Pituitary Adenylate-Cyclase-Activating Polypeptide (PACAP) Binding Sites and PACAP/Vasoactive Intestinal Polypeptide Receptor Expression in Human Pituitary Adenomas

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Pituitary adenylate-cyclase-activating polypeptide (PACAP) stimulates release of several anterior pituitary hormones by interacting with PACAP receptors on pituitary cells. To learn more about the distribution and possible regulatory roles of PACAP and its receptors in human pituitary adenomas, we investigated the expression of vasoactive intestinal polypeptide (VIP) and PACAP binding sites using receptor autoradiography, PACAP and PACAP/VIP receptor (PVR) mRNAs by reverse transcription polymerase chain reaction (RT-PCR), conventional *in situ* hybridization, and catalyzed reporter deposition *in situ* hybridization (CARD-ISH) analyses. PACAP mRNA was expressed in normal human hypothalamus, which was used as a positive control, but not in pituitary adenomas. Receptor autoradiography showed PACAP types I and II binding sites in all groups of pituitary adenomas, except prolactinomas. The highest levels were present in gonadotroph and null cell adenomas. PVR-2 mRNA was expressed in normal pituitaries and in all groups of pituitary adenomas by RT-PCR, whereas PVR-1 and -3 mRNAs were expressed in all groups of pituitary adenomas, except for most prolactinomas. Conventional *in situ* hybridization studies with digoxigenin-labeled probes demonstrated weak staining for PVR-1, -2, and -3 mRNAs in most tissues. The CARD-ISH technique, which increased the sensitivity of the *in situ* hybridization method, also revealed PVR-2 mRNA expression in all adenomas, whereas PVR-1 and -3 mRNAs were detected in nearly all adenomas except for prolactinomas. The presence of PACAP mRNA in the hypothalamus, but

not in normal anterior pituitary or in pituitary adenomas, and the differential expression of PVRs in adenomas indicate a selective regulatory endocrine and paracrine role of PACAP in normal and neoplastic anterior pituitary cells. (Am J Pathol 1998; 153:1787-1796)

Pituitary adenylate-cyclase-activating polypeptide (PACAP) was originally isolated from hypothalamic tissues by its ability to stimulate cAMP production in cultures of anterior pituitary cells with a potency 1000 times greater than that of vasoactive intestinal polypeptide (VIP).¹ PACAP exists as a 38-amino-acid peptide (PACAP-38) and as a shorter 27-amino-acid peptide (PACAP-27). These peptides share a 68% sequence homology with VIP^{1,2} and stimulate the release of several pituitary hormones/cytokines, including luteinizing hormone (LH),³ growth hormone (GH),⁴ and interleukin (IL)-6 from folliculostellate cells.⁵ Thus, PACAP fulfills the essential criteria of a hypophysiotropic factor. PACAP also stimulates adrenocorticotrophic hormone (ACTH) release from the AtT20 corticotroph cell line.⁶ It has been suggested that the variability in the action of PACAP between different pituitary cell types may be due to a functional expression of different PACAP/VIP receptor (PVR) subtypes.⁷

PACAP and VIP share binding sites in a variety of tissue types.^{8,9} These polypeptides bind to two major sites: type I sites, which show preferential binding to PACAP-38 and PACAP-27 over VIP, and type II sites, which have nearly equally high affinity for PACAP-38, PACAP-27, and VIP. cDNAs for three distinct human PVR subtypes, including PVR-1, PVR-2 (also known as VIP₁R), and PVR-3 (also known as VIP₂R) have been recently cloned, and represent seven transmembrane-spanning G-protein-coupled receptors that belong to the secretin/glucagon family of receptors.^{10,11} The type I binding site

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of PACAP is similar to PVR-1, and type II binding sites share PVR-2 and PVR-3.¹²

The identification of oncogenic mutations that constitutively activate adenylyl cyclase and cAMP formation in pituitary adenomas has provided further support for the view that pituitary cells proliferate in response to cAMP.^{13,14} In fact, both VIP and PACAP seem to be general activators of pituitary cell function, being able to activate adenylyl cyclase in pituitary adenomas.¹⁵

PACAP types I and II binding sites were previously investigated by binding assays in human pituitary adenomas¹⁶ and were found in most types of adenomas. PACAP-38 had a modest role in the regulation of GH, ACTH, and α -subunit secretion from human tumorous pituitary corticotrophs and somatotrophs in hormone assays.¹⁷

In situ hybridization (ISH) is useful in demonstrating gene expression in individual cells but is limited in its ability to detect low copy numbers of mRNAs. Recent studies have used biotinylated tyramide in a catalyzed reporter deposition ISH (CARD-ISH) amplification system to increase the sensitivity of assays detecting protein by immunohistochemistry¹⁸ and mRNA by ISH.¹⁹

We used reverse transcription polymerase chain reaction (RT-PCR) with Southern hybridization, conventional ISH, and CARD-ISH to analyze the expression of PACAP, PVR-1, -2, and -3 mRNAs, and PACAP binding sites in human pituitary tumors. Our studies localized PVR-1, -2, and -3 in pituitary adenomas and showed for the first time the *in situ* localization of PACAP receptors.

Materials and Methods

Three normal autopsy pituitaries and one hypothalamus were obtained within 5 hours postmortem from adult patients without endocrine abnormalities and used as positive controls, and 70 surgically resected pituitary adenomas were used in these studies.

Pituitary adenomas included 15 GH tumors, 10 prolactin (PRL) adenomas, 9 ACTH adenomas, and 36 clinically nonfunctioning adenomas with no evidence of hormone hypersecretion and serum PRL levels less than 100 μ g/L. Fourteen tumors, which stained for follicle-stimulating hormone (FSH) or luteinizing hormone (LH) β -subunits, were classified as gonadotroph adenomas. The remaining 22 tumors, which did not show hormone immunoreactivity or focal staining in which less than 25% of cells for gonadotropin β -subunits, were classified as null cell adenomas. Ultrastructural studies were done on some of the null cell and gonadotroph adenomas to confirm the immunohistochemical classification.

Portions of normal and neoplastic pituitary tissues frozen at -70°C were used for RNA extraction, immunohistochemistry, ISH studies, and PACAP binding studies. Frozen sections of both pituitary adenomas and non-neoplastic autopsy pituitaries were cut at 10 μm , fixed in 4% paraformaldehyde, washed in 2X standard saline citrate (SSC), dehydrated in alcohol, stored at -70°C ,

and then used for immunohistochemistry, ISH, and CARD-ISH experiments.

Receptor Autoradiograph with ¹²⁵I-Labeled VIP and ¹²⁵I-Labeled PACAP Radioligands

¹²⁵I-labeled VIP (2000 Ci/mmol; Anawa, Wangen, Switzerland) was used as the radioligand. Only the mono [¹²⁵iodo-Tyr¹⁰]-VIP, eluted as single peak from high-pressure liquid chromatography and analyzed by mass spectrometry, was used. The slide-mounted tissue sections were incubated for 90 minutes in a solution of 50 mmol/L Tris/HCL (pH 7.4) containing 2% bovine serum albumin, 2 mmol/L EGTA, 0.1 mmol/L bacitracin, and 5 mmol/L MgCl₂ to inhibit endogenous proteases in the presence of 30 pmol/L ¹²⁵I-labeled VIP at room temperature as described previously.^{20,21} To estimate nonspecific binding, paired serial sections were incubated as described above, except that 20 nmol/L VIP or PACAP-1-27 (Bachem, Bubendorf, Switzerland) were added to the incubation medium. After this incubation, the slides were washed twice in ice-cold 50 mmol/L Tris/HCL (pH 7.4) containing 0.25% bovine serum albumin, then in buffer alone, and quickly dried under a stream of cold air. The sections were subsequently exposed to a ³H-labeled hyperfilm (Amersham, Little Chalfont, UK) for 1 week. The autoradiograms were quantified using a computer-assisted image processing system previously described.²² Normally, a tissue was defined as receptor positive when the optical density measured in the total binding section was at least twice the optical density of the nonspecific binding section.

The same pituitary tumors were also evaluated with [¹²⁵I-Ac-His¹]PACAP-1-27 (2000 Ci/mmol; Anawa) for their receptor subtype specificity; displacement experiments under the same conditions as for VIP receptor autoradiography using increasing concentrations of unlabeled VIP and PACAP-1-27 were performed to differentiate PACAP type I and type II binding sites.^{8,23}

Oligonucleotide Primers and Probes

Oligonucleotide primers and hybridization probes were produced on a DNA oligonucleotide synthesizer (Applied Biosystems, Foster City, CA) (Table 1). Both primers and probes for human PACAP,²⁴ PVR-1,²⁵ PVR-2,^{26,27} and PVR-3^{27,28} were synthesized on the basis of published sequences and GenBank sequences. The sequences of the oligonucleotides were checked against the EMBL/GenBank sequence database, and no significant homology with other published sequences was found.

RT-PCR

Total RNA extraction was performed by the single-step methods (TRIzol reagent kit, Life Technologies) from 3 nontumorous pituitaries and 35 cases of pituitary adenomas.^{29,30}

Table 1. Sequences of Primers and Hybridization Probes for Human PACAP and PVR mRNAs

	Sequence
PACAP (Genbank X60435)	
Primers	SS: TGCAGTCGCTCGTGGCCCGGG (10491–10511) AS: GTTTGGATAGAACACACGAGC (11869–11889)
Probes	AS1*: ASCCTCCCTAGGACGCGCCCAAGTATTT (11712–11741) AS2: CCGTCCTCGCCGTACGCCCTTCTCTGGC (9813–9842) SS: GAAATACTTGGCGGCCGCTCCTAGGGAAGAG (11712–11741)
PVR-1 (Genbank D17516)	
Primers	SS: CTTGTGCAGAACTTCAGTCT (1235–1255) AS: TCGGTGCTTGAAGTCCACAGC (1517–1537)
Probes	AS1*: TAGTGGGATGAGCAGCAGGGTGGACCGGGC (1298–1327) AS2: CAGTCGCAAGTAGATGCTGGACTCATTGCC (1268–1297) AS3: GCTTCGCCATTTTCGCTTGATCTCCGCTTG (1466–1495) SS: GCCCGGTCCACCCTGCTGCTCATCCCACTA (1298–1327)
PVR-2 (Genbank X75299)	
Primers	SS: ATGTGCAGATGATCGAGGTG (127–146) AS: TGAGCCGGTCTTTCACAGAA (431–450)
Probes	AS1*: CTGCTGCTCATCCAACTCGCTGCCTTGTC (387–416) AS2: GGTGCAGCTGCGGCTTACATTGCGGCCTTG (289–318) AS3: GATGAGGGGACAGGCCAAGACAACCTACCTG (238–267) SS: GACAAGGCAGCGAGTTTGGATGAGCAGCAG (387–416)
PVR-3 (Genbank U18810)	
Primers	SS: CTTAGGAAGCTGCACTGC (617–6350) AS: CAAACACCATGTAGTGGACG (1181–1200) AS1*: AGACTGGTCGTTGCCGCCGACATCTGGGGA (1101–1130) AS2: GCGCCCCGAGCGCGGGGGCCGCCCTCCG (10–39) SS: TCCCCAGATGTCGCGGCAACGACCACTCT (1101–1130)

AS, antisense; SS, sense.
 *Used for Southern hybridization.

First-strand complementary DNA (cDNA) was prepared from total RNA by using a first-stand synthesis kit (Stratagene, La Jolla, CA). The RT reaction was performed at 37°C for 60 minutes in a final volume of 50 μ l with 5 μ g of total RNA, 300 ng of oligo dT primer, 1X RT buffer, 1.0 mmol/L each deoxyribonucleotide (dATP, dCTP, dTTP, and dGTP), 40 U of RNase inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase. The reaction product was then heated at 95°C for 5 minutes and immediately placed on ice.

The PCR was performed in 100- μ l final reaction volumes containing 5 μ l of RT reaction product as template DNA, corresponding to cDNA synthesized from 500 ng of total RNA, 1X PCR buffer (Promega, Madison, WI), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide (Boehringer Mannheim, Indianapolis, IN), 300 ng of each sense and antisense primer for PACAP and PVR-1, -2, -3, and 2.5 U of *Taq* DNA polymerase (Promega). Programmable temperature cycling (Perkin-Elmer/Cetus 480, Norwalk, CT) was performed with the following cycle profile: 95°C for 5 minutes, followed by 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes (30 cycles) for GAPDH and PACAP and 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes (40 cycles) for PVR-1, -2, and -3, respectively. After the last cycle, the elongation step was extended at 72°C for 10 minutes.

A 20- μ l aliquot of PCR product was analyzed by gel electrophoresis using a 2% agarose gel and was stained with ethidium bromide. PHx174 DNA/*Hae*III digest (Boehringer Mannheim) was used as the standard. The separated PCR products were transferred to

nylon membrane filters. Southern hybridization, with a single internal probe that hybridized to regions within the amplified sequences, was performed. Hybridization was performed with 1×10^6 cpm/ml [32 P]deoxyadenosine-diphosphate-labeled probe at 42°C for 18 hours. After washing with 6X SSC/0.1% SDS at 23°C for 20 minutes and at 42°C for 20 minutes, autoradiography was performed at –70°C with Kodak Omat-AR film (Eastman Kodak, Rochester, NY) with intensifying screens. In RT-PCR experiments, total RNAs from the human autopsy hypothalamus and non-neoplastic pituitaries were included as respective positive and negative controls for PACAP and PVR-1, -2, and -3.

Immunohistochemistry

Immunostaining for anterior pituitary hormones used the avidin-biotin peroxidase complex method (Vector Laboratories, Burlingame, CA). Primary antibodies against human anterior pituitary hormones included GH (1:1000 dilution), PRL (1:1000), LH- β (1:500), FSH- β (1:500), and thyroid-stimulating hormone (TSH)- β (1:1000), all rabbit polyclonal and obtained from the National Pituitary Agency, Bethesda, MD. Rabbit polyclonal ACTH (1:1000) was from Dako Corp., Santa Barbara, CA. The monoclonal antibody to the α -subunit of glycoprotein hormones (1:250) was purchased from Biogenex (San Ramon, CA). Chromogranin A antibody (LK2H 10, 1:1000) was produced in our laboratory, as previously described.³¹ The reaction products were visualized by 3,3'-diaminobenzidine tetrahydrochloride.

ISH

A cocktail of oligonucleotide probes for PACAP and for PVRs were labeled with digoxigenin-deoxyuridine 5-triphosphate (Boehringer Mannheim) by terminal deoxyribonucleotidyl transferase reaction, as previously reported.³¹ The ISH procedure was performed as described previously.^{31,32} In brief, the sections were treated with 1 μ g/ml proteinase K (Boehringer Mannheim) at 23°C for 10 minutes, followed by heat treatment, hydrochloride treatment, acetylation, and then prehybridization. Thereafter, the sections were hybridized with 1 ng/ml cocktail probe at 42°C for 18 hours. After hybridization, immunodetection was performed using antidigoxigenin at a 1:500 dilution (Boehringer Mannheim). The reaction product was visualized by nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Life Technologies). Control experiments were carried out using internal sense probes.

CARD-ISH

The CARD-ISH technique was modified (GenPoint kit, Dako, Carpinteria, CA) to detect mRNA and performed according to a modified protocol developed in our laboratory. The PACAP and PVR-1, -2, and -3 probes were the same as those used for ordinary ISH. A cocktail of oligonucleotide probes for PACAP and for PVR were labeled with biotin-11-dUTP (Boehringer Mannheim) by terminal deoxyribonucleotidyl transferase reaction, as previously reported.³¹ Target retrieval was performed by heating frozen tissue sections in 10 mmol/L citric acid (pH 6.0) in a microwave oven for 5 minutes (up to 95°C) and digesting with 1 μ g/ml proteinase K at 23°C for 10 minutes. Sections were then treated with 0.2 N HCL for 20 minutes followed by incubation with 0.25% (v/v) acetic anhydride in triethanolamine for 10 minutes.

To reduce background staining, slides were immersed in 3% H₂O₂ in methanol for 30 minutes and covered with prehybridization buffer for 1 hour at room temperature. Thereafter, the sections were hybridized with 1 ng/ μ l cocktail probe at 42°C for 18 hours. After stringent washing for 10 minutes at 42°C, the complexes were amplified with primary streptavidin/horseradish peroxidase complex (1/400) for 15 minutes, biotiny/tyramide solution (1/2), for 15 minutes, and secondary streptavidin/horseradish peroxidase (1/2) for 20 minutes with fresh 1X Tris-buffered saline/Tween 20 washes being performed after each step. The reaction product was visualized by developing the slides in diaminobenzidine chromogen/H₂O₂ solution for 5 minutes. Frozen sections of human hypothalamus were used as positive controls. Additional controls included 1) using sense probes for negative control and 2) omission of the biotiny/tyramide amplification during CARD-ISH.

Grading of the ISH and CARD-ISH was based on signal intensity as follows: -, negative; 1+, weak; 2+, moderate; 3+, strong, with more than 5% of the cells staining to be considered as positive.

Results

Receptor Autoradiography with ¹²⁵I-Labeled VIP and ¹²⁵I-Labeled PACAP Radioligands

The results of the *in vitro* receptor autoradiography are summarized in Table 2. Comparison of the same tumors analyzed by receptor autoradiography and for mRNA expression using aliquots of the same tumors showed similar results. Other than PRL adenomas, the majority of pituitary adenomas had PACAP binding sites. ¹²⁵I-labeled VIP binding was particularly high in gonadotroph and null cell adenomas, GH adenomas, and ACTH adenomas. ¹²⁵I-labeled VIP binding was characterized by high-affinity displacement by VIP and by the 27-amino-acid form of PACAP (PACAP-1-27), suggesting the presence of the PACAP type II binding site. Figure 1 shows the high density of those receptors in a GH tumor, an ACTH tumor, and a gonadotroph and null cell tumor. In all cases, ¹²⁵I-labeled PACAP receptor autoradiography was performed to evaluate the presence of the PACAP type I binding sites as well. Whereas a high-affinity displacement by PACAP-1-27 was seen in all cases, ¹²⁵I-labeled PACAP was displaced by VIP in most instances in a biphasic manner, with a high- and a low-affinity component, suggesting the presence of both PACAP types I and II binding sites. Figure 2 shows a GH tumor and gonadotroph and null cell tumor with ¹²⁵I-labeled PACAP binding fully displaced by 100 nmol/L PACAP but only partly displaced by 100 nmol/L VIP. Competition curves with ¹²⁵I-labeled PACAP showed the high- and low-affinity site of VIP binding compared with the single high-affinity site of PACAP binding (data not shown).

RT-PCR and Southern Hybridization Analysis

Results of RT-PCR and Southern hybridization studies are shown in Tables 2 and 3 as well as in Figure 3. Analysis of PACAP mRNA and PVR-1, -2, and -3 mRNAs demonstrated the expected 317-bp, 303-bp, 324-bp, and 584-bp PCR products in all positive specimens tested. PACAP mRNA was detected in the hypothalamus and in three non-neoplastic pituitaries but not in any of the 35 pituitary adenomas analyzed (Figure 3). PVR-1, -2, and -3 mRNAs were detected in the hypothalamus and in three non-neoplastic pituitaries. PVR-1 mRNA was detected in gonadotropin-secreting adenomas, null cell adenomas, GH adenomas, and ACTH adenomas. However, it was detected in only one of six prolactinomas (Table 3). PVR-2 mRNA was demonstrated in most adenomas (Table 3) whereas PVR-3 mRNA was detected in most adenomas except for prolactinomas where it was lacking in five of six tumors (Table 3).

ISH and CARD-ISH

Conventional ISH with a digoxigenin-labeled PACAP oligonucleotide probe showed strong staining for PACAP in hypothalamic neurons and weak focal staining in the

Table 2. Comparison of Receptor Autoradiography and RT-PCR in Pituitary Adenomas

	Autoradiography				RT-PCR		
	¹²⁵ I-VIP binding density (dmp/mg tissue)	¹²⁵ I displaced by PACAP	¹²⁵ I displaced by VIP	PACAP binding sites, receptor subtypes	PRV-1	PVR-2	PVR-3
GH adenomas							
GH1	0	0	0	—	—	2+	1+
GH2	660*	NT	NT	II	2+	2+	2+
GH3	0	0	0	—	—	3+	—
GH4	2120	High affinity	High + low affinity	I + II	2+	3+	2+
GH5	764*	NT			3+	3+	2+
PRL adenomas							
PRL1	0	0	0	—	—	2+	—
PRL2	0	0	0	—	—	—	—
Gonadotroph adenomas							
GTH1	1989	High affinity	High + low affinity	I + II	3+	3+	2+
GTH2	3721	High affinity	High + low affinity	I + II	3+	3+	3+
GTH3	4–12	High affinity	High + low affinity	I + II	3+	3+	3+
GTH4	4822	High affinity	High + low affinity	I + II	2+	3+	3+
Null cell adenomas							
NC1	2562	High affinity	High + low affinity	I + II	3+	3+	2+
NC2	0	High affinity	High affinity	I	—	2+	—
NC3	3438	High affinity	High + low affinity	I + II	3+	3+	3+
NC4	1731	High affinity	High + low affinity	I + II	3+	3+	3+
NC5	2666	High affinity	High + low affinity	I + II	3+	3+	—
NC6	3369	High affinity	High + low affinity	I + II	3+	3+	3+
NC7	3031	High affinity	High + low affinity	I + II	3+	3+	3+
NC8	1741	High affinity	High + low affinity	I + II	3+	3+	—
NC9	0	0	0	—	2+	3+	1+
NC10	2832	High affinity	High + low affinity	I + II	3+	3+	—

¹²⁵I-VIP binding was defined by high-affinity displacement with VIP and with PACAP (PACAP II-R). RT-PCR semiquantitative assessment was scored as follows: —, negative; 1+, weak band present; 2+, moderately strong band present; 3+, strong band present. Another 10 tumors analyzed by receptor autoradiography, but not by RT-PCR or ISH included GH adenomas (*n* = 3) with binding densities of 858, 2479, and 3346 dpm/mg; ACTH adenomas (*n* = 3) with 1175, 1360, and 1369 dpm/mg; gonadotroph adenomas (*n* = 3) with 878, 1514, and 3721 dpm/mg; and one prolactinoma with a binding density of 0 dpm/mg tissue. NT, not tested.

*Values for specific binding but representing less than twice the background due to a very high background activity of these tumors (does not correspond to the usual definition of a receptor-positive tumor; see Materials and Methods).

posterior but not in the anterior pituitary gland. All adenomas examined for PACAP were negative.

On conventional ISH, PVR mRNAs was detected in the cytoplasm of both non-neoplastic pituitary cells and hypothalamic neurons (data not shown). PVR-1, -2, and -3 mRNAs were weakly positive in some adenomas (Table 3). To enhance detection of these receptor mRNAs, CARD-ISH was used. The specificity of the CARD-ISH was checked by using a sense probe (Figure 4) and by omitting the biotinylated tyramide, both of which resulted in absent or weak staining.

The results of conventional ISH and CARD-ISH are shown in Table 3. PVR mRNAs were detected in many adenomas that were negative by conventional ISH. The signal intensity was increased for PVR mRNAs by CARD-ISH; moderate to strong signals were observed in tissues that expressed only weak or absent signal by conventional ISH. A positive signal for PVR mRNAs was usually present in most adenoma cells (Figure 4). PVR-1 and PVR-3 were usually negative in prolactinomas, but PVR-2 mRNA was moderately to strongly positive by CARD-ISH. In GH, ACTH, gonadotropin, and null cell adenomas, PVR-1, -2, and -3 mRNAs were consistently present by CARD-ISH. The results with RT-PCR were similar to those with CARD-ISH (Table 3).

Discussion

We analyzed VIP and PACAP binding sites by receptor autoradiography and detected PVR mRNAs by RT-PCR and ISH in a series of pituitary adenomas. The binding studies with PACAP-27 and VIP using autoradiography showed the presence of receptor proteins corresponding to PACAP binding sites. Competition experiments using ¹²⁵I-labeled VIP and ¹²⁵I-labeled PACAP indicated the presence of both PACAP type I and type II binding sites. One difference observed was the very high binding site incidence in gonadotroph and null cell tumors and the low incidence in GH adenomas. As parallel measurements of somatostatin receptors SS-R²² in the eight GH adenomas showed a very high density in GH adenomas (data not shown), the low PACAP binding site incidence was not attributed to poor tissue preservation or degradation of the receptor proteins. These results are in agreement with previously published data,^{16,20} demonstrating binding sites for PACAP-27 and VIP in most pituitary adenomas except prolactinomas. It should be mentioned, however, that the three tested prolactinomas were characterized by unusually high nonspecific binding, possibly masking the presence of a low number of receptors.

Figure 1. ^{125}I -labeled VIP receptor autoradiography in a GH adenoma (A to C), an ACTH adenoma (D to F), and a null cell adenoma (G to I). A, D, and G: H&E-stained sections. Bars, 1 mm. B, E, and H: Autoradiographs showing total binding of ^{125}I -labeled VIP. All tumors are VIP receptor positive. C, F, and I: Autoradiographs showing nonspecific binding (in presence of 20 nmol/L VIP).

VIP binding sites have been designated as PACAP type II binding sites, and these sites share two receptors, including PVR-2 and PVR-3.¹² PVR-3 (VIP₂R) was recently cloned.^{27,28,33} PVR-2 (VIP₁R) and PVR-3 (VIP₂R) have distinct distributions in the central nervous system, with high levels of PVR-2 (VIP₁R) mRNA in cortex, hippocampus, hypothalamus, and cerebellum³⁴ and PVR-3 (VIP₂R) in the hippocampus, thalamus, and the suprachiasmatic nucleus. The distribution of VIP binding sites in the central nervous system, determined by autoradiography, is consistent with the combined distributions of the PVR-2 (VIP₁R) and PVR-3 (VIP₂R) mRNAs.³³ In previous studies using binding assay and adenylate cyclase stimulation,¹⁶ it was suggested that VIP reacted mainly with PVR-3 (VIP₂R) and that either VIP is inactive in prolactinomas or PVR-2 mRNA in prolactinomas is not a functional receptor. As VIP has been shown to play a role in the regulation of PRL release from lactotrophs,^{35,36} our results suggest that PRL release from lactotrophs may be regulated mainly via PVR-2, but not PVR-3.

Solution RT-PCR and Southern hybridization analyses showed PACAP mRNA to be expressed in the hypothalamus, only weakly in non-neoplastic pituitary, and not at all in pituitary adenomas. These results were confirmed by ISH, in which hypothalamic neurons were strongly positive for PACAP mRNA, posterior pituitaries were only focally and weakly positive, and the anterior pituitary and the spectrum of pituitary adenomas were negative for PACAP. Our results are in agreement with previously published immunoassay studies.^{37,38}

In the present study, RT-PCR and Southern hybridization analyses showed PVR-1 mRNA to be strongly expressed in gonadotroph and null cell adenomas and variably expressed in GH and ACTH adenomas but not expressed in prolactinomas. PVR-2 mRNA was strongly expressed in all pituitary adenomas, including prolactinomas. PVR-3 mRNA was strongly expressed in most adenomas but was usually negative in prolactinomas. There was generally good agreement between the receptor autoradiography binding studies and RT-PCR and ISH

Figure 2. ^{125}I -labeled PACAP receptor autoradiography in a GH adenoma (A to D) and a null cell adenoma (E to H). A and E: H&E-stained sections. Bars, 1 mm. B and F: Autoradiograms showing total binding of ^{125}I -labeled PACAP. C and G: Autoradiograms showing nonspecific ^{125}I -labeled PACAP (in presence of 100 nmol/L PACAP). D and H: Autoradiograms showing ^{125}I -labeled PACAP binding in presence of 100 nmol/L VIP. The receptors in D and H are PACAP I receptors and PACAP II receptors.

studies. Detection of mRNA for PVR-2 in prolactinomas was more frequent than protein binding by receptor autoradiography. This may reflect low levels of mRNA amplified by RT-PCR, which may not translate into functional receptor proteins, as has been shown previously for somatostatin receptors in exocrine pancreatic cancers.³⁹

PACAP and VIP have multiple functions in a variety of tissues.^{9,40} For instance, it has been suggested that they act as hypothalamic hormones controlling anterior pituitary cell function. In support of this notion is the demonstration of PACAP- and VIP-immunoreactive neurons within the median eminence^{41,42} and specific binding sites for PACAP have been found on anterior pituitary cell membranes.⁴³

To date, only a few studies of PVRs in human pituitary adenomas have been published.^{16,17,44} One study using RT-PCR indicated that PVR-1 mRNA was highly expressed in all adenomas except prolactinomas.⁴⁴ Another report of the effects of PACAP on hormone secretion demonstrated that PACAP-38 had a modest role in

Figure 3. RT-PCR and Southern hybridization detection of PACAP mRNA and PVR mRNAs in normal human hypothalamus, normal pituitary, and human pituitary adenomas showing representative examples of the RT-PCR analysis. Lane 1, normal hypothalamus; lane 2, non-neoplastic human pituitary; lanes 3 and 4, GH-secreting adenomas; lanes 5 and 6, PRL-secreting adenomas; lanes 7 and 8, ACTH-secreting adenomas; lanes 9 and 10, gonadotropin-secreting adenomas; lanes 11 and 12, null-cell adenomas; lane 13, negative control without RT for human hypothalamus; lane 14, negative control without RT for human non-neoplastic pituitary. M, molecular size markers. The top part of each figure represents the RT-PCR results, and the bottom part is the Southern hybridization with internal probes.

the regulation of GH, ACTH, and α -subunit secretion from some tumorous pituitary corticotrophs and somatotrophs.¹⁷ Using a binding assay and adenylate cyclase

Table 3. Expression of PVR mRNAs in Pituitary Adenomas

Tumor type	RT-PCR			PVR-1		PVR-2		PVR-3	
	PVR-1	PVR-2	PRV-3	ISH	CARD	ISH	CARD	ISH	CARD
PRL adenoma	1/6	4/6	1/6	0/4	2/4	3/4	4/4	0/4	2/4
GH adenoma	5/7	7/7	5/7	1/7	7/7	6/7	7/7	0/7	6/7
ACTH adenoma	3/5	5/5	2/5	2/3	2/3	2/3	3/3	0/3	3/3
Gonadotroph adenoma	6/6	6/6	6/6	8/8	8/8	8/8	8/8	7/8	8/8
Null cell adenoma	10/11	11/11	7/11	11/13	13/13	13/13	13/13	4/13	13/13

Figure 4. Catalyzed reporter deposition *in situ* hybridization (CARD-ISH) detecting PVR-1, -2, and -3 mRNAs in pituitary adenomas. **A:** Gonadotroph adenoma positive for PVR-1 as indicated by brown cytoplasmic staining. The endothelial cells show no staining. **B:** The sense control probe for **A** is negative. The blue nuclear staining is from the hematoxylin counterstain. **C:** Gonadotroph adenoma positive for PVR-3. **D:** Prolactinoma showing focal staining for PVR-2 (arrows). **E:** ACTH adenoma positive for PVR-1 in many tumor cells (arrows). **F:** ACTH adenoma positive for PVR-2 in many tumor cells (arrows). Magnification, $\times 250$.

assays, Robberecht et al reported that PACAP-27 and PACAP-38 stimulated adenylate cyclase activity equally well and in all pituitary adenomas except prolactinomas.¹⁶ The expression of PVR-3 in human pituitary adenomas has not been previously reported, so our study links the expression of PVR-3 mRNA to specific pituitary adenoma subtypes. A schematic summary of our findings on the differential expression of PACAP and PACAP binding and PVR-1, -2, and -3 is shown in Figure 5.

Our results demonstrated that in prolactinomas PVR-1 and -3 mRNAs were usually absent by RT-PCR and

CARD-ISH. Recent studies using binding assays and adenylate cyclase stimulation¹⁶ indicated that in prolactinomas PACAP-27, PACAP-38, and VIP were inactive despite a response of the enzyme to guanosine 5'-triphosphate, Gpp(NH)p, forskolin, and fluoride. PACAP is considered to be the most potent activator of cAMP formation in nonfunctioning pituitary adenomas, including gonadotropin-secreting adenomas and null cell adenomas and suggests a possible modulatory action of this peptide on cell growth.⁴⁵ A recent study by Taupenot et al⁴⁶ showed that PACAP regulated the expression of the chro-

Figure 5. Schematic diagram summarizing the expression of PACAP, mRNA PACAP binding sites and PVR-1, -2, and -3 mRNAs in the hypothalamus and pituitary. The hypothalamus produces PACAP, which goes to the anterior pituitary via the hypophyseal portal system to stimulate cAMP in anterior pituitary cells. The posterior pituitary also expresses small amounts of PACAP. Prolactinoma cells are relatively unique as they do not express PVR-1 or PVR-3 and do not have PACAP type I and II binding sites. Gonadotroph adenoma (GTH) cells express all three PVR mRNAs and possess both PACAP types I and II binding sites. These findings indicate that the hypothalamus can regulate anterior pituitary hormone synthesis and secretion and possibly chromogranin A function by the secretion of PACAP.

mogranin A gene four- to fivefold in PC12 rat chromaffin cells by stimulation through protein kinase A, the cAMP response element, and CREB.⁴⁶ As chromogranin A is abundantly expressed in anterior pituitary cells,⁴⁷ PACAP may have a major role in regulating chromogranin A function in anterior pituitary cells.

With the CARD-ISH technique, mRNAs were readily detected in individual cells, and the signal intensity was increased when compared with conventional ISH. One advantage of performing CARD-ISH with nonisotopic probes is the excellent resolution obtained with biotin labeling after amplification by tyramide. This technique may approach the sensitivity of RT-PCR. With this increase in sensitivity, an *in situ* CARD technique has some advantage over RT-PCR or *in situ* RT-PCR, including reproducibility and ease of performance of the assay.^{18,19}

In summary, these studies show a differential distribution of PACAP binding sites and PVR-1, -2, and -3 mRNA expression in pituitary adenomas. The differences observed, especially with regard to prolactinomas, probably reflect different regulatory roles of PVR in these tumors. The high levels of PVR in gonadotroph and null cell adenomas indicate the importance of the cAMP regulatory system in these tumors.

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