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Molecular cloning and expression of a cDNA encoding a receptor for pituitary adenylate cyclase activating polypeptide (PACAP)

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We have cloned and sequenced a novel cDNA (RPR7) encoding a receptor for pituitary adenylate cyclase activating polypeptide (PACAP). RPR7 was identified by PCR of rat pituitary cDNA, and full-length clones were isolated from a rat olfactory bulb cDNA library. When expressed in COS cells, RPR7 was functionally coupled to increases in intracellular cyclic adenosine monophosphate (cAMP) in response to stimulation by PACAP-38, PACAP-27, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI). The order of potency of these ligands was PACAP-38 ~ PACAP-27 > VIP > PHI, suggesting that the receptor corresponds to the pharmacologically characterised PACAP Type I receptor.

Pituitary adenylate cyclase activating polypeptide (PACAP); G protein-linked receptor; Vasoactive intestinal peptide; Rat pituitary; Rat olfactory bulb; cDNA cloning

1. INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) was identified and purified on the basis of its ability to stimulate the accumulation of cAMP in rat pituitary cells in culture. Two forms of the peptide have been isolated from the hypothalamus, one of 38 amino acids (PACAP-38) [1] and a C-terminally truncated form of 27 amino acids (PACAP-27) [2]. PACAP is closely related to VIP (68% sequence homology within the N-terminal 28 residues) and more distantly related to glucagon, glucagon-like peptide I (GLP), PHI, secretin and growth hormone releasing hormone (GHRH). All of these peptides are thought to exert their actions through G-protein linked membrane receptors coupled to adenylyl cyclase. Recently, the receptors for VIP [3,4], glucagon [5–7], GLP [8], GHRH [9–11] and secretin [12] have been cloned. Together with receptors for calcitonin [13,14] and parathyroid hormone (PTH) [15–17] they form a protein family distinct from other G protein-linked receptors.

PACAP-38 and PACAP-27 have been shown to bind with high affinity to VIP receptors in a variety of tissues. However, specific PACAP receptors ('Type I PACAP receptors') to which VIP can bind with only modest affinity have also been described in brain and other tissues (for review see [18]). We report here the cloning and expression of a specific, adenylyl cyclase-linked Type I PACAP receptor (RPR7) from rat brain. RPR7 was identified by PCR of rat pituitary cDNA using degenerate oligonucleotide primers corresponding to the third and seventh transmembrane domains of the secretin family of G protein-linked receptors. Full-length cDNAs were isolated from an olfactory bulb cDNA library.

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Abbreviations: ACTH, adrenocorticotropic hormone; cAMP, adenosine 3′:5′-cyclic monophosphate; cDNA, complementary DNA; CGRP, calcitonin gene related peptide; CRF, corticotropin releasing factor; dNTP, deoxynucleotide triphosphate; DMEM, Dulbecco's modified Eagle's medium; GHRH, growth hormone releasing hormone; GLP, glucagon-like peptide I; mRNA, messenger RNA; PACAP, pituitary adenylate cyclase activating polypeptide; PCR, polymerase chain reaction; PHI, peptide histidine isoleucine; PTH, parathyroid hormone; RNase, ribonuclease; VIP, vasoactive intestinal peptide.

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no. Z23282

2. EXPERIMENTAL

2.1. *Cloning and sequence analysis of rat PACAP receptor*

Anterior pituitary glands from male rats (Cob Wistar, 250 g) were removed and total RNA was isolated by the method of Chomczynski and Sacchi [19]. Single-stranded cDNA synthesis and PCR were carried out using a commercial kit (Perkin Elmer Cetus). 1 μg RNA was annealed with 2.5 μM random hexanucleotide primers by heating to 95°C for 15 min, then rapidly cooling to 4°C. Single-stranded cDNA was synthesised by incubating the oligonucleotide–RNA solution at 42°C for 15 min in 20 μl 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM each dNTP, 20 U RNase inhibitor and 50 U reverse transcriptase. The reaction was terminated by heating to 99°C for 5 min. PCR was performed using a pair of degenerate 32-mer oligonucleotide primers (Fig. 1), corresponding to conserved regions in the third and seventh transmembrane domains of the rat secretin [12], pig calcitonin [14] and opossum PTH [16] receptors. Reactions (100 μl) contained 30 pmol of each primer, 20 μl reverse transcriptase reaction and 2.5 U Amplitaq DNA polymerase in 50 mM KCl, 10 mM Tris-HCl, pH 8.3 and 2 mM $MgCl_2$. 5 cycles of PCR (60 s denaturation

at 94°C, 60 s annealing at 45°C, 60 s extension at 60°C) were followed by a further 40 cycles (60 s denaturation at 94°C, 60 s+6 s per cycle annealing and extension at 60°C) followed by 7 min at 60°C. PCR products were precipitated with ammonium acetate and ethanol, after which one half of each reaction was run on a 1% agarose gel. Five bands ranging in size from 500 to 900 bp were excised from the gel and purified using the Sephaglas BandPrep kit (Pharmacia), and one quarter was used for a further round of PCR (5 cycles of 60 s denaturation at 94°C, 60 s annealing at 47°C, 60 s extension at 60°C followed by a further 40 cycles of 60 s denaturation at 94°C, 60 s+6 s per cycle annealing and extension at 60°C followed by 7 min at 60°C. PCR products were ethanol precipitated, digested with *Bam*HI and *Eco*RI, separated on a 1% agarose–TAE gel, purified using the Sephaglas BandPrep kit (Pharmacia), ligated into pBluescript (Stratagene) and used to transform competent *E. coli* DS941. Clones with inserts of the expected size were sequenced on both strands (Sequenase 2.0 kit; USB).

One of the clones isolated, RPR7, was used to screen a commercial cDNA library in the λZap II vector prepared from rat olfactory bulb (Stratagene). Screening and plaque purification were performed using standard methods [20]. Positive clones were excised with ExAssist helper phage (Stratagene) and re-circularised to generate subclones in the pBluescript SK^- vector prior to sequencing on both strands. For expression in transfected COS cells, the insert from the full-length clone (RPR7/9.1) was excised as an *Eco*RI fragment and ligated into the *Eco*RI site of the eukaryotic expression vector, pcDNA-1 (Invitrogen).

2.2. *RNA isolation and Northern blotting*

Total RNA was isolated from tissues using the guanidinium thiocyanate/caesium chloride method [21]. Approximately 20 μg of each RNA was separated by electrophoresis on denaturing 1% agarose/formaldehyde gels, transferred to a nitrocellulose membrane (Hybond-C, Amersham) and baked for 2 h at 80°C. The membrane was then hybridised with the insert from RPR7 that had been labelled with [^{32}P]dCTP using random hexanucleotide primers (Pharmacia) and the Klenow fragment of *E. coli* DNA polymerase. Hybridisation was performed overnight in 50% formamide, 25 mM KPO_4, pH 7.4, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 50 μg/ml salmon sperm DNA at 45°C. The membranes were subsequently washed twice for 20 min in 2× SSC/0.1% SDS at 50°C, then twice more in 0.5× SSC/0.1% SDS at 50°C, and exposed to Fuji RX film.

2.3. *Cell culture and transfection*

COS 7 cells (gift of Dr. Janet Allen) were grown in DMEM supplemented with 10% new-born calf serum and 100 U/ml each of penicillin and streptomycin, in a humidified atmosphere of 95% air/ 5% CO_2 at a constant temperature of 37°C, and were passaged every 3–4 days. Cells for transfection were trypsinised the day before the experiment and plated at a density of approximately 40–50% confluency in 75 cm^2 flasks.

For transfection, cells were washed twice with OptiMEM (Gibco) supplemented with 100 U/ml each of streptomycin and penicillin at 37°C before exposure to transfecting medium for 4 h. The transfecting medium consisted of OptiMEM/penicillin/streptomycin, 400 μg/ml DEAE dextran (Promega), 100 μM chloroquine phosphate (Sigma) and 10–20 μg plasmid per flask. This was replaced with 10% DMSO in PBS for 2 min, then DMEM/2% UltraSer G/penicillin/streptomycin. Cells were grown for 24 h, then trypsinised and re-plated. Cells were harvested 48 h later.

2.4. *cAMP measurement*

For screening purposes, cells transfected with RPR7/9.1 were seeded onto 12-well tissue culture dishes, and for dose–response experiments, onto 24-well dishes. Prior to incubation with peptides, the cultures were washed with DMEM containing 0.25% BSA, and pre-incubated at 37°C for 30 min in the presence of 0.5 mM isobutyl methylxanthine. Peptides were directly added at concentrations indicated in the figure legends, and incubated at 37°C for 15–30 min. The reaction was stopped by adding ice-cold 0.1 M HCl, and the cells homogenised by trituration. The levels of cAMP in the acidic extracts were measured by radioimmunoassay using antiserum cAB4 (courtesy of K.J. Catt, NICHD, NIH, Bethesda, MD) [22].

3. RESULTS

3.1. *Cloning and analysis of receptor cDNA clones*

The cloning of the calcitonin, secretin, and PTH receptors, and their recognition as a new sub-class of peptide receptors, has provided a structural basis for the cloning of other members of the secretin receptor family. Our strategy for the identification of new family members was based on the exceptionally high degree of sequence conservation found in the 3rd and 7th transmembrane regions of these proteins. We designed degenerate oligonucleotide primers encoding portions of these sequences (Fig. 1), and used them to amplify rat anterior pituitary cDNA. Several classes of PCR product were obtained, cloned into pBluescript, and the DNA sequence of several of the clones determined. The most abundant clone (7/17 clones sequenced: designated RPR7) was very similar in sequence to the VIP receptor [3,4], but distinct from all the known members of the family.

Northern blot analysis using RPR7 as a hybridisation probe (Fig. 2) revealed a transcript of approximately 9.5 kb expressed in the pituitary and regions of the brain, with the highest levels being observed in the olfactory bulb. Accordingly, we screened a rat olfactory bulb cDNA library with probes for RPR7, and isolated a large number (~ 80 from a total of 800,000) of positive clones. Four independent positive clones were characterised by restriction mapping and partial sequencing. The 5′ termini of all four clones were identical and each

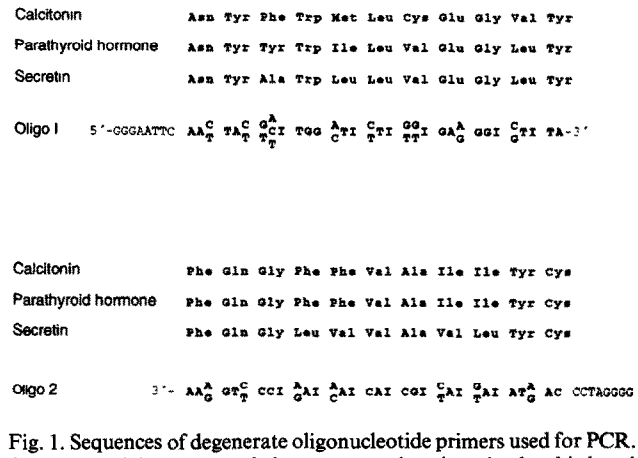

Fig. 1. Sequences of degenerate oligonucleotide primers used for PCR. Oligos 1 and 2 corresponded to conserved regions in the third and seventh transmembrane domains, respectively, of the secretin receptor family. Sequences containing restriction sites (*Eco*RI and *Bam*HI, respectively) were added to the 5′ ends of Oligo 1 and Oligo 2. Sequences of the two oligonucleotides are shown, aligned with the corresponding amino acid sequences in the rat secretin, pig calcitonin and opossum PTH receptors.

contained sequences corresponding to RPR7. The complete sequence of one clone (designated RPR7/9.1) was determined: it consisted of 2,468 bp that contained an open reading frame encoding a protein of 495 amino acids with a predicted molecular weight of 56,000 kDA (Fig. 3A). A hydropathy plot indicated that RPR7/9.1 encoded a protein with seven hydrophobic, putative membrane-spanning domains (Fig. 3B).

Comparison with other members of the secretin family (Fig. 4A) revealed that RPR7/9.1 is related to the calcitonin (31% amino acid identity), PTH (37%), glucagon (38%), GLP (37%), GHRH (41%) and secretin (47%) receptors, with greatest similarity being found with the rat VIP receptor (51%). The highest sequence identity is found in the putative transmembrane regions. The sequence of the amino-terminal extracellular domains are highly divergent, with the exception of specific amino acid residues. In particular, there are six cysteine residues that are completely conserved between the RPR7/9.1, VIP and GHRH receptors and are probably important for maintaining tertiary structure of the extracellular ligand binding domain. The carboxy-terminal cytoplasmic ends of each receptor are also highly divergent.

Examination of the amino acid sequence of RPR7/9.1 indicates a hydrophobic signal sequence at its amino-terminal end, with a predicted signal cleavage site between Ala^{19} and Met^{20} [23]. Potential sites of N-linked glycosylation are found in the amino-terminal extracellular domain at positions 47, 59, and 116, the putative 2nd extracellular loop (position 299), and 3rd intracellular loop (position 342). Potential sites for phosphorylation by protein kinase C are found at positions Ser^{365} and Thr^{375} of the 3rd intracellular loop.

Interestingly, the full-length clone (RPR7/9.1) was found to contain an additional 84 bp exon in the coding sequence when compared to RPR7 (Fig. 4B). This insertion, encoding a further 28 amino acids (including both sites for potential phosphorylation), occurs in the putative 3rd intracellular loop of the receptor polypeptide. Analysis of another clone (RPR7/9.8), which appeared to be derived from the cDNA of a partially processed transcript, indicated that the 5′ end of this insertion, at position 1,436, formed an almost perfect splice donor consensus sequence (data not shown); the 3′ end of this clone occurred within the ensuing intron. Preliminary PCR analysis of rat pituitary RNA indicated that the shorter form of the transcript was predominant, suggesting that these alternate forms of the receptor are the result of tissue-specific differential splicing.

3.2. *Expression of RPR7/9.1 in transfected COS cells*

In order to determine the pharmacological characteristics of the novel receptor, the RPR7/9.1 cDNA was cloned into the mammalian cell expression vector pcDNA-1 (Invitrogen), and transiently transfected into

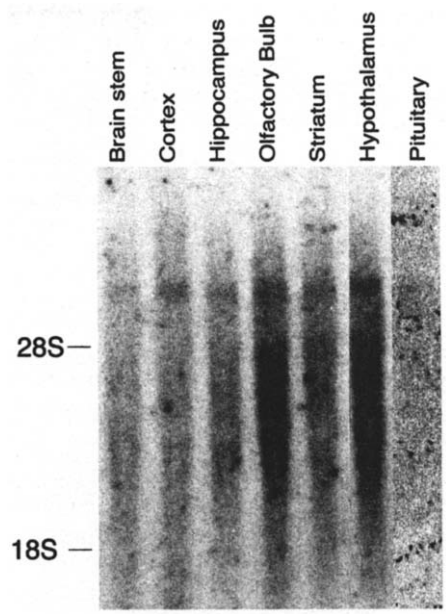

Fig. 2. Northern blot analysis of receptor mRNA in rat tissues. 20 μg of total RNA from the indicated tissues were probed with the cDNA clone, RPR7. A single transcript of approximately 9.5 kb is observed in each lane. The positions of the 28 S and 18 S ribosomal RNAs are indicated.

COS-7 cells. Since all members of the secretin receptor family so far identified are associated with activation of adenylyl cyclase, the transfected cells were stimulated with several potential ligands, and intracellular cAMP levels measured by radioimmunoassay. While treatment with GHRH, CRF, ACTH, CGRP and glucagon exhibited negligible effects (Fig. 5A), treatment with VIP, PHI and PACAP-38 resulted in a marked elevation of cAMP levels, with the latter showing the highest level of stimulation. PHI, VIP and PACAP-38 failed to stimulate cAMP levels in a control experiment where COS-7 cells had been transfected with the $5HT_{1A}$ receptor (data not shown). The stimulation of cAMP levels by PHI, VIP and PACAP was dose-dependent (Fig. 5B). The EC_{50} for cAMP accumulation was approximately 0.5 nM for PACAP-38, 0.8 nM for PACAP-27, and 48 nM for VIP. While the EC_{50} for PHI was not determined, Fig. 5B indicates that it was very much higher than that of VIP. We therefore conclude that the order of potency of these ligands is PACAP-38 ~ PACAP-27 > VIP > PHI, and that accordingly, RPR7/9.1 encodes a Type I PACAP receptor.

4. DISCUSSION

We report the cloning of a rat PACAP receptor from a rat olfactory bulb cDNA library. Evidence that the full-length clone (RPR7/9.1) we isolated encodes a PACAP receptor are: (i) the encoded protein includes many of the features expected of a G protein coupled receptor; (ii) it is highly homologous to receptors for

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                                                                                 CGGCACGAGCGCCTCTCCCGCAGACATAGCGGCCAA   36

GCAGCACGGGGTGGGCGACACAGGACGAGGGGCGCCTCGCTCCGATTCCCGGCTGCGACTGGCGGAGGCTCTGGAGCAACGTCCCGGTGTGCAATACGCACCAGCCGCGGCAGGGACAC  155

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ATG GCC AGA GTC CTG CAG CTC TCC CTG ACT GCT CTC CTG CTG CCT GTG GCT ATT GCT ATG CAC TCT GAC TGC ATC TTC AAG AAG GAG CAA   483
Met Ala Arg Val Leu Gln Leu Ser Leu Thr Ala Leu Leu Leu Pro Val Ala Ile Ala Met His Ser Asp Cys Ile Phe Lys Lys Glu Gln    30

GCC ATG TGC CTG GAG AGG ATC CAG AGG GCC AAC GAC CTG ATG GGA CTA AAC GAG TCT TCC CCA GGT TGC CCT GGC ATG TGG GAC AAT ATC   573
Ala Met Cys Leu Glu Arg Ile Gln Arg Ala Asn Asp Leu Met Gly Leu Asn Glu Ser Ser Pro Gly Cys Pro Gly Met Trp Asp Asn Ile    60

ACA TGT TGG AAG CCA GCT CAA GTA GGT GAG ATG GTC CTT GTA AGC TGC CCT GAG GTC TTC CGG ATC TTC AAC CCG GAC CAA GTC TGG ATG   663
Thr Cys Trp Lys Pro Ala Gln Val Gly Glu Met Val Leu Val Ser Cys Pro Glu Val Phe Arg Ile Phe Asn Pro Asp Gln Val Trp Met    90

ACA GAA ACC ATA GGA GAT TCT GGT TTT GCC GAT AGT AAT TCC TTG GAG ATC ACA GAC ATG GGG GTC GTG GGC CGG AAC TGC ACA GAG GAC   753
Thr Glu Thr Ile Gly Asp Ser Gly Phe Ala Asp Ser Asn Ser Leu Glu Ile Thr Asp Met Gly Val Val Gly Arg Asn Cys Thr Glu Asp   120

GGC TGG TCG GAG CCC TTC CCC CAC TAC TTC GAT GCT TGT GGG TTT GAT GAT TAT GAG CCT GAG TCT GGA GAT CAG GAT TAT TAC TAC CTG   843
Gly Trp Ser Glu Pro Phe Pro His Tyr Phe Asp Ala Cys Gly Phe Asp Asp Tyr Glu Pro Glu Ser Gly Asp Gln Asp Tyr Tyr Tyr Leu   150

TCG GTG AAG GCT CTC TAC ACA GTC GGC TAC AGC ACT TCC CTC GCC ACC CTC ACT ACT GCC ATG GTC ATC TTG TGC CGC TTC CGG AAG CTG   933
Ser Val Lys Ala Leu Tyr Thr Val Gly Tyr Ser Thr Ser Leu Ala Thr Leu Thr Thr Ala Met Val Ile Leu Cys Arg Phe Arg Lys Leu   180

CAT TGC ACT CGC AAC TTC ATC CAC ATG AAC CTG TTT GTA TCC TTC ATG CTG AGG GCT ATC TCC GTC TTC ATC AAG GAC TGG ATC TTG TAC  1023
His Cys Thr Arg Asn Phe Ile His Met Asn Leu Phe Val Ser Phe Met Leu Arg Ala Ile Ser Val Phe Ile Lys Asp Trp Ile Leu Tyr   210

GCC GAG CAG GAC AGC AGT CAC TGC TTC GTT TCC ACC GTG GAG TGC AAA GCT GTC ATG GTT TTC TTC CAC TAC TGT GTG GTG TCC AAC TAC  1113
Ala Glu Gln Asp Ser Ser His Cys Phe Val Ser Thr Val Glu Cys Lys Ala Val Met Val Phe Phe His Tyr Cys Val Val Ser Asn Tyr   240

TTC TGG CTG TTC ATT GAA GGC CTG TAC CTC TTT ACA CTG CTG GTG GAG ACC TTC TTC CCT GAG AGG AGA TAT TTC TAC TGG TAC ACC ATC  1203
Phe Trp Leu Phe Ile Glu Gly Leu Tyr Leu Phe Thr Leu Leu Val Glu Thr Phe Phe Pro Glu Arg Arg Tyr Phe Tyr Trp Tyr Thr Ile   270

ATC GGC TGG GGG ACA CCT ACT GTG TGT GTA ACA GTG TGG GCT GTG CTG AGG CTC TAT TTT GAT GAT GCA GGA TGC TGG GAT ATG AAT GAC  1293
Ile Gly Trp Gly Thr Pro Thr Val Cys Val Thr Val Trp Ala Val Leu Arg Leu Tyr Phe Asp Asp Ala Gly Cys Trp Asp Met Asn Asp   300

AGC ACA GCT CTG TGG TGG GTG ATC AAA GGC CCC GTG GTT GGC TCT ATA ATG GTT AAC TTT GTG CTT TTC ATC GGC ATC ATC ATC ATC CTT  1383
Ser Thr Ala Leu Trp Trp Val Ile Lys Gly Pro Val Val Gly Ser Ile Met Val Asn Phe Val Leu Phe Ile Gly Ile Ile Ile Ile Leu   330

GTA CAG AAG CTG CAG TCC CCA GAC ATG GGA GGC AAC GAG TCC AGC ATC TAC TTC AGC TGC GTG CAG AAA TGC TAC TGC AAG CCA CAG CGG  1473
Val Gln Lys Leu Gln Ser Pro Asp Met Gly Gly Asn Glu Ser Ser Ile Tyr Phe Ser Cys Val Gln Lys Cys Tyr Cys Lys Pro Gln Arg   360

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Ala Gln Gln His Ser Cys Lys Met Ser Glu Leu Ser Thr Ile Thr Leu Arg Leu Ala Arg Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly   390

ATC CAC TAC ACA GTA TTC GCC TTC TCT CCA GAG AAC GTC AGC AAG AGG GAA AGA CTT GTG TTT GAG CTT GGG CTG GGC TCC TTC CAG GGC  1653
Ile His Tyr Thr Val Phe Ala Phe Ser Pro Glu Asn Val Ser Lys Arg Glu Arg Leu Val Phe Glu Leu Gly Leu Gly Ser Phe Gln Gly   420

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Phe Val Val Ala Val Leu Tyr Cys Phe Leu Asn Gly Glu Val Gln Ala Glu Ile Lys Arg Lys Trp Arg Ser Trp Lys Val Asn Arg Tyr   450

TTC ACT ATG GAC TTC AAG CAC CGG CAC CCG TCC CTG GCC AGC AGT GGA GTA AAT GGG GGA ACC CAG CTG TCC ATC CTG AGC AAG AGC AGC  1833
Phe Thr Met Asp Phe Lys His Arg His Pro Ser Leu Ala Ser Ser Gly Val Asn Gly Gly Thr Gln Leu Ser Ile Leu Ser Lys Ser Ser   480

TCC CAG CTC CGC ATG TCC AGC CTC CCG GCC GAC AAC TTG GCC ACC TGAGGCCTGTCTCCCTCCTCCTTCTGCACAGGCTGGGGCTGCGGGCCAGTGCCTGAGCA  1937
Ser Gln Leu Arg Met Ser Ser Leu Pro Ala Asp Asn Leu Ala Thr                                                              495

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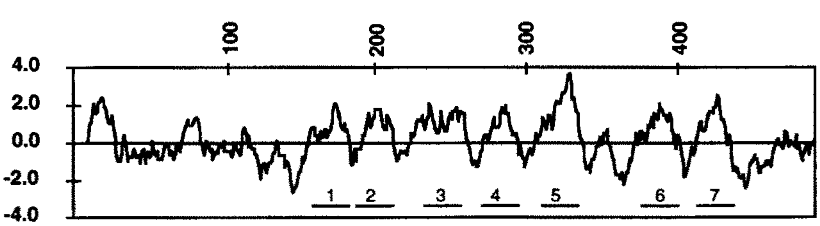

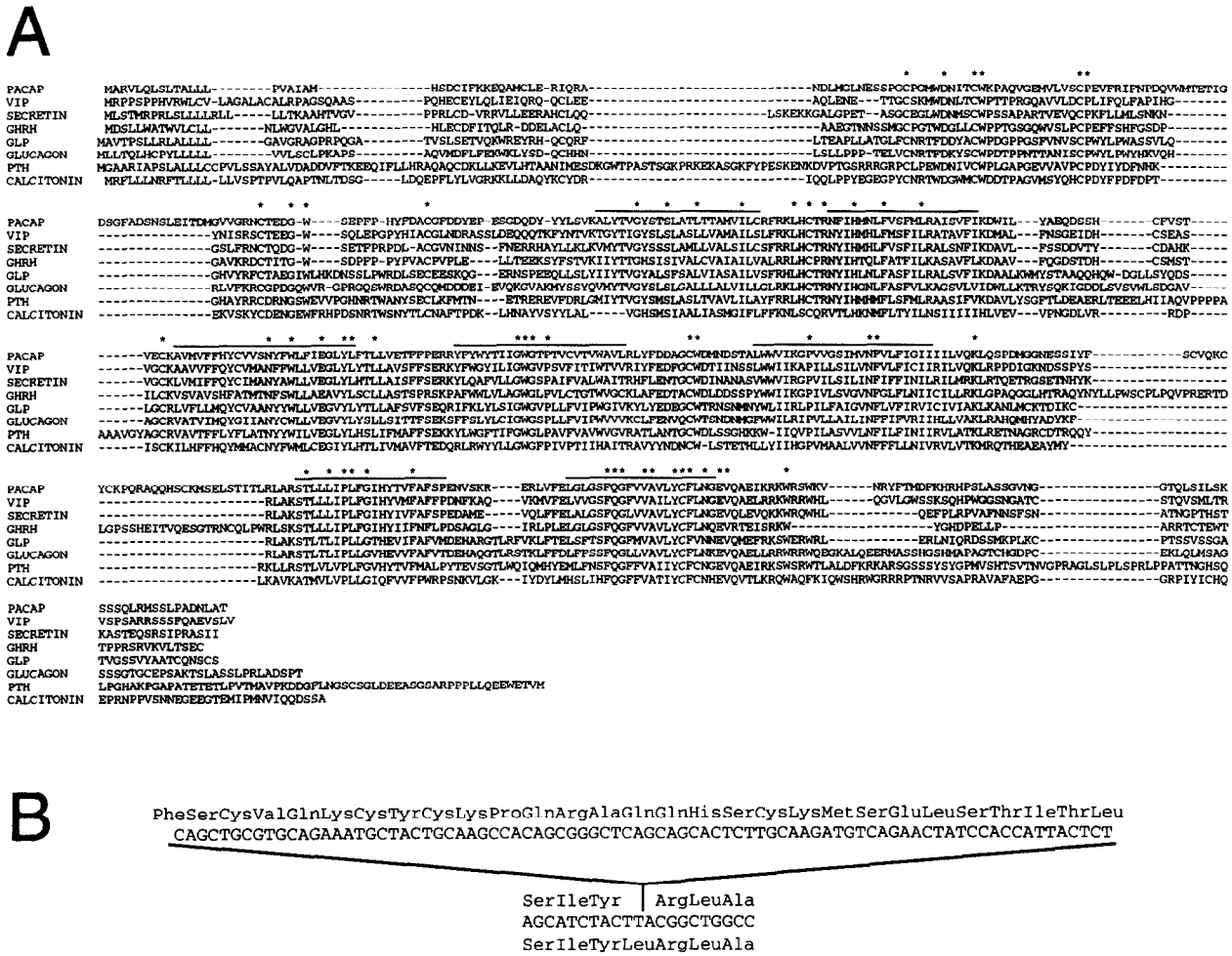

Fig. 4. (A) Alignment of the rat PACAP, VIP, secretin, GHRH, GLP, glucagon, PTH and calcitonin receptors. Amino acids identical in all eight receptors are indicated with an asterisk. Putative transmembrane regions are indicated by lines above the sequence. (B) Generation of isoforms of the rat PACAP receptor by differential RNA processing. The top line indicates the nucleotide sequence and translation of RPR7/9.1 between positions 1,436 and 1,521. This 84 base insertion is spliced out to give rise to the shorter isoform of the receptor shown below.

other secretin-like peptides which include PACAP; (iii) expression of the cDNA in transfected COS cells results in PACAP-stimulated production of cAMP.

Most sequence identity between the PACAP receptor and other members of the receptor family occurs in the putative transmembrane domains. The N-terminal domains, while highly divergent, exhibit substantial homology at specific amino acid positions. These include 6 cysteine residues which have been postulated to confer a general structure to the extracellular ligand binding domains of these receptors [10]. The PACAP receptor exhibits the highest degree of homology with the VIP receptor (51%), which reflects the high sequence identity of their respective ligands (68%).

While many genes encoding G protein-coupled receptors lack introns, there is increasing evidence that members of the secretin receptor family are large and interrupted by numerous introns. For instance, Mayo [10] identified two isoforms of the rat GHRH receptor which differed by the presence of a 124 base exon in the region encoding the putative third intracellular loop. Similarly, we have identified two isoforms of the

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Fig. 3. Sequence of the rat PACAP receptor cDNA and polypeptide. (A) DNA sequence and predicted amino acid sequence of rat cDNA clone RPR7/9.1. (B) Hydropathy plot of the rat PACAP receptor protein. The strongly hydrophobic region at the N-terminus corresponds to the signal sequence. A further seven hydrophobic putative membrane spanning regions are numbered from 1 to 7. The numbers on the horizontal scale refer to amino acid positions.

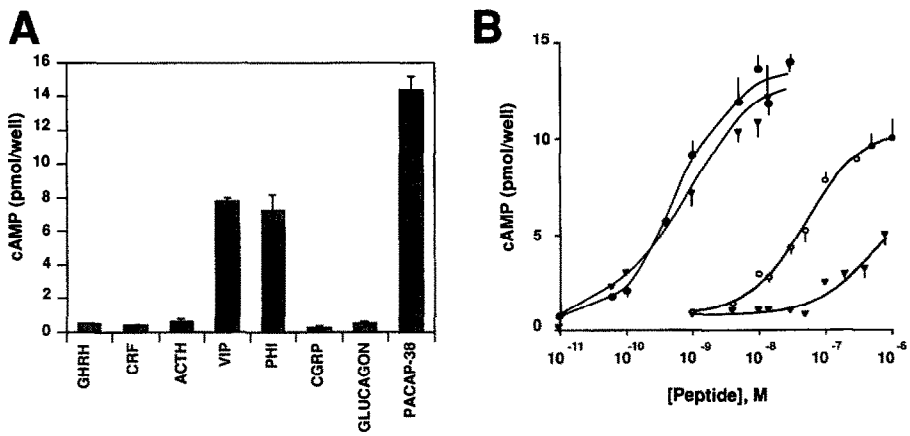

Fig. 5. Stimulation of cAMP production in COS cells transfected with RPR7/9.1. (A) Stimulation of cAMP production by various ligands. Cells transfected with RPR7/9.1 were stimulated with the ligands indicated, each at a concentration of 100 nM. Values of cAMP are expressed as the mean±S.E.M. (n = 3). (B) Dose–response curves of cAMP stimulation in COS cells stimulated by PACAP38 (●) PACAP27 (▽), VIP, (○) and PHI (▼). The values represent the mean ± S.E.M. (n = 4). Basal cAMP level was 0.42 ± 0.06 pmol/well.

PACAP receptor which differ by the presence or absence of an 84 base exon in an identical location. Analysis of other clones which appeared to be derived from cDNAs of unprocessed RNAs revealed the presence of other introns within the coding sequence (J.A. Morrow and K.M. West, unpublished data). This is analogous to the D_2 receptor where alternative splicing gives rise to two isoforms, one with a 29 amino acid insert in the third intracellular loop [24–26]. The longer form was found to predominate in the pituitary. Since the third intracellular loop is associated with the interaction with G proteins, it is possible that the longer and shorter isoforms of these proteins differentially interact with G proteins to activate different second messenger pathways.

Receptor binding studies have provided evidence for the presence in tissues of four types of membrane receptor capable of specific binding of PACAP. The Type II receptor [27] recognises VIP, PACAP-27 and PACAP-38 with very similar affinities, and may correspond to the cloned VIP receptor [3,4]. This receptor is present in a variety of peripheral tissues, including rat liver, rat lung, mouse splenocytes and human small intestinal epithelium [27–31]. The human lymphoblastic cell line, SUP-T1, contains a receptor which binds VIP and PACAP but for which helodermin is the most potent ligand [32]. Type I receptors which are highly specific for PACAP, but bind VIP with only modest affinity [27]) have been described in brain and pituitary [28,33,34], rat astrocytes in culture [35], testis [27] and adrenal medulla [27]. A variety of cell lines, including NB-OK neuroblastoma cells [36], PC12 phaeochromocytoma cells [37], the rat pancreatic acinar cell line AR 4–2J [38] and the THP-1 monocytic leukaemia cell line [39] have also been shown to possess Type I PACAP receptors.

Studies of PACAP binding to membranes from the rat central nervous system [33] and from the rat pancreatic tumour cell line AR4-2J [40,41] have led to the division of Type I PACAP receptors into two subclasses (PACAP-IA and PACAP-IB). PACAP-IA receptors bind PACAP-27 with slightly higher affinity than PACAP-38, whereas PACAP-IB receptors recognise PACAP-38 with high affinity and PACAP-27 with low affinity. PACAP-38-preferring receptors have also been described in hypothalamus [42] and pituitary [28].

Our data, which demonstrate that RPR7/9.1 encodes a receptor with high affinity for PACAP (EC_{50} = 0.5 nM for PACAP38 and 0.8 nM for PACAP27) and responds to ligands with the order of potency PACAP38 ~ PACAP27 > VIP > PHI, is consistent with RPR7/9.1 being a Type I receptor. It has yet to be established unambiguously, however, whether it is Type IA or IB.

While PACAP activates adenylyl cyclase and increases cAMP levels in various tissues, it has also been shown to stimulate the phosphatidyl inositol cascade and mobilise intracellular Ca^{2+} [43–45]. It will be interesting to determine if RPR7/9.1 can also stimulate the Ca^{2+}/inositol phosphate pathway, and whether the two receptor isoforms we have described differ in their ability to activate these intracellular effector systems.

The characterisation of the PACAP receptor will be of considerable importance for our understanding of the physiology of PACAP and may facilitate the development of specific PACAP agonists and antagonists. The presence of PACAP receptors in the central nervous system suggests that PACAP may play a role in the pathogenesis of psychiatric, neurological and neuroendocrine disorders. Type I PACAP binding sites have been described in the adrenal medulla [27], and PACAP has been implicated in the regulation of adrenal chromaffin cells, perhaps in the synthesis and secretion of catecholamines [46]. Type I receptors have also been described in pancreatic acinar cells [40] where PACAP

has been suggested to regulate pancreatic exocrine activity [47]. The presence of Type I receptors in the spermatozoa and other sites of the reproductive system [27] suggests that PACAP may play an important role in disorders and control of fertility. Finally, the presence of PACAP receptors in various tumour cell lines [36–40], and lung cancer cells [48] suggests the possibility of alterations in PACAP signal transduction being involved in oncogenesis.

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