one gene encoding four receptor splice variants, allow a more diverse repertoire for regulating intracellular signal transduction. Although this study focused on the regulation by hPACAP-R splice variants of the two predominant second messengers, phospholipase C and adenylate cyclase, PACAP has been shown to mediate other signal transduction pathways such as apamin-sensitive K^+ channels in colonic smooth muscle, L-type Ca^{2+} channels in pancreatic islets, non-cAMP-dependent Na^+ and Ca^{2+} channels in adrenal chromaffin cells, and nitric oxide pathways in gastrointestinal smooth muscle (47–50). Further study should be undertaken to determine whether these ion channel and nitric oxide-mediated signal transduction pathways may also be differentially regulated by individual 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. 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 whereby ligand-activation of a specific receptor splice variant may induce varying degrees of cellular responses.

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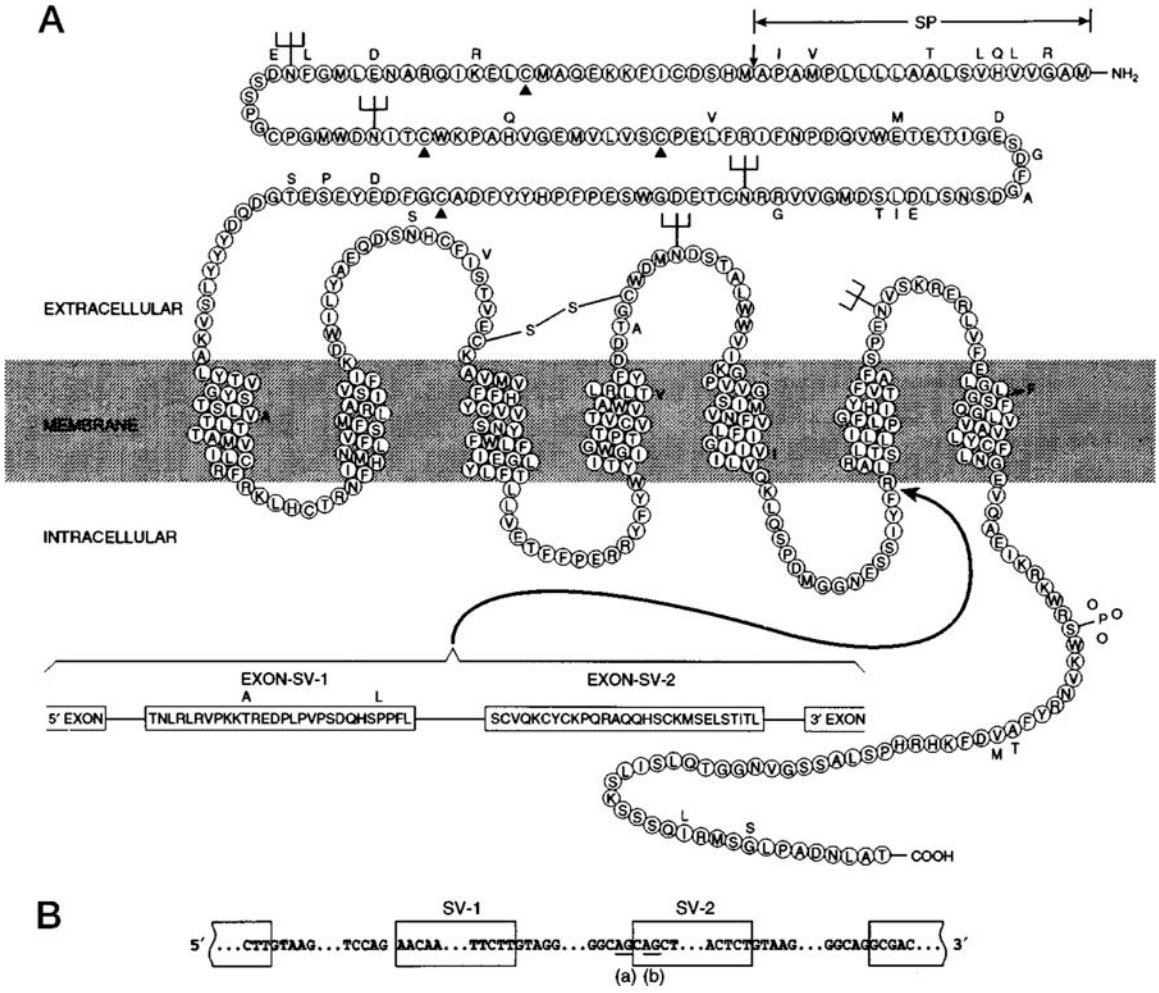

Fig. 1. Amino acid model and partial gene structure of the hPACAP-R.

A, model of the predicted amino acid sequence for the hPACAP-R showing seven transmembrane-spanning regions (*boxed* amino acids) (22), potential sites for *N*-linked glycosylation (*tridents*) signal peptide cleavage site (*arrow*) (23), seven highly conserved cysteine residues (*triangles*), and the non-homologous rPACAP-R amino acids (*letters* outside the *circles*) (5). Amino acid products of the alternatively spliced exons, SV-1 and SV-2, are shown in *brackets* with their location in the third intracellular loop indicated by the *arrow*. *B*, model of the partial gene structure of the hPACAP-R gene showing the consensus sequences for the intron-exon splice sites for the exons SV-1 and SV-2. Two consecutive splice sites for the SV-2 exon (*a* and *b*) give rise to two possible splice variants (SV-2a and SV-2b).

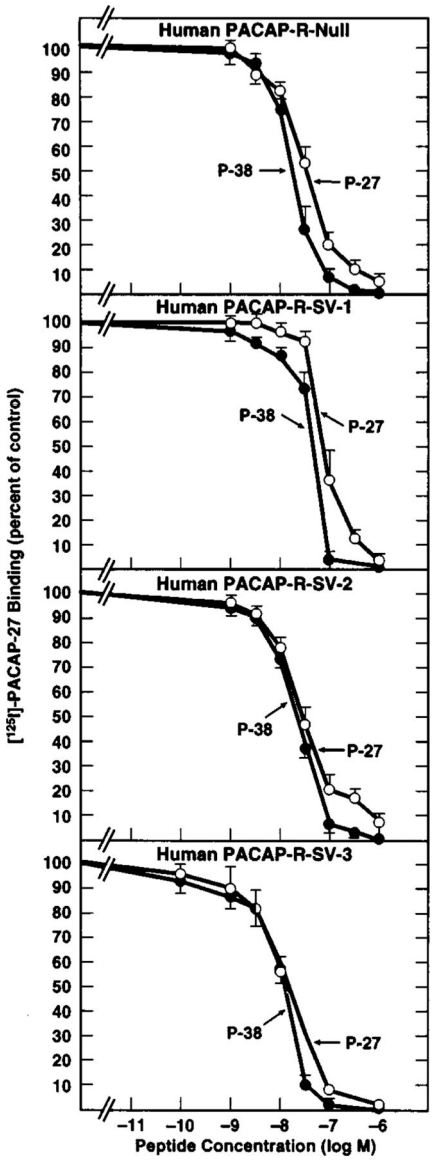

Fig. 2. Displacement of ^{125}I-PACAP-27 binding to NIH/3T3 cells stably expressing hPACAP-R splice variants.

NIH/3T3 cells stably expressing the indicated hPACAP-R splice variant were incubated with ^{125}I-PACAP-27 (50 pM) either alone or with the indicated concentrations of PACAP-38 or PACAP-27. Data are presented as percent of saturable binding in the absence of unlabeled ligand. Each value represents the mean ± standard error from at least five experiments performed in duplicate.

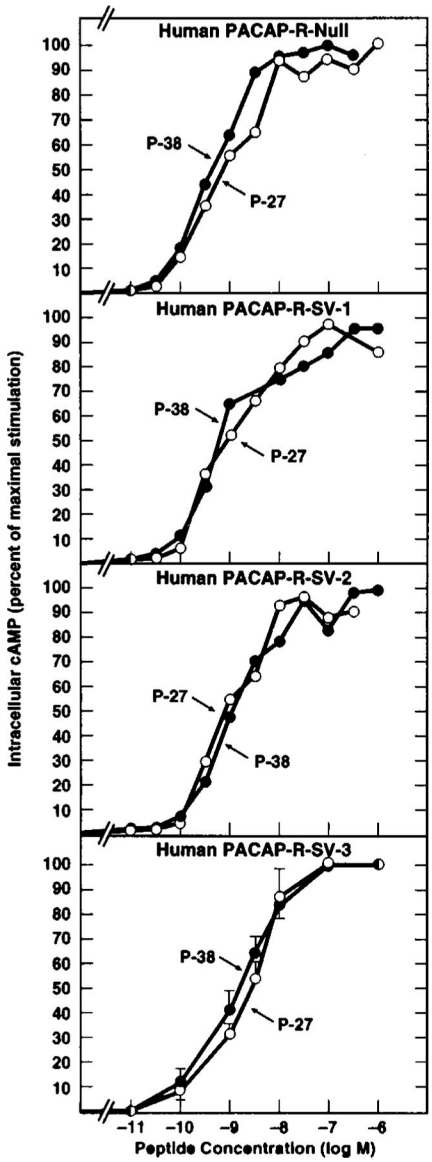

Fig. 3. Ability of PACAP to increase intracellular cAMP in NIH/3T3 cells stably expressing hPACAP-R splice variants.

NIH/3T3 cells stably expressing the indicated hPACAP-R splice variant cDNA were incubated either alone or in the presence of increasing concentrations of PACAP-38 and PACAP-27. Data are shown as percent of maximal stimulation (maximally stimulated cAMP minus nonstimulated cAMP) and represent the means of three experiments performed in triplicate.

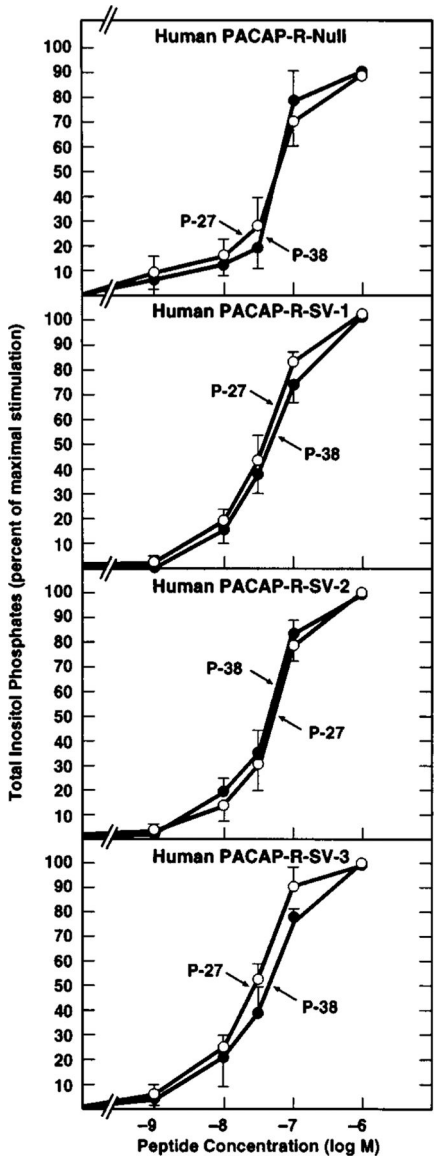

Fig. 4. Ability of PACAP to increase total inositol phosphates in NIH/3T3 cells stably expressing hPACAP-R splice variants.

NIH/3T3 cells stably expressing the indicated hPACAP-R splice variant cDNA were incubated either alone or in the presence of increasing concentrations of PACAP-38 and PACAP-27. Data are shown as percent of maximal stimulation (maximally stimulated IP minus nonstimulated IP) and represent the means of three experiments performed in triplicate.

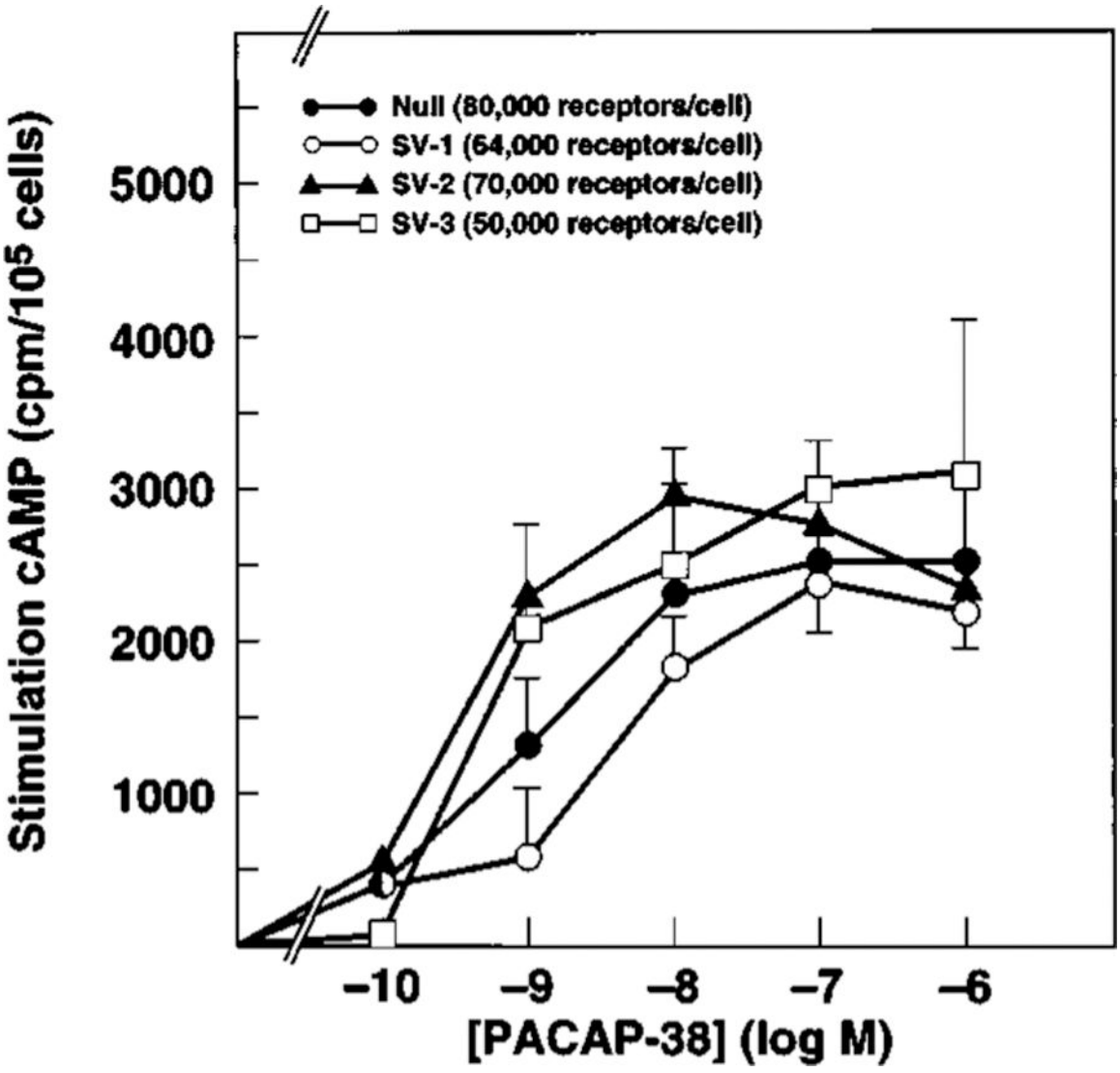

Fig. 5. Determination of the efficacy of the PACAP stimulated increase in cAMP for each of the hPACAP-R splice variants stably expressed in NIH/3T3 cells.

Clones of each of the hPACAP-R splice variants expressing similar receptor densities were incubated either alone or in the presence of increasing concentrations of PACAP-38. Data are presented as the increase in [^{3}H]cAMP in cpm/10^5 cells over basal (in the absence of added peptide) and represent the means of three experiments performed in triplicate. Similar results were observed for PACAP-27 (data not shown).

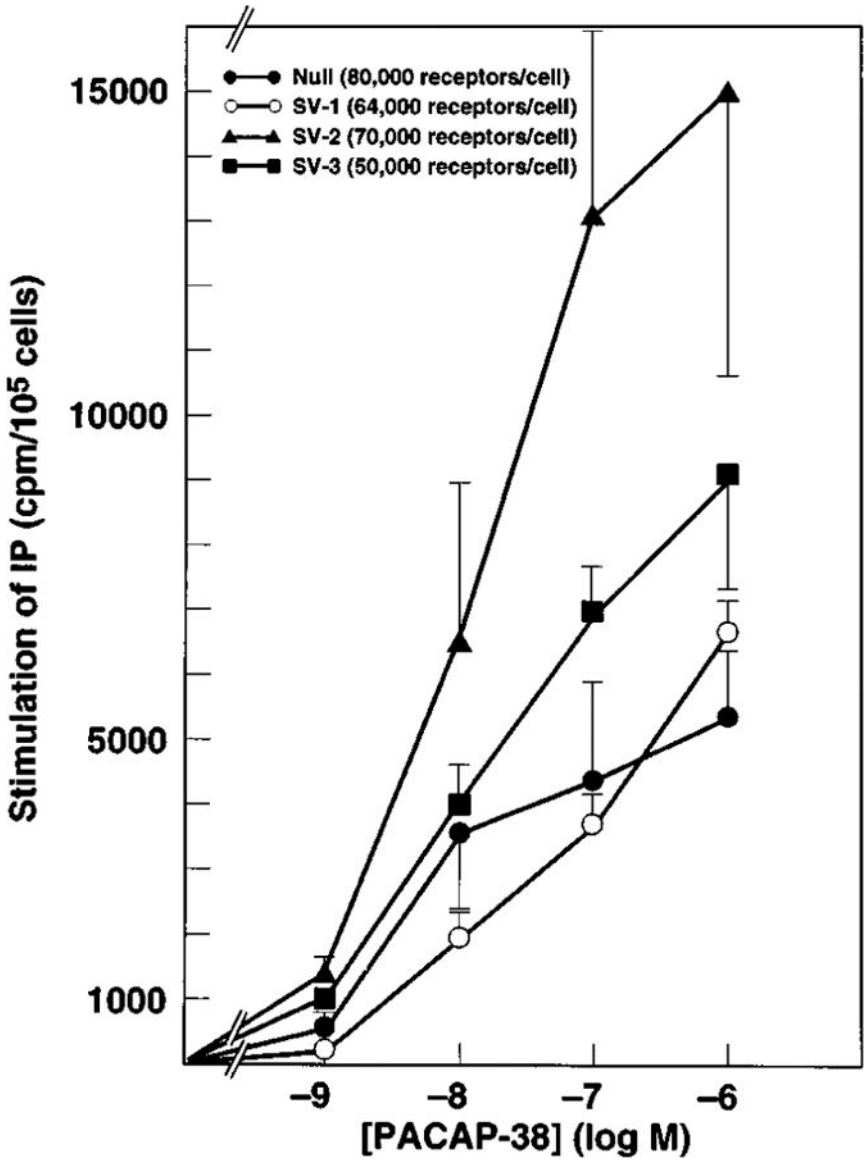

Fig. 6. Determination of the efficacy of the PACAP stimulated increase in total inositol phosphates for each of the hPACAP-R splice variants stably expressed in NIH/3T3 cells.

Clones of each of the hPACAP-R splice variants expressing similar receptor densities were incubated either alone or in the presence of increasing concentrations of PACAP-38. Data are presented as the increase in total [^{3}H]inositol phosphates in cpm/10^5 cells over basal (in the absence of added peptide) and represent the means of three experiments performed in triplicate. Similar results were observed for PACAP-27 (data not shown).

Table I

Comparison of PACAP affinity and ability to stimulate signal transduction for each of the hPACAP-R splice variants

Five separate clones varying in receptor density (50,000–200,000 receptors/cell) were evaluated for each hPACAP-R splice variant stably expressed in NIH/3T3 cells. Shown in this table are the IC_{50} values for radioligand binding, the EC_{50} values for the stimulation of cAMP and total IP, and the efficacies for the maximal stimulation of total IP (expressed as the increase in total [^{3}H]IP (cpm/receptor) for each hPACAP-R splice variant.

	EC_{50}						
	K_i		cAMP		Total IP		Total IP/Receptor
	P-38	P-27	P-38	P-27	P-38	P-27	P-38
	nM		nM		nM		*cpm*
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