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Specific Antibody Recognition of Rat Pituitary Adenylate Cyclase Activating Polypeptide Receptors

Min Li,[1] Seiji Shioda,[1] Aniko Somogyvári-Vigh,[1] Haruo Onda,[2] and Akira Arimura[1]

[1]*Department of Medicine and the US–Japan Biomedical Research Laboratories, Tulane University Medical Center, New Orleans, LA; and* [2]*Tsukuba Research Laboratories, Takeda Chemical Industries Ltd., Tsukuba, Ibaraki, Japan*

Pituitary adenylate cyclase activating polypeptide (PACAP) is a new member of the secretin/VIP family of peptides. The specific receptor for PACAP has been cloned in rat, human, and bovine tissues. The distribution of the transcripts of PACAP receptor genes has been studied in various tissues using *in situ* hybridization. However, the unavailability of a specific antibody against the PACAP receptor has hampered further study of the expression of receptor proteins. In the present study, rabbit antisera were generated against a synthetic 25-residue peptide corresponding to the C-terminal intracellular domain of the rat PACAP receptor. To validate the specificity of the antisera, CHO cells and cells stably transfected with rat PACAP receptor cDNA were prepared. Using one of these antisera, the membrane and soluble fractions of the transformants were examined by Western blot analysis. Three bands were observed in subcellular fractions from the transfected CHO cells, but no bands were found in similar preparations from the nontransfected cells. A distinct 57-kDa band, which corresponds to the size of cloned rat PACAP receptor, was detected. In addition, a less intense band, larger than 57 kDa, and a very weakly stained band, smaller than 57 kDa, were demonstrated. All of these bands disappeared or were considerably diminished when the antiserum was preabsorbed with the synthetic immunogen peptide. This suggests that these bands are PACAP receptor-related proteins. The membranes from the transfected CHO cells bound to [^{125}I]PACAP27. The size of the ligand/protein crosslinked product approximated 60 kDa, corresponding to the combined size of the PACAP receptor and PACAP27. No additional bands were observed, indicating that the immunopositive proteins larger or smaller than 57 kDa do not bind to the ligand and are not functional. Unlabeled PACAP27 and PACAP38, but not VIP, displaced the binding, suggesting that the receptors expressed in CHO cells are specific for PACAP. Solubilized membrane fractions prepared from rat brains were used for an immunoprecipitation study with [^{125}I]PACAP27 and [^{125}I]VIP. The PACAP receptor antiserum recognized [^{125}I]PACAP-, but not [^{125}I]VIP-bound proteins in the solubilized brain membrane fractions. Immunohistochemistry using this antiserum showed a distribution of PACAP receptor-like immunoreactivities similar to the distribution of the mRNA of PACAP receptor in the rat brain. Thus, the PACAP receptor antiserum is sufficiently specific to be used as a tool for studying the expression of PACAP receptors and related proteins.

Key Words: PACAP receptor; antibody; Western blot; transfection; CHO cell; rat brain.

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP38 and PACAP27) is a neuropeptide of the secretin/glucagon/vasoactive intestinal peptide (VIP) family *(1,2)*. PACAP is a pleiotropic neuropeptide expressed in the brain and peripheral tissues. High-affinity, specific binding sites for PACAP have been identified in the adrenal medulla, anterior pituitary, hypothalamus, and other extrahypothalamic regions of the brain *(3–7)*. Crosslinkage of the binding proteins with [^{125}I]PACAP27 indicated that the molecular mass of the major PACAP binding protein is around 57 kDa *(8–10)*, apparently larger than VIP receptors in the same tissues. Moreover, other tissues, such as the liver and lung, were found to contain high-affinity binding sites for PACAP, which were shared with VIP *(3)*.

PACAP specific binding sites were designated type 1 PACAP receptors; receptors shared with VIP were named type II PACAP receptors. More recently, type I and type II receptors were cloned, and type II PACAP receptors were found to be identical with VIP1 and VIP2 receptors *(8,11–19)*. There appear to be six splicing variants of rat PACAP-specific receptors *(6)*. Cloned PACAP receptor subtypes have displayed a phenotype for the regulation of receptor

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Author to whom all correspondence and reprint requests should be addressed: Akira Arimura, US–Japan Biomedical Research Laboratories, Tulane University Hebert Center, 3705 Main Street, Belle Chasse, LA 70037-3001. E-mail: arimura@mailhost.tcs.tulane.edu

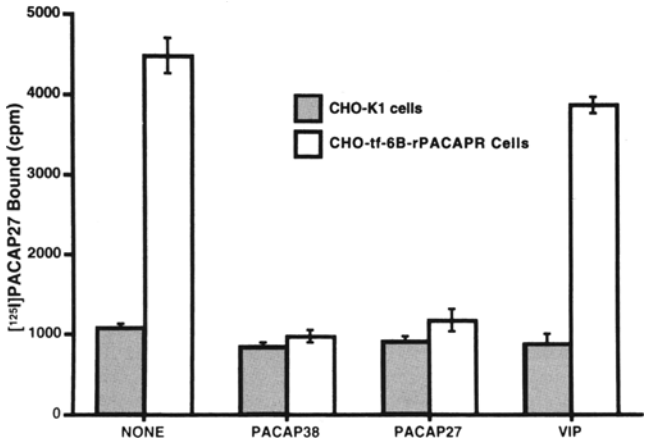

Fig. 1. Inhibition of [^{125}I]PACAP27 binding by PACAP38, PACAP27 or VIP in both CHO-K1 and CHO-tf-6B-PACAPR cells. Binding of [^{125}I]PACAP27 to the 20 μg of membranes from both cells was completed with 1 μ*M*-concentrations of unlabeled PACAP38, PACAP27, and VIP, respectively. Each bar shows the mean with standard error for triplicate experiments.

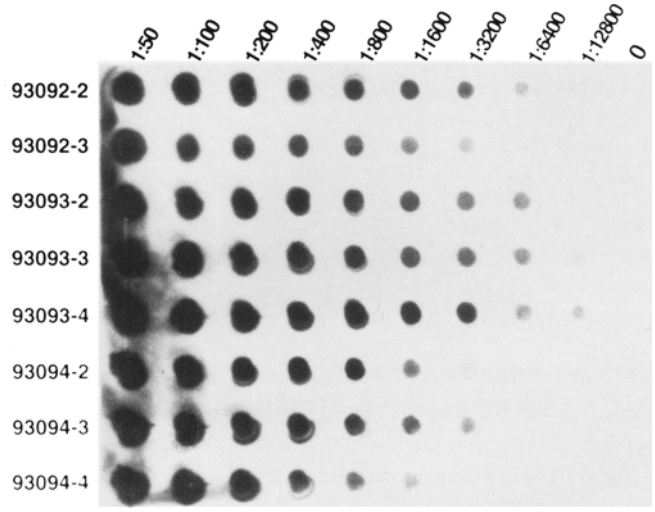

Fig. 2. Dot-blot analysis of the rat PACAP receptor antisera with CHO-tf-6B-PACAPR cells. Fifty-microgram membranes were incubated with various dilutions of rabbit antirat PACAP receptor antisera. Amersham's donkey antirabbit HRP conjugate and enhanced chemiluminescence reagent were used to visualize the signals.

function and transduction signal pathways *(6,20)*. More recently, an additional variant of PACAP receptor that lacks 21 amino acids in the N-terminal extracellular domain *(21)*, and another variant, with a different amino acid sequence in the fourth transmembrane domain, have been cloned *(22)*.

The tissue distribution of PACAP in the brain *(23–26)* and peripheral tissues *(27–30)* has been studied by immunohistochemistry, radioimmunoassay *(31)*, and sandwich-enzyme immunoassay *(32)*. Tissue distribution of PACAP binding sites has been examined using autoradiography with ^{125}I labeled PACAP27 *(7,33–35)*. Distribution of PACAP receptor mRNA has been investigated using *in situ* hybridization histochemistry *(36,37)*.

Although mRNA has been demonstrated in various tissues, the unavailability of a specific antibody against PACAP receptor has hampered study of the expression of receptor proteins. In the present study, an antiserum against PACAP specific receptor (or type I PACAP receptor) was generated in order to examine the distribution of PACAP receptor immunoreactivity in the rat brain.

Results

Confirmation of PACAP Receptor Expression in CHO Cells

In order to obtain expressed rat PACAP receptors, CHO-K1 cells were stably transfected with the expression vector pRPR4-B containing rat PACAP receptor cDNA, and the transformants were selected using G418. To verify that the cDNA insert of pRPACAPR12 encoded the functional PACAP receptor, the transformant cells were examined for specific binding to PACAP using [^{125}I]PACAP27. Figure 1 indicates that the membrane preparations from the transformants bound to a significant amount of [^{125}I]PACAP27, and that the binding was completely displaced by 1 μ*M* of PACAP38 and PACAP27, but not by the same concentration of VIP. Nontransfected CHO cells did not show any specific binding (Fig. 1). This transformant cell line (CHO-6B-PACAPR) was used for further experiments.

Specificity of PACAP Receptor Antibody

Rabbits were immunized with a synthetic 25 amino acid peptide conjugate corresponding the intracellular C-terminal amino acid sequence of the rat PACAP receptor *(6)*. Screening for the antibody titer was conducted by Dot-blot analysis. The results indicated that three antisera had significant titers as shown in Fig. 2. Antisera no. 93093-2 and 93093-3 showed the highest titers of any antibodies that reacted with the membrane preparations from transfected CHO cells, suggesting that the antisera generated against the synthetic peptide recognize rat PACAP receptor.

The specificity of the antiserum to PACAP receptor was examined by Western blot analysis using transfected cells (CHO-tf-6B-PACAPR) and nontransfected CHO cells. As shown in Fig. 3A, both the membrane and soluble fractions of the transfected cells specifically reacted with the antiserum (93093-3). Three immunoreactive bands were demonstrated, the most prominent being an approx 57 kDa band, which corresponds to the molecular size of cloned rat PACAP receptor *(9)*. The intensity of these bands increased when samples were obtained from 1 h of ultracentrifugation of the membrane preparation (Fig. 3A, Lane 2). A less-intense band, larger than 57 kDa, and a very weakly stained band, smaller than 57 kDa, may represent the PACAP receptor precursor and a degraded product, respectively. All these bands disappeared or were consid-

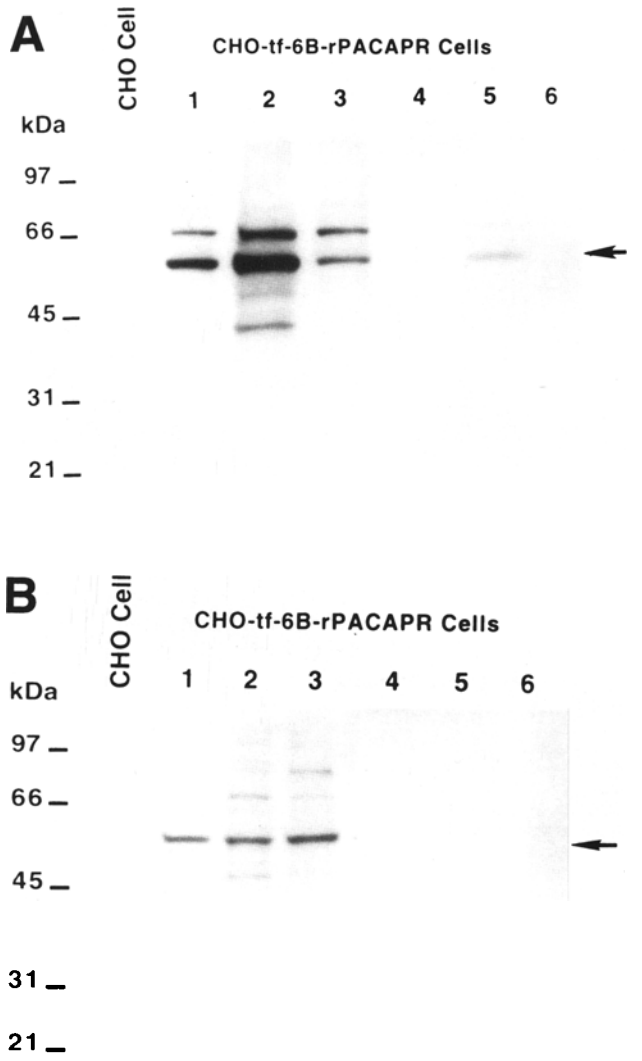

Fig. 3. Western blot analysis of rat PACAP receptor in CHO and CHO-tf-6B-PACAPR cells. Fifty micrograms of proteins were reacted with unpurified **(A)** and affinity-purified **(B)** PACAP receptor antibody (93093-3). Lanes 1–3: membrane 1 (30 min of ultracentrifugation), membrane 2 (60 min of ultracentrafugation), and soluble fraction. Lanes 4–6 represent analysis of the samples from lanes 1–3 by the antibody preincubated with the immunogen peptides. Arrows in (A) and (B) indicate the band of the approximate 57 kDa corresponding to the molecular size of cloned rat PACAP receptor.

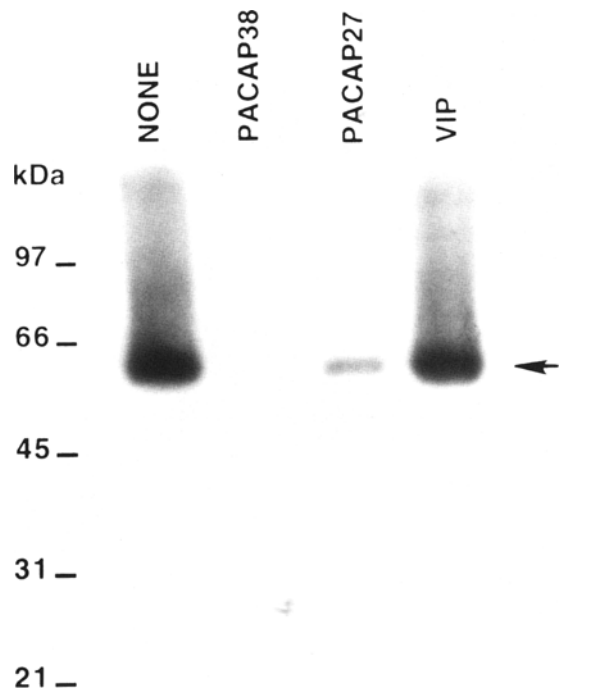

Fig. 4. Crosslinking of [^{125}I]PACAP27 to CHO-tf-6B-PACAPR cell membranes. The cells were incubated with [^{125}I]PACAP27 in the absence or presence of 1 μ*M* unlabeled PACAP38, PACAP27, or VIP. Crosslinking was performed by DSS, and after washing, membranes were solubilized and submitted to SDS-PAGE under reducing conditions. Autoradiographies were conducted for 1 wk at –80°C.

erably diminished when the antiserum was preabsorbed with the synthetic immunogen peptide, suggesting that they are related to the PACAP receptor protein. Moreover, the affinity-purified antibody (93093-3) reacted specifically with the 57-kDa protein from membrane preparations of transfected CHO cells (Fig. 3B), but did not react with similar membrane preparations from nontransfected CHO cells. The lower molecular protein band appears to be a PACAP receptor-degraded product. Staining of all these bands was prevented by the presence of the immunogen peptide. The results indicate that this antibody specifically recognizes the PACAP receptor or its related proteins.

Characterization of PACAP Receptor Antibody

After crosslinking the membrane fractions of transfected CHO cells with [^{125}I]PACAP27, it was subjected to SDS-PAGE gel electrophoresis and autoradiography. The results revealed the presence of a band with a M_r of 60 kDa, approximately corresponding to the sum of 57 kDa (rat PACAP receptor) and the molecular size of PACAP27 (Fig. 4). The labeling of this band was markedly reduced when membranes were incubated in the presence of 1 μ*M* of PACAP38 or PACAP27, but was unaffected by the presence of 1 μ*M* of VIP.

The molecular complex of the PACAP receptor and biotinyl-PACAP27 was examined using both immunostaining of Western blot analysis and detection of biotin-containing complex with ABC reagent (Vectastain Elite ABC kit, Vector Lab., Burlingame, CA) followed by visualization with 3,3'-diaminobenzidine. The biotinylated PACAP27/membrane complexes were run on the same SDS-PAGE and electroblotted onto the same nitrocellulose membrane, and the membrane was cut in half. The left half was examined for biotin-positive materials, and the right half for immunoreactivity. Only one immunopositive band corresponding to the M_r of the PACAP receptors was demonstrated (Fig. 5, right panel). However, there were several biotin-positive bands (Fig. 5, left panel). Two

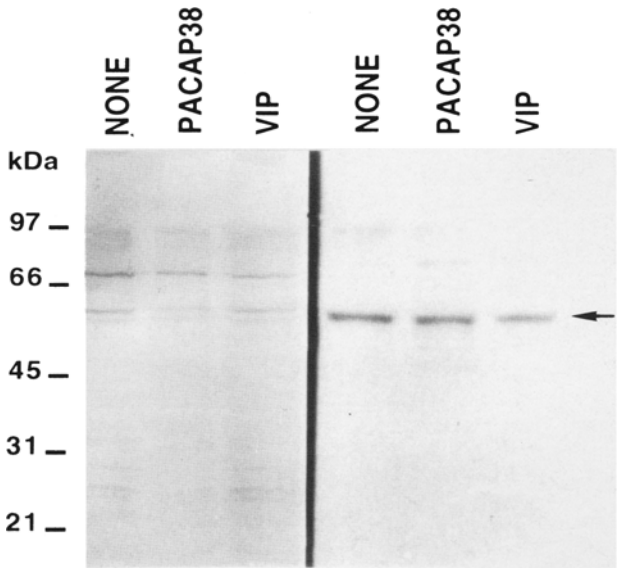

Fig. 5. Affinity labeling of rat PACAP receptor from CHO-tf-6B-PACAPR cell membranes with biotinyl-PACAP27. Affinity labeling of PACAP receptors was carried out using DSS in the absence or presence of unlabeled PACAP38 or VIP (10^{-6} *M*). The left half of blots were detected with ABC-DAB (*see* Materials and Methods); the right half of blots were subjected to Western blot analysis as described in the text, and similar results were obtained through ECL detection.

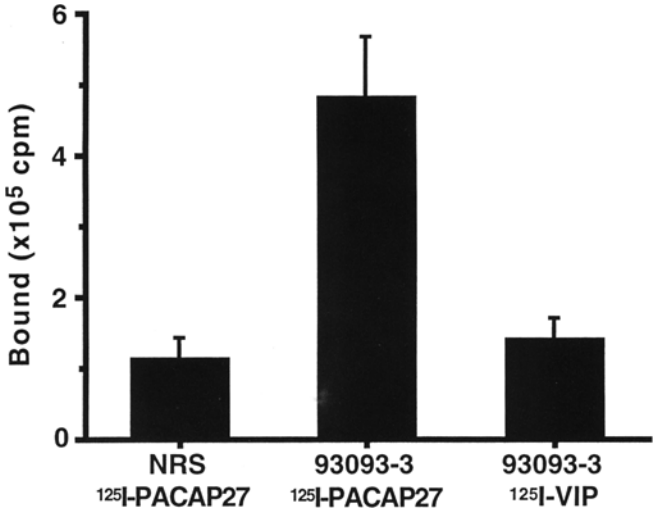

Fig. 6. Specific binding of [^{125}I]PACAP27 or [^{125}I]VIP to the membrane proteins (50 μg) of the rat brain recognized by the PACAP receptor antiserum (93093-3). Rat brain membrane fractions were solubilized and immunoprecipitated with 93093-3. As a control, normal rabbit serum (NRS) was used instead of 93093-3. Specific binding was determined by incubation with [^{125}I]PACAP27 or [^{125}I]VIP after correction for nonspecific binding of the respective radioligands. Nonspecific binding was estimated by the specific binding of the ligands after addition of 10^{-6} *M* unlabeled PACAP38 or VIP, respectively. Each bar shows the mean with standard error for triplicate experiments.

biotin-positive bands, at approx 57–60 kDa, appear to correspond to the immunoreactive band. Only these two bands diminished in staining intensity after coincubation with unlabeled PACAP38 (10^{-6} *M*), but neither diminished with VIP (10^{-6} *M*). It is possible that these two bands represent the products of chemical crosslinking. The staining intensity of the other bands remained unchanged after coincubation with PACAP38, suggesting that these bands represent nonspecific binding with biotinylated PACAP27. Immunostaining was not diminished after coincubation with 10^{-6} *M* of PACAP38 (Fig. 5, right panel). The results of affinity labeling of the PACAP receptor in combination with specific PACAP receptor-like immunoreactivity (PACAPR-li) through Western blot analysis indicate that this antibody recognizes PACAP receptor.

Immunoprecipitation of Solubilized Rat Brain Membrane with the PACAP Receptor Antibody

To examine whether the proteins in the rat brain membrane preparations recognized by the rat PACAP receptor antiserum (93093-3) bind PACAP, the membranes (including the cerebellum, which is known to express abundant PACAP receptor) were solubilized with digitonin and immunoprecipitated with PACAP antiserum 93093-3 or treated with normal rabbit serum. As shown in Fig. 6, the protein in the solubilized rat brain membrane preparation precipitated by the PACAP receptor antiserum (93093-3) showed considerably greater binding to [^{125}I]PACAP27 compared to the proteins treated with normal rabbit serum. The protein recognized by the PACAP receptor antibody did not show appreciable binding to [^{125}I]VIP.

Localization of PACAPR-li in the Brain

The rabbit antiserum (93093-3) against rat PACAP receptor immunostained the rat brain with low background. Immunoreactivity was completely eliminated by preabsorption with the immunogen peptide. The distribution of PACAP receptor immunoreactivity was similar to that of mRNA as studied by *in situ* hybridization histochemistry *(6,11)*.

PACAPR-li staining with antiserum 93093-3 was the most intense in neurons in the internal granular layer of the olfactory bulb (Fig. 7A) and the dentate gyrus of the hippocampus (Fig. 7B). In the cerebellar cortex, PACAPR-li was observed in the granule cells of the granular layer and in the Purkinje cells, whereas dot-like structures were occasionally seen in the molecular layer. PACAPR-li was infrequently found in the medulla (Fig. 7C,D). In the hypothalamus, PACAPR-li was strongly demonstrated in the supraoptic nucleus and medial parvocellular area of the paraventricular nucleus (data not shown). Many PACAP receptor-positive cells were found in the anterior and intermediate lobes of the pituitary (Fig. 7), whereas none was found in the posterior lobe. These results are in agreement with those obtained by *in situ* hybridization histochemistry for PACAP receptor mRNA *(36)* and radioreceptor assay *(4,38,39)*.

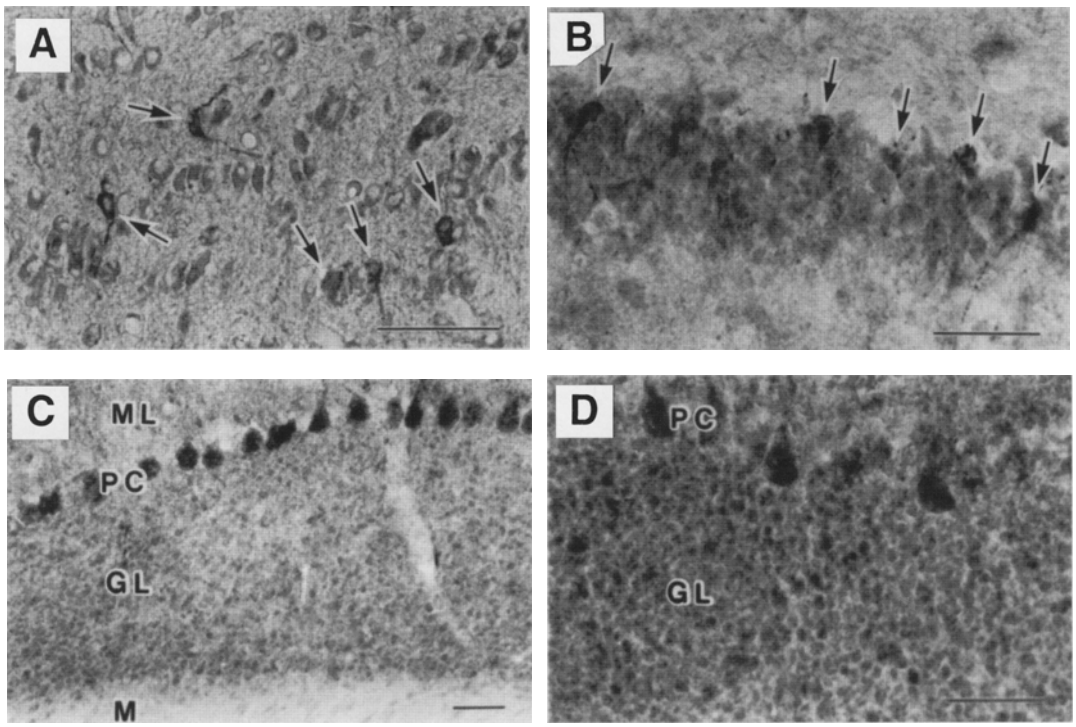

Fig. 7. Light photomicrographs of frozen sections of the olfactory bulb **(A)**, hippocampus **(B)**, and cerebellum **(C, D)** with rat PACAP receptor antibody (93093-3). (A) Many immunoreactive neurons with processes are visible (arrows) in the internal granular of the olfactory bulb. (B) Many immunoreactive granule cells are visible (arrows) in the dentate gyrus of the hippocampus. (C, D) PACAP receptor-like immunoreactivities are visible in the granules cells of the granular layer (GL) and Purkinje cells (PC). A few such immunoreactivities are seen in the molecular layer (ML), but rarely in the medulla (M). Scale bars: 50 μm.

Discussion

Receptor antibodies are inevitably an important tool for investigating the localization of a specific receptor protein. Precise knowledge of the cellular and subcellular sites of expression of a receptor protein is important for understanding cell–cell interaction, cell structure and function, receptor precursor processing, and many other biological activities. Although a number of antisera against PACAP receptors have become available, they are often used without conducting systematic examination of their specificity. Thus, reports of studies using uncharacterized antisera have resulted in considerable confusion, misinterpretation, and disagreement.

In the present study, a rabbit antiserum 93093, generated against a synthetic peptide with 25 residues corresponding to the intracellular C-terminal domain of type I or specific rat PACAP receptors, was rigorously examined for its specificity for the PACAP receptors. This antibody does not recognize any protein in membrane and soluble fractions from the normal CHO cells, but detects the proteins in similarly prepared fractions from CHO cells transfected with rat PACAP receptor cDNA. The PACAP receptor immunoreactivity detectable by the antiserum was demonstrated in both membrane and soluble fractions of the transfected CHO cells. The antibody also recognized the PACAP receptor as well as its related proteins, possibly including the precursor and degraded products containing an epitope fragment present in the intracellular C-terminus of the receptor.

Western blot analysis of the subcellular fractions prepared from the transfected CHO cells demonstrated three bands, one corresponding to 57 kDa (the size of PACAP receptor), and two less intensely stained bands, one larger and one smaller than 57 kDa. These bands appear to represent PACAP receptor precursor protein and possibly degraded products. Interestingly, the binding study with [^{125}I]PACAP27 showed only one positive band on the same SDS-polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to 57 kDa. This indicates that only matured PACAP receptor is capable of binding to the ligand, but neither the precursor nor the degraded fragment can bind to PACAP. However, immunostaining using this antiserum may demonstrate that the examined tissue contains PACAP receptors, precursor and/or products, or that translation of the PACAP receptor mRNA has occurred.

The 25 residue peptide corresponding to the intracellular C-terminus was used as the antigen peptide for PACAP receptor, because this sequence shows the least homology compared to other portions of the corresponding sequence of similar G protein-coupled receptors, particularly the receptor for VIP1 *(40,41)*. This PACAP receptor antibody could be used for the specific immunoprecipitation of PACAP receptor. Spengler and his associates *(6)* have

cloned five subtypes of rat PACAP receptors resulting from alternative splicing. These subtypes vary in the third intracellular domain by the absence or presence of a 27 or 28 residue cassette. Recently, a shorter PACAP receptor subtype, with the deletion of 21 residues in the extracellular N-terminal domain, has been cloned *(21)*. Another variant, which has alternative amino acids in the fourth transmembrane domain, has also been cloned *(22)*. All of these subtypes of PACAP receptor contain the same sequence in the C-terminal intracellular domain and, therefore, may be recognized by the PACAP receptor antibody. However, it is unlikely that this antibody recognizes the proteins that do not contain the same or similar sequence, including VIP1 or VIP2 receptors and their receptor proteins. Immunoprecipitation clearly showed that the rat brain membrane proteins, which are recognized by this antibody, bind to [^{125}I]PACAP27, but not to [^{125} I]VIP.

In summary, this study presents evidence for the specificity of the rabbit antiserum (93093-3) against rat PACAP receptors. Although this antiserum was generated against a 25-residue peptide corresponding to the C-terminal intracellular region of the rat PACAP receptor, the data indicate that its specificity is sufficient to make it a useful tool for studying the expression of various PACAP receptor subtypes and their related proteins.

Materials and Methods

Cell Culture and Transfection

CHO-K1 cells (ATCC, Rockville, MD) were cultured in a F12K nutrient mixture (Kaighn's Modification) medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY) and 1% antibiotic-antimycotic (Gibco BRL) under 5% CO_2/95% air at 37°C.

To produce the cloned cDNA of rat PACAP receptor, the *Nco*I-*Apo*I cDNA fragment (1.7 kb) containing a protein coding region of pRPACAPR12 was inserted into the expression vector pRC/CMV (Invitrogen, San Diego, CA) and designated pRPR4-B. The resultant expression plasmids were introduced into CHO-K1 cells by the calcium phosphate transfection method using the ProFection Mammalian Transfection kit (Promega, Madison, WI). The transfected cells (CHO-tf-6B-PACAPR) were selected in the medium containing 500 µg/mL geneticin (G418, Sigma, St. Louis, MO), and cloned by colony formation.

Preparation of Primary Antibody

The primary antisera were raised in rabbits against a synthetic peptide that corresponded to the sequence of 25 amino acids from Lys^{411} to Ala^{435} of the rat PACAP receptor, which is presumed to be its carboxy-terminal intracellular domain. The amino acid sequence of the peptide (KRKWRSWKVNRYFTMDFKHRHPSLA) was synthesized by the solid-phase method (Peptide Research Laboratory, Tulane University School of Medicine). The peptide was coupled to bovine thyroglobulin (type I, Sigma) using 0.02 *M* glutaraldehyde (Polysciences, Warrington, PA). The conjugate was dialyzed against 0.9% NaCl overnight. It was emulsified with complete Freund's adjuvant (Difco Labs., Detroit, MI) and injected into mixed-breed female rabbits subcutaneously in the nuchal area. After each booster injection, blood from the marginal ear vein was separated to collect serum by centrifugation. The titer was monitored with an enzyme-linked immunosorbant assay (ELISA) *(42,43)*. Dot-blot analysis was performed to determine optimal concentrations of the primary antisera (93092, 93093, and 93094) for Western blot analysis, which was used to detect PACAP receptors expressed in CHO cells. A portion of the antisera was purified by Affi-Gel 10 column (Pierce, Rockford, IL) according to the affinity chromatography method using a synthetic peptide from Lys^{411} to Ala^{435} of rat PACAP receptor.

Membrane Preparation and Western Blotting

The CHO-K1 cells and their transformants were homogenized in ice-cold 10 m*M* Tris-HCl (pH 8.0 at 0°C) containing 0.25 *M* sucrose, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 µ*M* pepstatin A, 10 µ*M* leupeptin, and 1 m*M* 2-mercaptoethanol, and centrifuged at 2000*g* for 5 min at 4°C. The supernatants were further ultracentrifuged at 55,000*g* for 30 or 60 min at 2°C. The supernatants (soluble cytosolic fraction) were collected, and the resultant pellets (membrane fraction) were resuspended in 50 m*M* of Tris-HCl (pH 8.0 at 0°C) containing 1 m*M* of EDTA and 1 m*M* of 2-mercaptoethanol. The membrane and soluble fractions in the sample buffer (8% SDS, 20% β-mercaptoethanol, 0.25 *M* Tris-HCl, pH 6.8, and 40% glycerol) were subjected to a 12% gel SDS-PAGE, and then electroblotted onto a nitrocellulose membrane (Hybond-ECL, Amersham, UK). The blots were blocked with skim milk and further incubated with rabbit PACAP receptor antibody (93093-3). To check for specificity, antibodies preincubated with 50 µg/mL of their immunized peptide and bovine thyroglobulin were used. The bound antibodies were detected using an enhanced chemiluminescence system (ECL, Amersham, UK).

[^{125}I]PACAP27 Binding Assay

Competitive binding experiments were conducted with membranes prepared from both CHO and CHO-tf-6B-PACAPR cells. The membrane fractions, which contained 20 µg of protein, were incubated with [^{125}I]PACAP27 in a total volume of 300 µL of 50 m*M* Tris, pH 7.4, 5 m*M* $MgCl_2$, 0.5 mg/mL bacitracin, 2 µg/mL PMSF, 200 U/mL Trasylol, and 1.0% BSA at 22°C for 90 min. The assay was terminated, and bound/free ligands were separated by rapid filtration method as previously described *(9)*. Radioactivity in each filter was measured using an automatic γ-counter. Nonspecific binding was determined in the presence of 10^{-6} *M* of PACAP38, PACAP27, and VIP. The concentra-

tion of protein was determined using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

Affinity Labeling Experiments

Transfected CHO cells were grown in a medium containing 500 μg/mL geneticin in 100-mm culture dishes. The cells were then washed with 50 m*M* HEPES buffer (pH 7.5) containing 128 m*M* NaCl, 5 m*M* KCl, 5 m*M* $MgSO_4$, 1.2 m*M* $CaCl_2$, and 2 mg/mL BSA, and incubated overnight at 4°C with [^{125}I]PACAP27 in the same buffer. The cells were washed twice with ice-cold HEPES buffer (no BSA). Chemical crosslinking with 0.1 m*M* of disuccinimidyl suberate (DSS, Pierce, Rockford, IL) was carried out at 4°C for 30 min. After incubation, the cells were washed twice in ice-cold Tris-HCl buffer (10 m*M*, pH 7.4) containing 0.25 *M* sucrose, 1 m*M* EDTA, and 0.3 m*M* PMSF, and treated with a Tris buffer (pH 7.0) containing 125 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, and protease inhibitors. After the cells were homogenized with a Polytron homogenizer and centrifuged, the resultant supernatants were subjected to electrophoresis on 12% of SDS-PAGE in a sample buffer composed of 8% SDS, 20% β-mercaptoethanol, 0.25 *M* Tris-HCl, pH 6.8, and 40% glycerol. The gel was fixed and dried, and autoradiography was conducted for 1 wk at –80°C. The PACAP receptor was affinity-labeled with 10 n*M* biotinyl-PACAP27 in the same manner.

Immunoprecipitation

Preparation of rat brain membrane fractions and solubilization of membrane proteins were performed as described previously *(44)*. Brain membranes obtained from adult male rats (CD strain, Charles River Breeding Labs., Welmington, MS) were solubilized in a 20 m*M* Tris buffer (pH 7.4) containing 0.25 *M* sucrose, 10 m*M* EDTA, 0.03% NaN_3, and a mixture of protease inhibitors with a high concentration of digitonin (1.5%, digitonin to protein ratio of 5:1, Aldrich, Milwaukee, WI). The mixture was stirred gently at 4°C for 1 h and then ultracentrifuged at 55,000*g* for 20 min. A 10-fold dilution of the supernatant fraction was used for immunoprecipitation.

The solubilized membranes in the reaction buffer (0.1% digitonin, 50 m*M* Tris-HCl [pH 7.5], 5 m*M* EDTA, 0.1 m*M* PMSF) were incubated with 1/20 vol. of normal rabbit serum for 1 h at 4°C and then precleared with protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ) for 1 h at 4°C. Two hundred microliters of supernatant containing 50 μg protein were incubated with 10 μL undiluted PACAP receptor antibody (93093-3) for 2 h at 4°C. Five hundred microliters of protein A-Sepharose beads were added, and the reaction was incubated for an additional 1 h at 4°C. The beads were washed with the reaction buffer and incubated with 10 n*M* [^{125}I]PACAP27 or [^{125}I]VIP. To estimate nonspecific bindings, 10^{-6} *M* of unlabeled PACAP38 or VIP were added to half of these reaction tubes for 3 h at 4°C. After centrifugation at 10,000*g* for 5 min, the radioactivity of the beads was counted by an autoγ-counter. The mean of the results of triplicate determinations after correction for nonspecific bindings was used for comparison between treatments.

Immunohistochemical Staining

Under nembutal anesthesia (50 mg/kg, ip), young adult male rats of the Wistar strain were intracardially perfused with physiological saline (37°C) followed by freshly prepared 2% paraformaldehyde fixative in 0.1 *M* phosphate-buffered saline (PBS, pH 7.2). The brain tissues were dissected and postfixed with the same fixative at 4°C for 12 h, and then infiltrated with 20% sucrose. The brain sections (10-μm thick) were cut on a cryostat (Mikrom, Heidelberg, Germany) and mounted on gelatin-coated slides. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in 2% normal goat serum, followed by 0.2% Triton X-100 for 30 min. Cryosections were incubated with 10% normal goat serum in PBS for 30 min, and incubated overnight with the primary antibody (93093-3) against rat PACAP receptor proteins at 4°C. The sections were sequentially reacted with antirabbit IgG avidin–biotinylated HRP complex (ABC, Vector Labs., Burlingame, CA). Immunoreactivities were visualized with 3,3'-diaminobenzidine. The sections were counterstained with veronal acetate-buffered 1% methyl green (pH 4.0). The antigen preabsorption control produced the same results as when the primary antibody was omitted.

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References

1. Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D., and Coy, D. H. (1989). *Biochem. Biophys. Res. Commun.* **164,** 567–574.
2. Miyata, A., Jiang, L., Dahl, R. D., Kitada, C., Kubo, K., Fujino, M., Minamino, N., and Arimura, A. (1990). *Biochem. Biophys. Res. Commun.* **170,** 643–648.
3. Gottschall, P. E., Tatsuno, I., Miyata, A., and Arimura, A. (1990). *Endocrinology* **127,** 272–277.
4. Lam, H. C., Takahashi, K., Ghatei, M. A., Kanse, S. M., Polak, J. M., and Bloom, S. R. (1990). *Eur. J. Biochem.* **193,** 725–729.
5. Cauvin, A., Robberecht, P., DeNeef, P., Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., and Christophe, J. (1991). *Regul. Pept.* **35,** 161–173.
6. Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journot, L. (1993). *Nature* **365,** 170–175.
7. Tatsuno, I., Somogyvari-Vigh, A., and Arimura, A. (1994). *Peptides* **15,** 55–60.

8. Ohtaki, T., Watanabe, T., Ishibashi, Y., Kitada, C., Tsuda, M., Gottschall, P. E., Arimura, A., and Fujino, M. (1990). *Biochem. Biophys. Res. Commun.* **171,** 838–844.
9. Gottschall, P. E., Tatsuno, I., and Arimura, A. (1991). *FASEB* **J5,** 194–199.
10. Tatsuno, I., Gottschall, P. E., and Arimura, A. (1991). *Peptides* **12,** 617–621.
11. Hashimoto, H., Ishihara, T., Shigemoto, R., Mori, K., and Nagata, S. (1993). *Neuron* **11,** 333–342.
12. Hosoya, M., Onda, H., Ogi, K., Masuda, Y., Miyamoto, Y., Ohtaki, T., Okazaki, H., Arimura, A., and Fujino, M. (1993). *Biochem. Biophys. Res. Commun.* **194,** 133–143.
13. Morrow, J. A., Lutz, E. M., West, K. M., Fink, G., and Hamar, A. J. (1993). *FEBS Lett.* **329,** 99–105.
14. Ogi, K., Miyamoto, Y., Masuda, Y., Habata, Y., Hosoya, M., Ohtaki, T., Masuo, Y., Onda, H., and Fujino, M. (1993). *Biochem. Biophys. Res. Commun.* **196,** 1511–1521.
15. Pisegna, J. R. and Wank, S. A. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 6345–6349.
16. Svoboda, N., Tastenoy, M., Ciccarelli, R., Stievenart, M., and Christophe, J. (1993). *Biochem. Biophys. Res. Commun.* **195,** 881–888.
17. Svoboda, N., Tastenoy, M., Van Rampelbergh, J., Goossens, J. F., De Neef, P., Waelbroeck, M., and Robberecht, P. (1994). *Biochem. Biophys. Res. Commun.* **205,** 1617–1624.
18. Miyamoto, Y., Habata, Y., Ohtaki, T., Masuda, Y., Ogi, K., Onda, H., and Fujino, M. (1994). *Biochem. Biophys. Acta* **1218,** 297–307.
19. Hashimoto, H., Yamamoto, K., Hagiwara, N., Ogawa, N., Nishino, A., Aino, H., Nogi, H., Imanishi, K., Matsuda, T., and Baba, A. (1996). *J. Biochem. Biophys. Acta* **1281,** 129–133.
20. Pisegna, J. R. and Wank, S. A. (1996). *J. Biol. Chem.* **271,** 17,267–17,274.
21. Pantaloni, C., Brabet, P., Bilanges, B., Dumuis, A., Houssami, S., Spengler, D., Bockaert, J., and Journot, L. (1996). *J. Biol. Chem.* **271,** 22,146–22,151.
22. Chatterjee, T. K., Sharma, R. V., and Fisher, R. A. (1996). *J. Biol. Chem.* **271,** 32,226–32,232.
23. Koves, K., Arimura, A., Somogyvari-Vigh, A., Vigh, S., and Miller, J. (1990). *Endocrinology* **127,** 264–271.
24. Koves, K., Arimura, A., Gorcs, T. G., and Somogyvari-Vigh, A. (1991). *Neuroendocrinology* **54,** 159–169.
25. Vigh, S., Arimura A., Koves, K., Somogyvari-Vigh, A., Sitton, J., and Fermin, C. D. (1991). *Peptides* **12,** 313–318.
26. Yon, L., Freuilloley, M., Chartrel, N., Arimura, A., Conlon, J. M., Fournier, A., and Vaudry, H. (1992). *J. Comp. Neurol.* **324,** 485–499.
27. Uddman, R., Luts, A., Arimura, A., and Sundler F. (1991). *Cell Tissue Res.* **265,** 197–201.
28. Fridolf, T., Sundler, F., and Ahren, B. (1992). *Cell Tissue Res.* **269,** 275–279.
29. Sundler, F., Ekblad, E., Absood, A., Hakanson, R., Koves, K., and Arimura, A. (1992). *Neuroscience* **46,** 439–454.
30. Tabain, A., Chen, D., Hakanson, R., and Sunder, F. (1994). *Neuroendocrinology* **59,** 113–119.
31. Arimura, A., Somogyvari-Vigh, A., Miyata, A., Mizuno, K., Coy, D. H., and Kitada, C. (1991). *Endocrinology* **129,** 2787–2789.
32. Masuo, Y., Suzuki, N., Matsumoto, H., Tokito, F., Matsumoto, Y., Tsuda, M., and Fujino, M. (1993). *Brain Res.* **602,** 57–63.
33. Shivers, B. D., Gorcs, T. J., Gottschall, P. E., and Arimura, A. (1991). *Endocrinology* **128,** 3055–3065.
34. Masuo, Y., Ohtaki, T., Masuda, Y., Nagai, Y., Suno, M., Tsuda, M., and Fujino, M. (1991). *Neurosci. Lett.* **126,** 103–106.
35. Masuo, Y., Tokito, F., Matsunoto, Y., Shimanoto, N., and Fujino, M. (1994). *Neurosci. Lett.* **170,** 43–46.
36. Shioda, S., Shuto, Y., Somogyvari-Vigh, A., Legradi, G., Onda, H., and Arimura, A. (1994). *Soc. Neurosci. Abstracts* **20,** 516, 221.14.
37. Hashimoto, H., Nogi, H., Mori, K., Ohishi, H., Shigemoto, R., Yamamoto, K., Matsuda, T., Mizuno, N., Nagata, S., and Baba, A. (1996). *J. Comp. Neurol.* **371,** 567–577.
38. Suda, K., Smith, D. M., Ghatei, M. A., Murphy, J. K., and Bloom, S. R. (1991). *J. Clin. Endocrinol. Metab.* **72,** 958–964.
39. Basile, M., Gonzalezk, B. J., Leroux, P., Jeandel, L., Gourner, A., and Vaudry, H. (1993). *Neuroscience* **57,** 329–338.
40. Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K., and Nagata, S. (1992). *Neuron* **8,** 811–819.
41. Mayo, K. E. (1992). *Mol. Endocrinol.* **6,** 1734–1744.
42. Engvall, E. and Perlmann, P. (1971). *Immunohistochemistry* **8,** 871–874.
43. Kruseman, A. N. (1983). *J. Clin. Pathol.* **36,** 406–410.
44. Ohtaki, T., Masuda, Y., Ishibashi, Y., Kitada, C., Arimura, A., and Fujino, M. (1993). *J. Biol. Chem.* **268,** 26,650–26,657.