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Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor

(neuropeptide receptor/gastrointestinal peptide receptor/G-protein-coupled receptor)

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ABSTRACT **Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide belonging to the vasoactive intestinal peptide (VIP)/secretin/glucagon family of peptides, interacts with a distinct high-affinity receptor (type I receptor) on a number of tissues. These PACAP type I receptors have a high affinity for PACAP and a low affinity for VIP and are present in the hypothalamus and anterior pituitary, where they regulate the release of adrenocorticotropin, luteinizing hormone, growth hormone, and prolactin, and in the adrenal medulla, where they regulate the release of epinephrine. Type I PACAP receptors are also present in high concentrations in testicular germ cells, where they may regulate spermatogenesis, and some transformed cell lines, such as the rat pancreatic acinar carcinoma cell AR4-2J. Here we report the molecular cloning and functional expression of the PACAP type I receptor isolated from an AR4-2J cell cDNA library by cross-hybridization screening with a rat VIP receptor cDNA. The cDNA sequence encodes a unique 495-amino acid protein with seven transmembrane domains characteristic of guanine nucleotide-binding regulatory protein-coupled receptors. A high degree of sequence homology with the VIP, secretin, glucagon-like peptide 1, parathyroid, and calcitonin receptors suggests its membership in this subfamily of G_s-coupled receptors. Results of binding studies and stimulation of cellular cAMP accumulation in COS-7 cells transfected with this cDNA are characteristic of a PACAP type I receptor. Cloning of the PACAP type I receptor will enhance our understanding of its distribution, structure, and functional properties and ultimately increase our understanding of its physiological role.**

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide recently isolated from ovine hypothalamus (1) and occurs in two amidated forms, PACAP-38 and PACAP-27, sharing the same N-terminal 27 amino acids. The high degree of amino acid identity of these peptides with vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), secretin, and glucagon (30–68%) suggests its membership in this family of peptides (1, 2). The highest concentration of PACAP occurs in the central nervous system (CNS). Outside the CNS, PACAP is found in moderate concentrations in the testis and adrenal medulla and at lower concentrations in the ovary, lung, gastrointestinal tract, and pancreas (3). Both forms of PACAP stimulate adenylate cyclase with equal efficacy and high potency (1000-fold greater than VIP) in cultured rat pituitary cells (1).

A majority of studies suggest that there are at least two types of high-affinity receptors for PACAP on the basis of their relative affinities for PACAP and VIP. Type I PACAP receptors have high affinity only for PACAP, and type II PACAP receptors have high affinity for both PACAP and VIP, consistent with a classical VIP receptor (2, 4). Type I PACAP receptors are present in the hypothalamus and anterior pituitary, where they may regulate the release of adrenocorticotropin, growth hormone, prolactin, and luteinizing hormone (5–8); on the chromaffin cells of the adrenal medulla, where they mediate catecholamine release (9, 10); on germinal cells of the testis and epididymis, where they may play a role in spermatogenesis and sperm motility (4); and in the gastrointestinal tract, where they cause smooth muscle relaxation and secretion (11–13). Type I receptors have also been described on cultured rat astrocyes (14), a rat adrenal pheochromocytoma cell line, PC12H (9), a rat pancreatic acinar carcinoma cell line, AR4-2J (15), and a human neuroblastoma cell line, NB-OK-1 (16).

Affinity crosslinking studies of type I PACAP receptors on bovine brain membranes demonstrate a 57-kDa molecular species that is modulated in its affinity for PACAP by guanine nucleotides (17) and suggest its membership in the guanine nucleotide-binding regulatory protein (G protein)-coupled family of receptors similar to the recently cloned VIP (18) and secretin receptors (19).

Further knowledge of the molecular structure of the PACAP type I receptor and its gene would enhance our understanding of its distribution, function, and regulation. We describe here the identification of cDNA clones resulting from cross-hybridization screening of a cDNA library constructed from the rat pancreatic carcinoma cell line AR4-2J with a rat VIP receptor cDNA probe.† These clones encode a protein that has an affinity for PACAP-38, PACAP-27, and VIP consistent with the type I PACAP receptor pharmacology, has a high degree of homology to both the VIP and secretin receptors, and mediates PACAP-stimulated accumulation of intracellular cAMP.

MATERIALS AND METHODS

cDNA Library Construction and Isolation of cDNA Clones. A cDNA library was constructed from AR4-2J cells as described previously (20). The library ($\approx 7 \times 10^5$ plaques) was screened with a ^{32}P-labeled, randomly primed probe (21) corresponding to the complete coding region of the rat VIP receptor cDNA (18) that was PCR cloned from rat pancreatic cDNA. The library was initially screened under conditions of low and later high stringency [three 20-min washes at 37°C with 2× SSC/0.1% SDS for low-stringency screening and three 20-min washes at 42°C with 0.1× SSC/0.1% SDS for high-stringency screening (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)] (22). Several clones that hybridized only at low stringency were plaque-purified from the AR4-2J cell library.

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Abbreviations: PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; PTH–PTHrP, parathyroid hormone–parathyroid hormone-related protein.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L16680).

PCR Cloning. Approximately 5 ng of double-stranded cDNA from the AR4-2J cDNA library served as a template for PCR amplification. The clones isolated from cross-hybridization screening of the AR4-2J cDNA library described above provided the sequence used to design primers from the receptor 5′ and 3′ untranslated regions. The oligonucleotide sequences (0.5 μM) 5′-ATAGCCAGAGATAGTGGCTGGG-3′ (nucleotides 2–23, Fig. 1) and 5′-CTGAGCTGCATCCCTAGCCAAC-3′ (nucleotides 1975–1996, Fig. 1) were used as the 5′ sense and 3′ antisense primers, respectively. The following cycle temperatures and times were used under standard PCR (Perkin–Elmer/Cetus) conditions: 34 cycles of denaturation at 94°C for 45 sec, annealing at 61°C for 25 sec, and extension at 72°C for 2 min with a final extension duration of 15 min. This PCR product (0.02%) was used as the target for a nested PCR amplification for an additional 35 cycles as described above using 0.5 μM 5′ and 3′ untranslated oligonucleotide sequences 5′-ACTGACTAGTCTAGATGGGAAGCACCATGGCCAGAG-3′ (nucleotides 20–40, Fig. 1) and 5′-ACTGACTAGTCTAGACTGTGCAGAAGGAGGAGGGAG-3′ (nucleotides 1526–1546, Fig. 1), respectively, as nested primers (each containing an *Xba* I site and a 9-bp cap on the 5′ end). The final PCR product was digested with *Xba* I and subcloned in the mammalian expression vector pCDL-SRα at the *Xba* I site (23).

DNA Sequencing. Plaque-purified clones in λgt10 were sequenced using the dsDNA Cycle Sequencing System (Bethesda Research Laboratories). The PCR products, subcloned in pCDL-SRα as described above, were sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* (24), using Sequenase 2.0 (United States Biochemical).

DNA and Protein Sequence Analysis. Nucleotide and amino acid sequences were analyzed by the Genetics Computer Group (Madison, WI) software package, using the PILEUP program (25).

Northern Blot Analysis of mRNAs. Poly(A)$^+$ RNA was isolated as previously described (20) from rat pancreas, the rat pancreatic acinar carcinoma cell line AR4-2J, brain, lung, duodenum, liver, kidney, and striated muscle. Two micrograms of poly(A)$^+$ RNA per lane was electrophoretically separated on a 1.2% agarose/formaldehyde gel and blotted onto Nytran (Schleicher & Schuell). The blot was hybridized with the PACAP receptor full-length cDNA coding region, which had been ^{32}P-labeled as described previously (22). The blot was washed under conditions of high stringency (three 20-min washes at 55°C with 0.1× SSC/0.1% SDS) and exposed for 48 hr in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to prepare an autoradiograph.

Transfection of PACAP Receptor cDNA into Mammalian Cells. Two micrograms of pCDL-SRα containing the PACAP receptor cDNA insert subcloned at an *Xba* I site in the sense orientation was transfected into near-confluent COS-7 cells by using a DEAE-dextran method (26). Approximately 48 hr after transfection, cells were washed twice at 4°C with sodium phosphate-buffered saline (PBS), pH 7.4, containing bovine serum albumin at 1 mg/ml, scraped from the plate in Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin at 1 mg/ml at 4°C, centrifuged (400 × *g*), and resuspended in the appropriate medium at 4°C for use in either radiolabeled binding or cAMP assays.

Binding of ^{125}I-Labeled PACAP-27 (^{125}I-PACAP-27) to Transfected COS-7 Cells. Transfected COS-7 cells suspended in 500 μl of DMEM with bovine serum albumin at 1 mg/ml were incubated for 60 min at 37°C with the radiolabeled hormone ^{125}I-PACAP-27 (2200 Ci/mmol; 1 Ci = 37 GBq) at 50 pM, either with or without the indicated concentrations of the various unlabeled peptide hormones and antagonists. Cells were subsequently washed three times at 4°C with 2 ml of PBS containing bovine serum albumin at 1 mg/ml by filtration on glass fiber filters (Whatman GF/A) using a suction manifold (Millipore). Filters were assayed for γ radioactivity (Packard, AutoGamma).

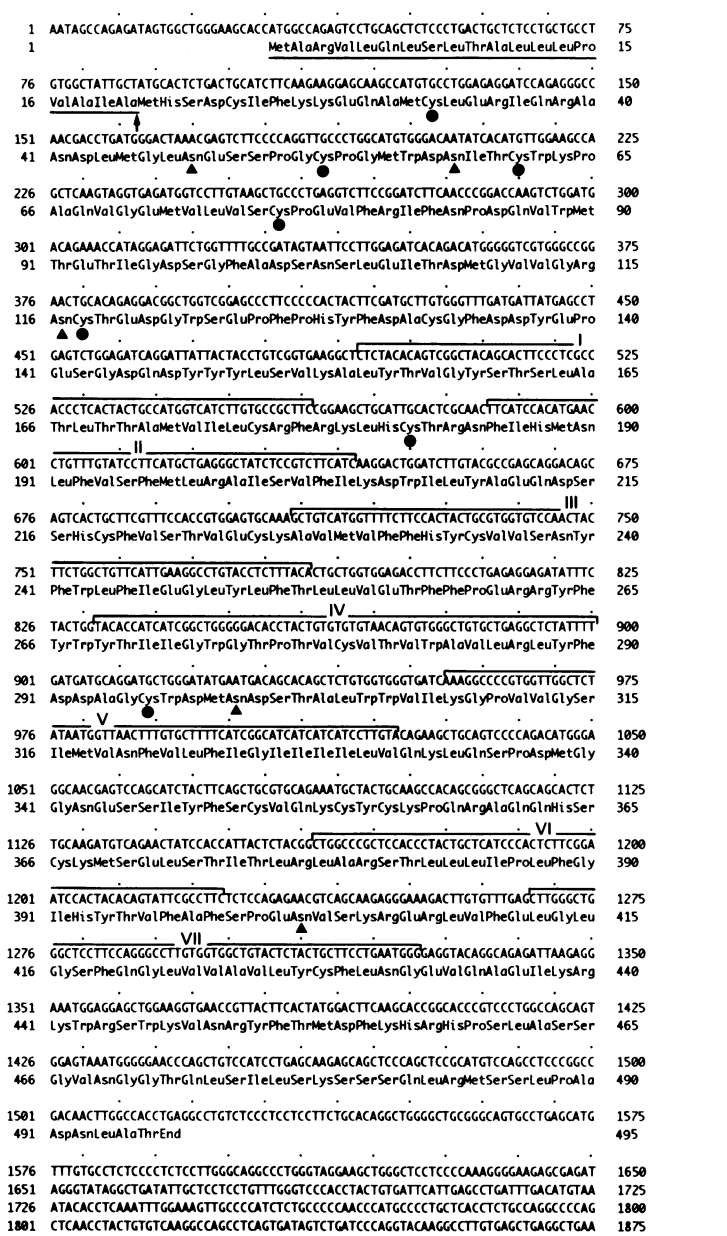

FIG. 1. Nucleotide and deduced amino acid sequences of the rat PACAP receptor cDNA. Solid lines labeled with roman numerals I–VII delineate the putative transmembrane domains predicted by Kyte–Doolittle criteria (27), using a 19-residue window. The solid line below the first 19 amino acids and the arrow between amino acids 19 and 20 indicate a potential signal peptide and cleavage site (28), respectively. Solid triangles indicate five potential sites for N-linked glycosylation. Solid circles indicate the seven cysteine residues conserved in all five cloned receptors belonging to this receptor family [PACAP, VIP, secretin, parathyroid hormone/parathyroid hormone-related protein (PTH–PTHrP), and calcitonin receptors].

Assay of Intracellular cAMP in Transfected COS-7 Cells. Transfected COS-7 cells were suspended in 500 μl of DMEM containing bovine serum albumin at 1 mg/ml and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) alone or with the indicated concentrations of the various peptide hormones for 45 min at 37°C. The reaction was terminated by the addition of 1 ml of iced ethanol and centrifugation at 14,000 × *g*. Supernatants were assayed for cAMP by using a cAMP radioimmunoassay system (NEN/DuPont).

RESULTS AND DISCUSSION

Relying on the high degree of nucleotide sequence homology among members of receptor families (20), we used the rat VIP receptor coding sequence cDNA as a radiolabeled probe to screen an AR4-2J cell cDNA library to identify PACAP receptor clones. After screening approximately 7×10^5 cDNA clones, we identified four clones that hybridized only under conditions of low stringency. Two of the longest clones, numbered 6 and 31 (2.4 and 3.6 kb, respectively) were sequenced. Clone 6 contained the 3′ untranslated region and all but 452 bp of the 5′ coding sequence (nucleotides 481–1518, Fig. 1) and clone 31 contained the 5′ untranslated region and 680 bp of the 5′ coding sequence (nucleotides 30–709, Fig. 1). Unable to isolate a single clone containing the complete coding sequence, we used nested PCR to clone the full-length coding sequence. PCR primers were derived from clones 6 and 31 (nucleotides 2–23 and 1975–1996, first round PCR, and nucleotides 20–40 and 1526–1546, second round nested PCR, Fig. 1) and the same AR4-2J cDNA library served as the target DNA. Nested PCR primers contained an *Xba* I site used to subclone the product in the vector pCDL-SRα (23).

The first in-frame ATG of the cDNA consistent with a consensus translation initiation site (29) represents the start codon of a 1485-bp single long open reading frame that encodes a unique 495-amino acid protein. The protein has a calculated molecular mass of 57 kDa, which is in close agreement with previously reported affinity crosslinking studies of the PACAP receptor in rat astrocyte cultures and bovine brain membranes (17, 30, 31). The sequence allows for five potential N-linked glycosylation sites, three in the N terminus and one in the second extracellular loop, similar to the VIP and secretin receptors (18, 19) and one on the third extracellular loop. A hydropathy plot of the predicted amino acid sequence using the criteria of Kyte and Doolittle (27) identifies eight regions of hydrophobic residues, seven corresponding to putative transmembrane domains, suggesting its membership in the G-protein-coupled superfamily of receptors (32). The eighth hydrophobic region, 19 N-terminal amino acids, is consistent with a putative signal sequence and cleavage site (indicated by the arrow between residues Ala-19 and Met-20 in Fig. 1) according to the criteria of von Heijne (28), similar to VIP, secretin, PTH–PTHrP, and calcitonin receptors (18, 19, 33, 34).

An alignment of the deduced amino acid sequence with all known deduced protein sequences (GenBank release 73.1) showed the highest homology with rat VIP (50% identity and 68% similarity), secretin (46% identity and 63% similarity), glucagon-like peptide 1 (37% identity and 57% similarity), PTH–PTHrP (41% identity and 63% similarity), and porcine calcitonin (28% identity and 54% similarity) receptors (Fig. 2) confirming its membership in this family of receptors (18, 19, 33–35). The greatest degree of homology is within the transmembrane domains and the least is in the N and C termini and third intracellular loop. Similar to the other members of this receptor family, the PACAP receptor has a long extracellular N terminus with five conserved cysteines and a sixth and seventh conserved cysteine in the first and second extracellular loops, some of which may form disulfide bridges required for the stabilization of tertiary receptor structure, as has been shown for rhodopsin (36), β-adrenergic (37), and muscarinic (38) receptors. All six members of this receptor

FIG. 2. Alignment of the rat PACAP receptor (RATPACAPR), rat VIP receptor (RATVIPR), rat secretin receptor (RATSECR), rat glucagon-like peptide 1 receptor (RATGL1RCP), rat PTH-PTHrP receptor (RATPTHR), and porcine calcitonin receptor (PORCTR) protein sequences. Using the PILEUP program in the sequence analysis package of the Genetics Computer Group (25), we aligned the PACAP receptor deduced amino acid sequence for maximal homology to the deduced protein sequences of the five nucleotide sequences [rat VIP receptor (18), rat secretin receptor (19), rat glucagon-like peptide 1 receptor (35), rat PTH–PTHrP receptor (33), and porcine calcitonin receptor (34)] found to be the most homologous upon searching GenBank (release 73.1). Shown here, using single-letter abbreviations for amino acids, is the result of this alignment, with boxed areas denoting amino acids conserved between the PACAPR and at least two other receptors. The numbers of residues in the variable C terminus (not displayed) are in parentheses. Solid lines labeled with roman numerals indicate the seven putative transmembrane domains (Fig. 1).

family can mediate the activation of adenylate cyclase following ligand binding (18, 19, 33–35), and all five have conserved basic amino acids in the third intracellular loop which may be important for coupling to G_s, similar to the β_2-adrenergic receptor (39).

To determine the identity of the receptor protein sequence encoded by the cDNA, pharmacological characterization of the recombinant receptor expressed in COS-7 cells was performed. COS-7 cells transfected with the full-length cDNA insert (nucleotides 19–1546, Fig. 1) in the vector pCDL-SRα were incubated with ^{125}I-PACAP-27 alone or in the presence of increasing concentrations of unlabeled PACAP-38, PACAP-27, VIP, or secretin (Fig. 3). PACAP-38 (IC_{50} = 8 nM) was approximately 2.5-fold more potent than PACAP-27 (IC_{50} = 20 nM) (Fig. 3). VIP and secretin did not inhibit binding of ^{125}I-PACAP-27 at doses of up to 1 μM. These results are in close agreement with the pharmacological profile of type PACAP receptors reported for an adrenal pheochromocytoma cell line, PC12H (9), and hypothalamic and anterior pituitary membranes (30, 40, 41). In addition, the cloned receptor can be subclassified as a type IA, having nearly equal affinity for PACAP-27 and PACAP-38, similar to the type IA described in AR4-2J cell membranes (42).

To further demonstrate that the protein sequence encoded by the cDNA is a functional type IA PACAP receptor, we examined the ability of PACAP and related peptides to stimulate adenylate cyclase activity in COS-7 cells expressing the cloned receptor. COS-7 cells transfected with the PACAP cDNA insert in the vector pCDL-SRα were incubated either alone or with increasing concentrations of PACAP-38, PACAP-27, VIP, or secretin. PACAP-38 and PACAP-27 stimulated the accumulation of intracellular cAMP with nearly the same efficacy (cAMP concentration almost 3 times basal) in a dose-dependent manner, with PACAP-38 (EC_{50} = 0.6 nM) being 3-fold more potent than PACAP-27 (EC_{50} = 1.6 nM) (Fig. 4). VIP and secretin had a negligible effect on cAMP generation (Fig. 4). The PACAP

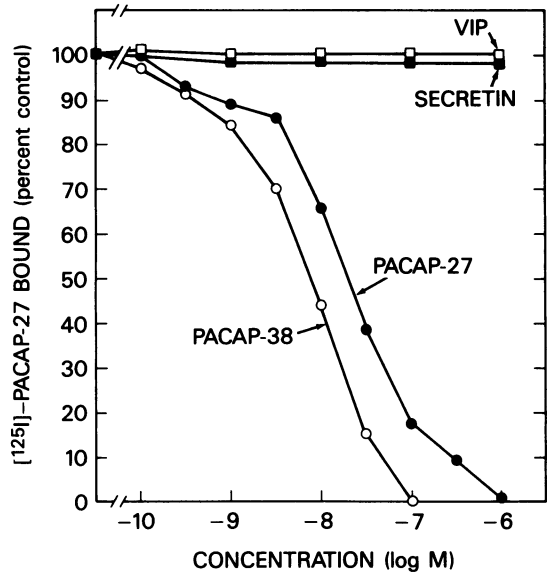

FIG. 3. ^{125}I-PACAP-27 binding to transfected COS-7 cells expressing the rat PACAP receptor. COS-7 cells were transiently transfected with the expression vector pCDL-SRα (23) containing the PACAP receptor cDNA sequence. ^{125}I-PACAP-27 (50 pM) was incubated either alone or with increasing concentrations of PACAP-38, PACAP-27, VIP, or secretin. Data are presented as percent saturable binding (total binding in the presence of radiolabeled hormone alone minus binding in the presence of 1 μM PACAP-38). The results given are means of values from at least five experiments performed in duplicate. Cells transfected with vector alone showed no binding.

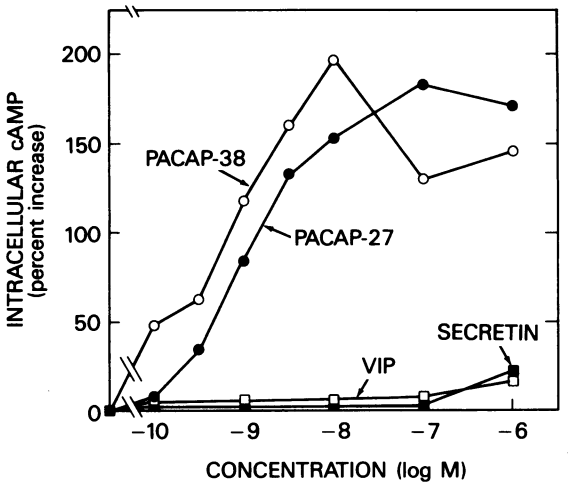

FIG. 4. Increase of intracellular cAMP in transfected COS-7 cells expressing the rat PACAP receptor. COS-7 cells were transiently transfected with the expression vector pCDL-SRα (25) containing the PACAP receptor cDNA sequence. Transfected COS-7 cells were incubated either alone or in the presence of increasing concentrations of PACAP-38, PACAP-27, VIP, or secretin. Data are presented as the percent increase in intracellular cAMP over basal (in the absence of added peptide). The results given are means of values from at least five experiments performed in triplicate.

receptor antagonist PACAP-(6–27) (42) at 10 μM caused a 46% and 38% inhibition in the stimulation of cAMP by 10 nM PACAP-27 and 3.0 nM PACAP-38, respectively. These results are similar to those reported previously for native receptor, when brain (40), anterior pituitary (2), AR4-2J cells (42), and NB-OK-1 human neuroblastoma cell (16) membranes as well as cultured astrocytes (14) were used and further support the idea that the cloned cDNA encodes a functional type IA PACAP receptor.

To determine whether the tissue distribution of the cloned receptor mRNA was consistent with ligand binding data for type IA PACAP receptors, high-stringency Northern blot analysis using 2 μg of rat tissue specific polyadenylylated mRNA was performed. A PACAP receptor cDNA coding region probe hybridized to a single 7.5-kb transcript from only the rat brain and AR4-2J cells (Fig. 5), both known to possess type IA PACAP receptors. This transcript size is

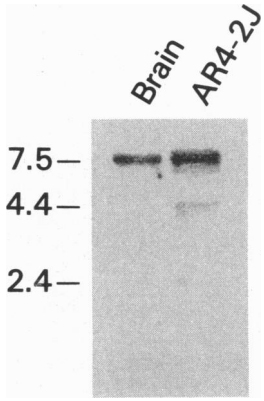

FIG. 5. Northern blot analysis of poly(A)$^+$ RNA from rat tissues. Poly(A)$^+$ RNA was prepared from rat brain and the rat pancreatic acinar carcinoma cell line AR4-2J. Four micrograms of poly(A)$^+$ RNA from each source per lane was probed under conditions of high stringency with a randomly primed ^{32}P-labeled probe of the PACAP receptor coding region. An ≈7.5-kb hybridizing transcript from rat brain and the rat pancreatic acinar carcinoma cell line AR4-2J was detected. Positions of RNA molecular size markers are indicated in kb.

larger than the 5.5- and 2.5-kb transcripts detected for the VIP and secretin receptors, respectively (18, 19). Tissues previously shown to possess only type II PACAP receptors, including pancreas (43), lung, liver, and kidney (44), were negative for hybridizing transcripts, as was the negative control, striated muscle (data not shown). Duodenum, previously shown to possess small numbers of both type I and type II (44) PACAP receptors, was also negative for a hybridizing transcript (data not shown), most likely because of the relatively small mass of neuronal cells expressing type I PACAP receptors.

In the relatively short period of time since the discovery of PACAP-38 from ovine hypothalamus in 1989 (1), a tremendous amount of progress has been made in the understanding of its genetic structure (45), peptide processing (45), tissue distribution (3), and corresponding receptor localization in the brain, pituitary, adrenal, and testis as well as its presence in nerve fibers in the respiratory and gastrointestinal tract (3, 11, 12, 44, 46). Already three types of receptors for PACAP have been identified, of which one has high affinity and specificity (type I) and may be subdivided into subtypes with various abilities to activate adenylate cyclase and the phosphatidylinositol/Ca^{2+} cascade (40–42). Despite these rapid advances, little is known about the physiologic actions of PACAP and its receptors. Our results, describing the molecular cloning and functional expression of type IA PACAP receptors, will further the identification of PACAP receptor subtypes, aid in the search for potent and specific agonists and antagonists, and allow dissection of ligand/receptor coupling to intracellular mediators. This should hasten our understanding of the physiologic actions of PACAP in both health and disease.

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