

## MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF RAT cDNAs ENCODING THE RECEPTOR FOR PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)<sup>#</sup>

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Received May 25, 1993

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**SUMMARY:** Two types of cDNA encoding PACAP receptors were isolated from the rat brain cDNA library by homology screening with a cDNA probe for VIP receptor. Nucleotide sequence analysis indicated that these two types of receptor mRNA were generated by alternative splicing mechanisms. These two cloned cDNAs were introduced into CHO cells respectively. Resultant transformants showed specific binding to [<sup>125</sup>I]PACAP27 which was displaced by unlabeled PACAP27 but not by VIP. Thus, these receptors are two subtypes of Type I PACAP receptor (Type I-A and Type I-B). The amino acid sequences of rat PACAP receptors deduced by the cDNAs showed a remarkable similarity with rat receptors for VIP, secretin, glucagon, and GHRH. A 6.5 kb significant hybridizing signal of the PACAP receptor mRNA was detected in the rat brain, and slight signal was also detected in the lung and the liver. © 1993 Academic Press, Inc.

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Pituitary adenylate cyclase activating polypeptides (PACAPs) are a 38 amino acid (PACAP38) and 27 amino acid peptides (PACAP27, which corresponds to N-terminal 27 amino acid residues of PACAP38) (1,2). They were found in the ovine hypothalamus as novel peptides which stimulate the synthesis of cAMP in cultured rat pituitary cells (1,2). The numerous biological actions of PACAP such as the stimulation of the secretion of GH, prolactin, ACTH and LH from the superfused pituitary cells (1), catecholamine from adrenal medullary cells (3), pancreatic amylase (4), insulin (5) and endothelium-independent vasodilation (6,7) were

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<sup>#</sup> The nucleotide sequences reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession numbers of D14908 and D14909.

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Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BSA, bovine serum albumin.

reported. Based on the structural features, PACAP (PACAP38 and PACAP27) belongs to a family of peptides, which consist of vasoactive intestinal peptide (VIP), glucagon, secretin, growth hormone releasing hormone (GHRH) and peptide histidine isoleucine (PHI) (1). The isolated cDNAs for ovine (8), human (8,9) and rat (10) PACAP show completely identical sequences, suggesting the structural significance of the peptide against the evolutionary pressures. PACAP was originally isolated from hypothalamic tissues (1,2), however, it was also detected in central nervous system (CNS) and peripheral tissues (11). In addition, the specific binding sites for PACAP have also been observed in those tissues (12). So PACAP is speculated to have not only endocrine but also neurotransmitter/neuromodulator role in vivo (13). By the specificity of the binding to the ligands, PACAP receptor was classified into two types, Type I (specific to PACAP) and Type II (similar affinities to PACAP and VIP) (13). Various biological actions of PACAP were supposed to be mediated by these receptors, and especially the PACAP specific action by Type I receptor.

The function of various biologically active peptides are mediated by their interaction with specific receptors. However, the peptides similar in structure have structurally related receptors, and their bindings significantly compete against each other. This is also the case for VIP (14), secretin (15), glucagon (16) and GHRH (17) receptors. The amino acid sequences deduced from the cloned receptor cDNAs revealed the similar structure of the receptors which shared the feature of G protein coupling. Since PACAP also showed a related structure and stimulated the intracellular cAMP accumulation, it was expected to have a structurally related receptor for VIP, glucagon, secretin and GHRH receptors. The VIP receptor so far cloned has a similar affinities to both VIP and PACAP (14), so we supposed that the VIP receptor plays a role as Type II receptor in binding and mediating the PACAP action.

In this study, we represented here first the cloning of the cDNAs encoding rat PACAP specific receptors (Type I) from rat brain by cross-hybridization with rat VIP receptor cDNA. The amino acid sequence of rat PACAP receptors showed a high degree of similarity with those for rat VIP, secretin, glucagon and GHRH receptors.

## MATERIALS AND METHODS

***Poly(A)<sup>+</sup>RNA Preparation, cDNA Synthesis and Library Construction.*** Total RNA was prepared from rat brain, lung, liver, kidney and testis, and poly(A)<sup>+</sup>RNA fractions were selected by oligo(dT)-cellulose column chromatography. A rat brain cDNA library consisting of about  $3.0 \times 10^6$  independent clones was constructed with the cDNA synthesis and cloning kits

(Amersham, UK). The rat VIP receptor cDNA (14) used as a probe for screening was prepared by polymerase chain reaction (PCR) using rat lung poly(A)<sup>+</sup>RNA preparation and appropriate primers.

**Cloning and Sequence Analysis.** The rat VIP receptor cDNA fragment was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by the multiprime DNA labeling kit (Amersham) and used as a hybridization probe for screening. Filters containing plaque lifts of rat brain cDNA library were hybridized with the probes overnight at 55°C in a buffer containing 2 x SSC, 0.1% SDS, 10 x Denhard's solution and 0.17% yeast RNA. The filters were then washed with 2 x SSC, 0.1% SDS at 50°C before autoradiography. One of the phage clones with a significant intensity of signal was plaque purified, subcloned into plasmid and named pRPACAPR18. The rat brain 5' stretch cDNA library (Clontech, CA) was screened with the cDNA insert of pRPACAPR18 (42°C with a buffer containing 50% formamide, 5 X SSPE, 5 x Denhard's solution, 0.1% SDS and 100  $\mu$ g/ml of denatured salmon sperm DNA). Three of 26 positive clones were plaque purified, the cDNA inserts were subcloned into plasmids and named pRPACAPR5, pRPACAPR12 and pRPACAPR46, respectively. The subcloned cDNA inserts were sequenced by dideoxy nucleotide chain termination method with [ $\alpha$ -<sup>32</sup>P]dCTP and also with an ABI 370A DNA sequencer. The sequences were connected and analyzed by DNASIS (Hitachi, JAPAN).

**Expression of Cloned cDNA and Binding Studies.** The NcoI-ApoI cDNA fragments containing protein coding region of pRPACAPR46-5 and pRPACAPR12 were inserted in the expression vector pRC/CMV (Invitrogen) and named pRPR3-A and pRPR4-B respectively. The resultant expression plasmids were introduced into CHO cells by the calcium phosphate transfection method with CellPfect Transfection Kit (Pharmacia). Transformants were selected in the culture medium (Ham's F12 with 10% fetal calf serum) containing 500  $\mu$ g/ml of Geneticin (GIBCO). The transfected or untransfected CHO cells grown confluent in 6-well tissue culture plates (Falcon) were incubated with 100 pM [<sup>125</sup>I]PACAP27 (2200 Ci/mmol)(18) in 5 mM Hepes buffered Krebs-Ringer solution containing 0.05% CHAPS and 0.1% BSA at 37 °C for 1hr. The cells were washed with the same medium three times, harvested with 0.5N NaOH-0.1% SDS, and subjected to gamma-counting. Competitive binding experiments were done with the membranes prepared from the transfected cells. The membrane was incubated with 100 pM of [<sup>125</sup>I]PACAP27 in 20 mM Tris, 1 mM EDTA, 0.05% CHAPS, 0.1% BSA and protease inhibitors (pH7.4) at 25°C for 1hr. Bound and free ligands were separated by the filtration method as described previously (19). The non-specific binding was determined in the presence of 1  $\mu$ M of unlabeled PACAP27.

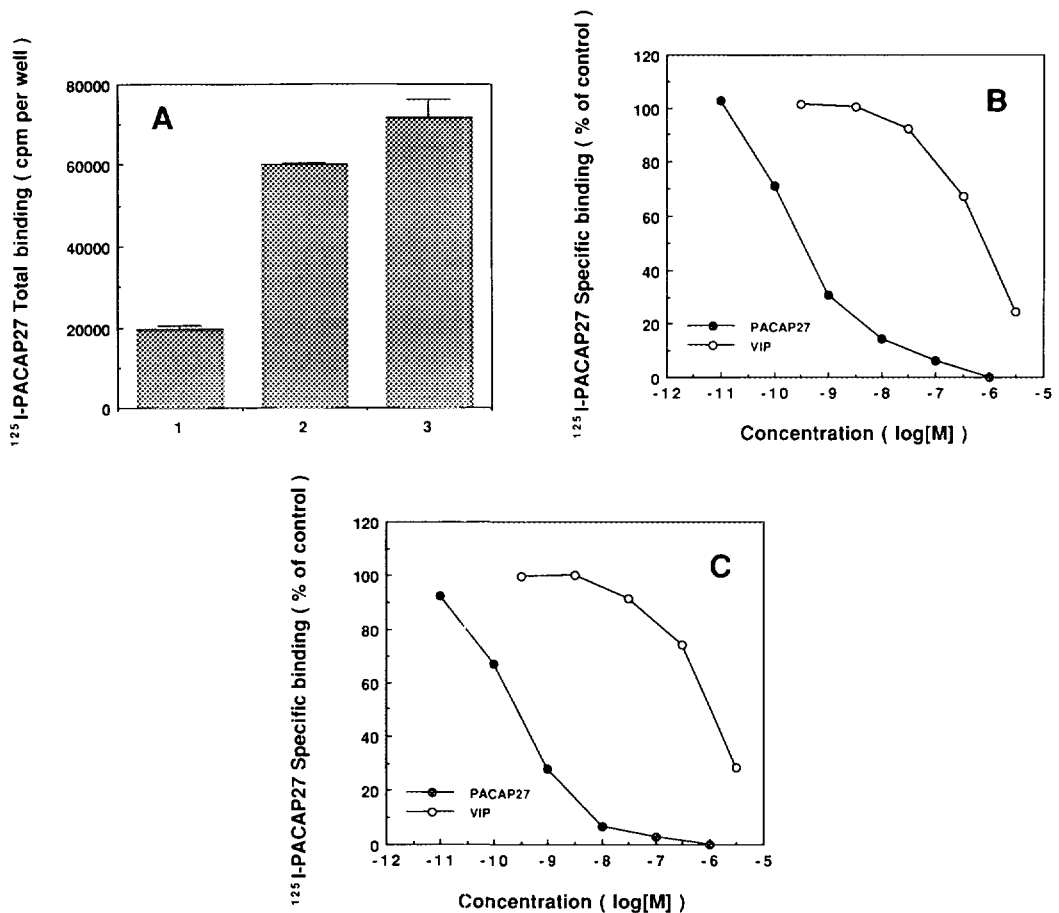
**Northern Blot Analysis.** As a probe, the 0.4 kb NcoI-BanII DNA fragment of pRPACAPR12 corresponding to the N-terminal region of both cDNAs was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by the multiprime DNA labeling Kit. Five  $\mu$ g of rat brain, lung liver, kidney and testis poly(A)<sup>+</sup>RNA preparations were denatured, electrophoresed onto 1.2% agarose gel (20) and transferred to a nylon membrane filter (PALL). Hybridization was carried out overnight with radiolabeled probe in the buffer containing 50% formamide at 42°C. The condition of final washing of the filter was 0.1% SSC, 0.1% SDS at 50°C.

## RESULTS

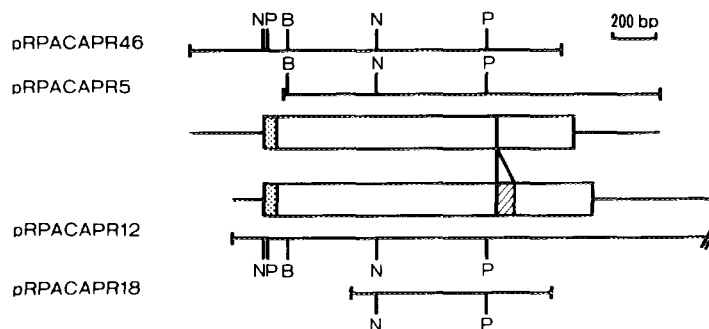
### *Isolation of Rat PACAP Receptor cDNA.*

By screening a rat brain cDNA library with the cDNA fragment of rat VIP receptor, we isolated a clone of significant signal, subcloned its cDNA insert into plasmid pUC118 and sequenced. The nucleotide and deduced amino acid sequences of the clone named pRPACAPR18 showed significant homology with not only rat VIP receptor but also secretin and GHRH receptor cDNAs. A search of the GeneBank database revealed that the sequence of pRPACAPR18 was a novel one, however, we could not obtain its complete translation unit. So we further screened the rat brain library and obtained the clones named pRPACAPR5,

pRPACAPR12 and pRPACAPR46 (Fig. 2). To confirm that the cDNA inserts of pRPACAPR46-5 (a recombinant cDNA at BamHI sites of pRPACAPR46 and pRPACAPR5 to obtain a complete open reading frame) and pRPACAPR12 encode the PACAP receptor, the cDNAs were introduced into CHO cells. Figure 1 shows the properties of [ $^{125}$ I]PACAP27 binding to CHO cells transfected with the PACAP receptor cDNAs. The transformants showed the significant increase of [ $^{125}$ I]PACAP27 binding (Fig. 1A). The bound [ $^{125}$ I]PACAP27 was displaced effectively by PACAP27, whereas it was only slightly displaced by VIP at higher concentrations (Fig. 1B,C).



**Fig. 1.** The binding of [ $^{125}$ I]PACAP27 to CHO cells transfected with the PACAP receptor cDNA. A. Comparison of [ $^{125}$ I]PACAP27 binding to nontransfected (1) and transfected (2, pRPR3-A and 3, pRPR4-B) CHO cells. Each bar shows the mean with standard error of triplicated experiments. B and C. Displacement of [ $^{125}$ I]PACAP27 binding by PACAP27 and VIP. Each reaction mixtures contained the 10 (pRPR3-A) or 15 (pRPR4-B)  $\mu$ g of proteins with specific binding (100%) about 4100 and 3900 cpm, respectively. Bindings of [ $^{125}$ I]PACAP27 to the membrane preparation from pRPR3-1 (B) and pRPR4-B (C) transfected CHO cells were plotted versus variable concentrations of unlabeled PACAP27 (closed circle) and VIP (open circle), respectively.



*Fig. 2. Schematic illustration of the structure of the cloned rat PACAP receptor cDNAs and the postulated structure of corresponding mRNA. Restriction endonuclease cleavage sites used for mapping are indicated (N, NcoI; P, PstI; B, BamHI). Boxes indicate the protein coding region and the dotted areas are signal sequence. The alternative exon pRPACAPR12 is denoted by the shaded area.*

### **Structure of the Rat PACAP Receptor.**

Figure 3 shows nucleotide sequences of pRPACAPR46-5 and pRPACAPR12 together with the deduced amino acid sequences. The sequences contained open reading frames encoding the proteins of 467 and 495 amino acids (M.W. = 53.2 kD and 56.4 kD) respectively. The difference between the two sequences is explained by the alternative splicing of an exon, encoding the 28 amino acid residues in pRPACAPR12 (Fig. 3B). The first 19 amino acid residues in N-termini apparently served as a signal sequence (21). The characterization of the hydrophobicity of the sequences represented the expected feature of G protein coupled receptors, the seven hydrophobic segments arranged in tandem (Fig.4), and we suspected the segments to be possible transmembrane regions of the receptors. The alternative region in pRPACAPR12 turned out to supply a hydrophilic motif in postulated cytoplasmic region between fifth and sixth hydrophobic segments (Fig.4). The amino acid sequence of the alternative exon showed no significant homology with the other receptor proteins so far reported. The overall amino acid sequences of the two receptor proteins contained six potential N-glycosylation sites (Asn-X-Ser/Thr)(Fig.1).

### **Northern Blot Analysis.**

Figure 5 shows the Northern blot analysis of rat poly(A)<sup>+</sup>RNA with the cDNA fragment corresponding to the N-terminal region of a rat PACAP receptor. The size of hybridizing bands was about 6.5 kb and larger than that of rat VIP receptor (14). The brain mRNA gave a strong signal while those of lung and liver emitted weak signals. Kidney had been reported to have no

<b>A</b>	1	CGAGTGGACAGTGGCAGGCGGTGACTGAATCTCCAAGTCTGGAAACAATAGCCAGAGA	58
	59	TAGTGGCTGGGAAGCCACATGGCCAGAGTCTCGACGCTCTCCCTGACTGCTCTCCTGCTG	118
	1	MetAlaArgValLeuGlnLeuSerLeuThrAlaLeuLeuLeu	14
	119	CCTGTGGCTATTGCTATGCACTCTGACTGCATCTTCAAGAAGGAGCAAGCCATGTGCCTG	178
	15	ProValAlaIleAlaMetHisSerAspCysIlePheLysLysGluGlnAlaMetCysLeu	34
	179	GAGAGGATCCAGAGGCCAACCGACCTGATGGGACTAAACGAGTCTTCCCCAGGTTGCCCT	238
	35	GluArgIleGlnArgAlaAsnAspLeuMetGlyLeuAsnGluSerSerProGlyCysPro	54
	239	GGCATGTGGGCAATATCACATGTTGGAAAGCCAGCTCAAGTAGGTGAGATGGCTCTGTA	298
	55	GlyMetTrpAsp <u>AsnIleThrCysTrpLysProAlaGlnValGlyGluMetValLeuVal</u>	74
	299	AGCTGCCCTGAGGTCTTCCGGATCTTCAACCCGGACCAAGTCTGGATGACAGAAACATA	358
	75	SerCysProGluValPheArgIlePheAsnProAspGlnValTrpMetThrGluThrIle	94
	359	GGAGATTCTGGTTTTGCCGATAGTAATTCCTTGGAGATCACAGACATGGGGTCTGGGC	418
	95	GlyAspSerGlyPheAlaAspSerAsnSerLeuGluIleThrAspMetGlyValValGly	114
	419	CGGAACGACAGAGGACGGCTGGTCGGAGCCCTCCCCACTACTTCGATGCTTGTGGG	478
	115	ArgAsnCysThrGluAspGlyTrpSerGluProPheProHisTyrPheAspAlaCysLeu	134
	479	TTTGATGATTATGAGCCTGAGTCTGGAGATCAGGATTATTACTACCTGTCCGGTAGAGCT	538
	135	PheAspAspTyrGluProGluSerGlyAspGlnAspTyrTyrTyrLeuSerValLysAla	154
	539	CTCTACACAGTCGGCTACAGCACTCCCTCGCCACCCTCACTACTGCCATGGTCTCTTG	598
	155	LeuTyrThrValGlyTyrSerThrSerLeuAlaThrLeuThrThrAlaMetValIleLeu	174
	599	TGCCGCTCCGGAAAGTGCATTGCCTCGCAACTTCATCCACATGAACCTGTTGTATCC	658
	175	CysArgPheArgLysLeuHisCysThrArgAsnPheIleHisMetAsnLeuPheValSer	194
	659	TTCATGCTGAGGGCTATCTCCGCTCTTCAAGGACTGGATCTTGTACGCCGAGCAGGAC	718
	195	PheMetLeuArgAlaIleSerValPheIleLysAspTrpIleLeuTyrAlaGluGlnAsp	214
	719	AGCAGTCACCTGCTTCGTTCCACCGTGGAGTGCAAGCTGTCATGGTTCCTCCACTAC	778
	215	SerSerHisCysPheValSerThrValGluCysLysAlaValMetValPhePheHisTyr	234
	779	TGCGTGGTGTCAACTACTTCTGGCTGTTTATTGAGGCGCTGTACCTCTTACACTGCTG	838
	235	CysValValSerAsnTyrPheTrpLeuPheIleGluGlyLeuTyrLeuPheThrLeuLeu	254
	839	GTGGAGCCTTCTCCCTGAGAGGAGATATTTCTACTGGTACACCATCATCGGCTGGGG	898
	255	ValGluThrPhePheProGluArgArgTyrPheTyrTrpTyrThrIleIleGlyTrpGly	274
	899	ACACCTACTGTGTGTAAACAGTGTGGGCTGTGCTGAGGCTCTATTTGATGATGCAGGA	958
	275	ThrProThrValCysValThrValTrpAlaValLeuArgLeuTyrPheAspAspAlaGly	294
	959	TGCTGGGATATGAATGACAGCACAGCTCTGTGGTGGGTGATCAAAGGCCCGGTGGTGGC	1018
	295	CysTrpAspMetAsnAspSerThrAlaLeuTrpTrpValIleLysGlyProValAlaGly	314
	1019	TCTATAATGGTTAACTTTTGCTTTTTCATCGGCATCATCATCTCTGTACAGAAGCTG	1078
	315	SerIleMetValAsnPheValLeuPheIleGlyIleIleIleIleLeuValGlnLysLeu	334
	1079	CAGTCCCCAGACATGGGAGGCCAAGGAGTCCAGCATCTACTTACGGCTGGCCGCTCCACC	1138
	335	GlnSerProAspMetGlyGlyAsnGluSerSerIleTyrLeuArgLeuAlaArgSerThr	354
	1139	CTACTGCTCATCCACTCTTCGCAATCCACTACACAGTATTGCGCTTCTCTCCAGAGA	1198
	355	LeuLeuLeuIleProLeuPheGlyIleHisTyrThrValPheAlaPheSerProGluAsn	374
	1199	GTGAGCAAGAGGAAAGACTTGTGTTTGAGCTTGGGCTGGGCTCCTTCCAGGGCTTTGTG	1258
	375	ValSerLysArgGluArgLeuValPheGluLeuGlyLeuGlySerPheGlnGlyPheVal	394
	1259	GTGGCTGACTCTACTGCTTCTCGAATGGGGAGGTACAGGCAGAGATTAAAGAGAAATGG	1318
	395	ValAlaValLeuTyrCysPheLeuAsnGlyGluValGlnAlaGluIleLysArgLysTrp	414
	1319	AGGAGCTGGAAGGTGAACCGTTACTTCACTATGGACTTCAAGCACCCGGCACCCGCTCCCTG	1378
	415	ArgSerTrpLysValAsnArgTyrPheThrMetAspPheLysHisArgHisProSerLeu	434
	1379	GCCAGCAGTGGAGTAAATGGGGCAACCCAGCTGCCATCTGAGCAAGAGCAGCTCCAG	1438
	435	AlaSerSerGlyValAsnGlyGlyThrGlnLeuSerIleLeuSerLysSerSerSerGln	454
	1439	CTCCGATGTCACCGCTCCCGCCGCAACTTGGCCACCTGAGGCGTGTCTCCCTCTCTCC	1498
	455	LeuArgMetSerSerLeuProAlaAspAsnLeuAlaThr**	467
	1499	TTCTGCACAGGCTGGGGCTGGGGCCAGTGCCTGAGCATGTTTGTGCCTCTCCCTCTCC	1558
	1559	TTGGCCAGCCCTGGGTAGGAAGCTGGGCTCCTCCCAAAGGGGAAGAGAGATAGGGT	1618
	1619	ATAGGCTGATATTGCTCTCTCTGTTGGGTCCCACCTACTGTGATTTCATTGAGCCTGATT	1678
	1679	TGACATGTAATACACCTCAAATTTGGAAAGTTGCCCATCTCTGCCCAACCCATGCC	1738
	1739	CCTGCTCACCTCTGCCAGGCCCAAGCTCAACCTACTGTGTCAAGGCCAGCCTCAGTGATA	1798
	1799	GTCTGATCCCAAGGTACAAGGCCCTGTGAGCTGAGGCTGAAAGGCCCTGTTTGGAGAGGCT	1858
	1859	GGGGTAGTGCC	1869

**Fig. 3.** Nucleotide and predicted amino acid sequences of the rat PACAP receptor cDNAs. The numbers on the right refer to the amino acid and nucleotide positions of pRPACAPR46-5 (A) and pRPACAPR12 (B), respectively. The arrowhead indicates the postulated cleavage site of the signal sequence. The inserted sequence which was observed in pRPACAPR12 was underlined. The potential N-glycosylation sites are doubly underlined.

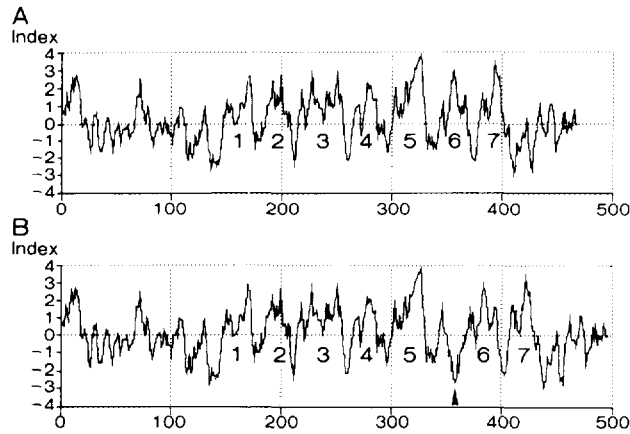
PACAP specific binding sites, however, testis also gave no detectable signal despite the presence of specific binding sites reported so far (23). We also hybridized the mRNAs with the cDNA probe of rat VIP receptor. The probe gave a smaller band in size (about 5.0 kb) with a strong signal in the lane of lung mRNA (data not shown).

<b>B</b>	1	CGAGTGGACAGTGGCAGGCGGTGACTGAATCTCCAAGTCTGGAAACAATAGCCAGAGA	58
	59	TAGTGGCTGGGAAGCACCATGGCCAGAGTCTCGAGCTCTCCCTGACTGCTCTCCTGCTG	118
	1	MetAlaArgValLeuGlnLeuSerLeuThrAlaLeuLeuLeu	14
	119	CCTGTGGCTATTGCTATGCACTCTGACTGCATCTTCAAGAAGGAGCAAGCCATGGCGCTG	178
	15	ProValAlaIleAlaMetHisSerAspCysIlePheLysLysGluGlnAlaMetCysLeu	34
	179	GAGAGGATCCAGAGGGCCAAACGACCTGATGGGACTAAACGAGTCTTCCCCAGGTGGCCCT	238
	35	GluArgIleGlnArgAlaAsnAspLeuMetGlyLeuAsnGluSerSerProGlyCysPro	54
	239	GGCATGTGGGCAATATACATGTTGGAAGCCAGCTCAAGTAGGTGAGATGGTCTCTGTA	298
	55	GlyMetTrpAspAsnIleThrCysTrpLysProAlaGlnValGlyGluMetValLeuVal	74
	299	AGCTGCCCTGAGGCTTCCGGATCTTCAACCCGGACCAAGTCTGGATGACAGAAACCCATA	358
	75	SerCysProGluValPheArgIlePheAsnProAspGlnValTrpMetThrGluThrIle	94
	359	GGAGATTCTGGTTTTGCCGATAGTAATTCCTTGGAGATCACAGACATGGGGTCCGTGGGC	418
	95	GlyAspSerGlyPheAlaAspSerAsnSerLeuGluIleThrAspMetGlyValValGly	114
	419	CGGAACGCACAGAGGACGGCTGGTCCGAGCCCTTCCCCACTACTTCGATGCTGTGGG	478
	115	ArgAsnCysThrGluAspGlyTrpSerGluProPheProHisTyrPheAspAlaCysGly	134
	479	TTTGATGATTATGAGCTGAGTCTGGAGTCAAGATTATTACTACCTGCTGGTGAAGCT	538
	135	PheAspAspTyrGluProGluSerGlyAspGlnAspTyrTyrTyrLeuSerValLysAla	154
	539	CTCTACACAGTCCGGCTACAGCACTTCCCTCGCCACCCTCACTACTGCCATGGTCACTTG	598
	155	LeuTyrThrValGlyTyrSerThrSerLeuAlaThrLeuThrThrAlaMetValIleLeu	174
	599	TGCCGCTTCCGGAAGCTGCATTGCCTCGCAACTTCATCCACATGAACCTGTTTGTATCC	658
	175	CysArgPheArgLysLeuHisCysThrArgAsnPheIleHisMetAsnLeuPheValSer	194
	659	TTCATGCTGAGGGCTATCTCCGCTTTCATCAAGGACTGGATCTTGTACGCCGAGCAGGAC	718
	195	PheMetLeuArgAlaIleSerValPheIleLysAspTrpIleLeuTyrAlaGluGlnAsp	214
	719	AGCAGTCACTGCTTCGTTTCCACCGTGGAGTGCAGAGCTGTCATGGTTTTCTTCCACTAC	778
	215	SerSerHisCysPheValSerThrValGluCysLysAlaValMetValPheHisTyr	234
	779	TGCGTGGTGTCCAACTACTTCTGGCTGTTTCATTGAAGCCCTGTACCTCTTTACACTGGTG	838
	235	CysValValSerAsnTyrPheTrpLeuPheIleGluGlyLeuTyrLeuPheThrLeuLeu	254
	839	GTGGAGACCTTCTTCCCTGAGAGGAGATATTTCTACTGGTACACCATCATCGGCTGGGG	898
	255	ValGluThrPhePheProGluArgArgTyrPheTyrTrpTyrThrIleIleGlyTrpGly	274
	899	ACACCTACTGTGTGTAACAGTGTGGGCTGTGCTGAGGCTCTATTTTGTATGATCAGGA	958
	275	ThrProThrValCysValThrValTrpAlaValLeuArgLeuTyrPheAspAspAlaGly	294
	959	TGCTGGATATGAATGACAGCACAGCTCTGTGTGGTGTATCAAAGGCCCGGTGTGGC	1018
	295	CysTrpAspMetAsnAspSerThrAlaLeuTrpTrpValIleLysGlyProValValGly	314
	1019	TCTATAATGGTTAACTTTGTGCTTTTCATCGGCATCATCATCTCTGTACAGAAAGCTG	1078
	315	SerIleMetValAsnPheValLeuPheIleGlyIleIleIleIleLeuValGlnLysLeu	334
	1079	CAGTCCCCAGACATGGGAGGCCAAGAGTCCAGCATCTACTTCACTGCGGTGAGAAATGC	1138
	335	GlnSerProAspMetGlyGlyAsnGluSerSerIleTyrPheSerCysValGlnLysCys	354
	1139	TACTGCAAGCCACAGGGGCTCAGCAGCACTTTGCAAGATGTCAGAAGTATCCACCATT	1198
	355	TyrCysLysProGlnArgAlaGlnGlnHisSerCysLysMetSerGluLeuSerThrIle	374
	1199	ACTCTACGGCTGGCCCGCTCCACCCTACTGCTCATCCCACTCTTCCGAAATCCACTACACA	1258
	375	ThrLeuArgLeuAlaArgSerThrLeuLeuLeuIleProLeuPheGlyIleHisTyrThr	394
	1259	GTATTCCGCTTCTCTCCAGAGAACGTCAGCAAGAGGGAAAGACTTGTGTTGAGCTTGGG	1318
	395	ValPheAlaPheSerProGluAsnValSerLysArgGluArgLeuValPheGluLeuGly	414
	1319	CTGGGCTCCTTCCAGGGCTTGTGGTGGCTGTACTCTACTGCTTCCCTGAATGGGGAGGTA	1378
	415	LeuGlySerPheGlnGlyPheValValAlaValLeuTyrCysPheLeuAsnGlyGluVal	434
	1379	CAGGCAGAGATTAAGAGGAAATGGAGGAGCTGGAAGGTGAACCGTTACTTCACTATGGAC	1438
	435	GlnAlaGluIleLysArgLysTrpArgSerTrpLysValAsnArgTyrPheThrMetAsp	454
	1439	TTCAAGCACCCGGCACCCGCTCCCTGGCCAGCAGTGGAGTAAATGGGGAAACCCAGCTGTCC	1498
	455	PheLysHisArgHisProSerLeuAlaSerSerGlyValAsnGlyGlyThrGlnLeuSer	474
	1499	ATCCTGAGCAAGAGCAGCTCCAGCTCCGATGTCCAGCCTCCCGGCCGCAACTTGGCC	1558
	475	IleLeuSerLysSerSerSerGlnLeuArgMetSerSerLeuProAlaAspAsnLeuAla	494
	1559	ACCTGAGGCCTGTCTCCCTCCTCCTTCTGCACAGGCTGGGGCTGCGGGCCAGTGCTGAG	1618
	495	Thr***	
	1619	CATGTTTGTGCTCTCCCTCTCCTTGGGCAGGCCCTGGGTAGGAAGCTGGGCTCCTCC	1678
	1679	CAAAGGGGAAGAGAGAGATAGGGTATAGGCTGATATTGCTCCTCTGTTGGGTCACC	1738
	1739	TACTGTGATTATTGAGCCTGATTTGACATGTAAATACACCTCAAATTTGGAAAGTTGCC	1798
	1799	CCATCTCTGCCCCCAACCCATGCCCTGCTCACCCTCTGCCAGGCCCAAGCTCAACCTACT	1858
	1859	GTGTCAAGGCCACCCCTCACTGATGCTGTATCCAGGTACAGGCCCTGTGAGCTGAGGC	1918
	1919	TGAAGGCCCTGTTTGGAGAGGCTGGGGTGTGCCACCCAGCAGCCCTTTCAGCAAT	1978
	1979	GACTTTGGATGTGGACCCCTTCTCAGCCTGTACCAAGTACTGCGAGTTGGCTAGGGATGCG	2038
	2039	CTCAGTTTCTCTGAGCATCCTTTGGAGCAGGTCAACCTGAGGCTCCTTTTGGCTTACCCGAC	2098
	2099	ATCTAAGTTGTCCAGGTGCTCGGCTCCTGTGTGCTGGATGACGGGAGGGCTCCGGGGTCC	2158
	2159	TTTCACTCAAAGACTTACATTGAGGTGGGTGAGAGTCAAGAGAAAAGTTCTGGTCTTTT	2218
	2219	CATTTGTTCTAAGAGCTGAGAGCCAGGAATGCAGAGTCAATTTGGGAAGGAGATGGGATG	2278
	2279	CTGATGATCTTACCATGGCCATGACTGTGCCCTGATTCAAGACCCGATCATGTGGTGGC	2338
	2339	TTTATTTCTACACTTCTGTGCCCAATGGACAGTCTGAGGAGGCTCTCTTTACGCCACA	2398
	2399	ACAAACCCAGAAAGCCCTTCTTCCCTCTGTCTCCATAGCTCAAAGCCATGTT	2458
	2459	AGAACGGACCGCCACCTTCCGATGAATCTGTAGTCTGAGCCACTTTCAAATTTCCA	2518
	2519	CGAGCCAAAGTCTGGTCCAGGACGCCCC	2548

*Fig. 3. - Continued.*

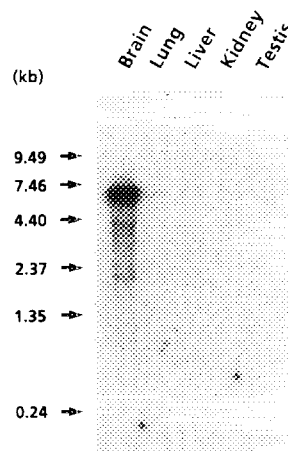
## DISCUSSION

In this study, rat PACAP receptor cDNA was isolated by cross-hybridization with rat VIP receptor cDNA. The deduced amino acid sequence of rat PACAP receptor (which is encoded



**Fig.4.** Hydropathy plot of the rat PACAP receptor proteins. The hydropathy plots of the deduced amino acid sequence of pRPACAPR46-5 (A) and pRPACAPR12 (B) were generated using the DNASIS with Kyte matrix (window = 6 amino acids). Seven strongly hydrophobic regions presumed to be transmembrane segments are numbered 1-7. The arrowhead indicates the alternative region found in pRPACAPR12.

ed by pRPACAPR46-5) is markedly homologous to not only rat VIP receptor (58.8%)(Fig.6) but also secretin (50.0%), glucagon (32.3%) and GHRH (46.8%) receptors of rat. The result suggests that PACAP receptors also constitute the subfamily of the G protein-coupled receptor superfamily as proposed by Ishihara et al. (14). The PACAP receptor that was encoded by pRPACAPR12 had an additional 28 amino acid sequence which was thought to be derived from an alternative exon (Fig.3B). The insertion site of alternative exon in pRPACAPR12 is corre-



**Fig.5.** Northern blot analysis of the rat PACAP receptor. Poly(A)<sup>+</sup>RNA was prepared from rat brain, lung, liver, kidney and testis, and 5.0  $\mu$ g of the RNAs was electrophoresed on 1.2% agarose gel. Northern hybridization was performed using the 0.4 kb NcoI-BanII fragment of pRPACAPR12 as a probe. The sizes of the markers (BRL) are indicated.



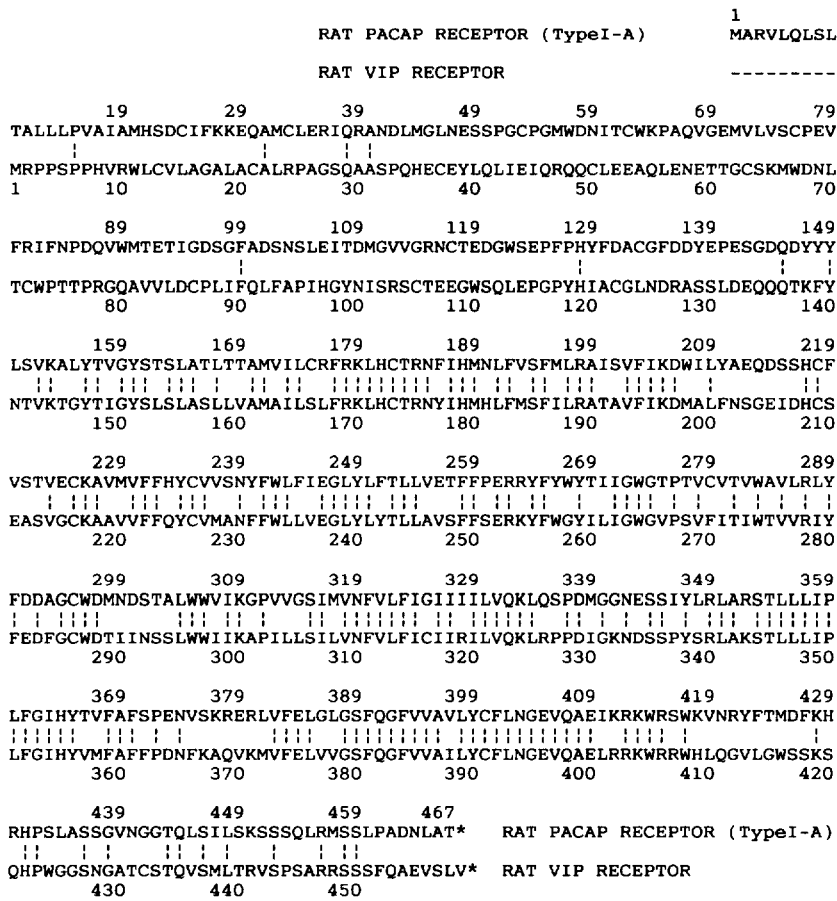


Fig. 6. Amino acid sequence alignment of the rat PACAP and VIP receptors. The upper sequence is for PACAP receptor and the lower is for VIP receptor. The amino acid residues identical in two sequences are marked(!).

sponded well with those observed in GHRH receptor (17). In a recent report, Okamoto et al. identified the third inner loop of the  $\beta_2$ -adrenergic receptor as a Gs activator region (22). The positions of basic amino acid residues in the region were thought to be important in interacting with Gs protein, and regions with similar characteristics could be found in PACAP, VIP and other neuropeptide receptors. The PACAP receptor encoded by pRPACAPR12 cDNA clone have a hydrophilic motif in such region which might cause the different interaction with intracellular effector systems. Therefore, we distinguished and named the PACAP receptor that is encoded by pRPACAPR46-5 for PACAP receptor Type I-A and pRPACAPR12 for Type I-B. The detailed comparison of the two receptors will help us to understand the biological significance of such variations of PACAP receptors.

Although the PACAP specific binding site was reported in testis, the mRNA for Type I PACAP receptor could not be detected (Fig.5). The PACAP binding sites were localized on germinal cells (23) and the content of mRNA of the cells might be far less than that of other cells. In other tissues such as brain, lung and liver, the content of Type I and Type II receptors (23) corresponded well with the results in the Northern blot analysis (Fig.5). Since brain tissue contained mostly Type I binding site (23), a strong hybridization signal was observed. VIP receptor probe gave a strong signal in lung and liver tissues which was dominated by Type II binding sites (23), but PACAP receptor probe emitted faint signals (Fig.5). So, we expect that PACAP plays a specific role in brain and its action is mediated by Type I receptor.

These studies on the distribution and binding character of PACAP receptors using the cloned cDNA will help us to further understand the physiological significance of PACAP. In addition, the highly related pairs of ligands and receptors such as PACAP and VIP will be suitable models in studying the mechanisms underlying the recognition of ligands by receptors.

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