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REGULAR ARTICLE

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Expression of pituitary adenylate cyclase-activating polypeptide (PACAP) and the PACAP-selective receptor in cultured rat astrocytes, human brain tumors, and in response to acute intracranial injury

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is a bioactive peptide with diverse activities in the nervous system. In addition to its more classic role as a neurotransmitter, PACAP functions as a neurotrophic factor. PACAP exerts these activities by binding to PACAP-selective (PAC1) or nonselective (VPAC1, VPAC2) receptors (-R). Glial cells also exhibit PACAP binding, which is associated with the increased proliferation of astrocytes. The present report demonstrates a distinct spatiotemporal regulation of PACAP, PAC1-R, VPAC1-R, and VPAC2-R expression in primary cultured rat astrocytes. To determine the role of PACAP and PAC1-R expression on glial proliferation, two in vivo models were examined – human brain tumors of glial origin and the reactive gliosis induced by a penetrating stab wound to the mature rat brain. Relative to normal human brain, PAC1-R expression is significantly upregulated in glioma, particularly oligodendrogliomas. While similar polymerase chain reaction (PCR) analysis does not detect PACAP expression, in situ hybridization studies reveal PACAP expression in a limited number of cells within the tumor. In sharp contrast, neither PACAP nor PAC1-R expression are upregulated consequent to injury. These results suggest a distinct role for PACAP and PAC1-R in glioma development and nervous system response to injury.

Key words Glia · Glioma · Pituitary adenylate cyclase-activating polypeptide · Reactive gliosis · Receptor · Vasoactive intestinal peptide · Traumatic brain injury · Rat (Sprague Dawley) · Human

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon family of bioactive peptides (Arimura 1992). PACAP is expressed throughout the central and peripheral nervous systems, where it exerts effects on neurite outgrowth (Deutsch et al. 1993), neuropeptide and neurotransmitter production (May and Braas 1995), and neuronal proliferation (Lu and DiCicco-Bloom 1997; Villalba et al. 1997). The diverse activities of PACAP are mediated by multiple-target tissue receptors. The PACAP-selective receptor (PAC1-R) exhibits high affinity for PACAP but also binds VIP, although with 1000-fold lower affinity. VPAC1 and VPAC2 receptors (VPAC1-R and VPAC2-R, formerly VIP_1/PACAP and VIP_2/PACAP) bind PACAP and VIP with similar affinity (Hashimoto et al. 1993; Ishihara et al. 1992; Lutz et al. 1993). The PACAP receptors belong to the family of seven transmembrane-domain G protein-coupled receptors. Splice variants of the PAC1-R differ in the presence or absence of two cassettes, termed HIP and HOP, in the region of the gene encoding the third cytoplasmic loop, the primary site of interaction between the receptor and G proteins (Spengler et al. 1993). The five splice variants of the PAC1-R exhibit different patterns of adenylyl cyclase and phospholipase C (PLC) stimulation, suggesting a cell-specific mechanism for differential intracellular signaling (Pantaloni et al. 1996; Spengler et al. 1993).

Numerous studies have demonstrated a role for glial cells in the regulation of VIP and PACAP expression. In addition to their more classic roles as neurotransmitters, PACAP and VIP function as neurotrophic factors. The neuroprotective effects of VIP are mediated by astroglial-derived molecules (Brenneman et al. 1987). Rat brain astrocytes exhibit PACAP binding (Tatsuno et al. 1991), which is associated with the proliferation of astrocytes (Tatsuno et al. 1996). Therefore, studies were undertaken to investigate PACAP and PACAP receptor mRNA expression in primary cultured rat astrocytes and in two in vivo models of glial proliferation – human brain tumors

and the reactive gliosis induced by a penetrating stab wound to the mature rat brain.

The present report demonstrates the differential regulation of PACAP and PAC1-R mRNA in glial tumors and reactive gliosis. Relative to normal human brain, PAC1-R expression is significantly upregulated in tumors of glial origin, particularly oligodendrogliomas. While similar PCR analysis does not detect PACAP expression, in situ hybridization studies reveal expression in a limited number of cells within the tumor. In sharp contrast, neither PACAP nor PAC1-R expression are upregulated consequent to injury. These results suggest a distinct role for PACAP and PAC1-R in glioma development and nervous system response to injury.

Materials and methods

Animal care and human tissue procurement

All studies followed the *Principles of laboratory animal care* (NIH publication No. 86–23, revised 1985) and were performed in accordance with approved university institutional animal care and use protocols. Untimed pregnant Sprague-Dawley rats, obtained from Charles Rivers (Canada), were housed in the university vivarium and provided with food and water ad libitum. For developmental studies, animals were killed, rapidly and humanely by decapitation, within 24 h of the time of birth (designated postnatal day 0, P0) and at weekly intervals thereafter. Dams were used as adult animals (at least 6 months of age).

Human surgical tissue samples were obtained from the University of Vermont (kindly supplied by Dr. Paul Penar) or Yale University (kindly supplied by Dr. Joseph Piepmeier). Studies were approved by the human research committees at both universities, which requires patient's consent prior to inclusion in the study.

Cell culture

Primary astrocyte cultures were prepared according to Cornell-Bell and Finkbeiner (1991). Individual brain areas (cortex, brainstem, and cerebellum) were dissected from appropriatly aged rats (P0, P7, and P14) and finely minced prior to digestion with 20 U/ml papain (Worthington Biochemicals; Lakewood, N.J.) in complete saline (137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 25 mM dextrose, 3 mM $CaCl_2$,10 mM HEPES) supplemented with 1.5 mM $CaCl_2$, 0.5 mM EDTA, 20 µg/ml DNase, and 0.2 mg/ml L-cysteine at 37°C for 45 min. The tissue pieces were rinsed twice in trituration solution (media containing 1.5 mg/ml each trypsin inhibitor and bovine serum albumin) and gently triturated. Dissociated cells were diluted in either complete media [minimal essential media with Earle's salts (Life Technologies; Grand Island, N.Y.) supplemented with 10% fetal calf serum, 20 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin] or serum-free media (Opti MEM supplemented with 20 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin) and plated (10,000 cells/cm^2) on untreated tissue culture plates. Within 24 h the plates were shaken vigorously to remove loosely adherent nonglial cells. The plates were shaken and media replenished on the 3rd day and 5th day in vitro and RNA extracted on the 6th day in vitro.

Reverse transcription polymerase chain reaction

Total cellular RNA was prepared using a modification of the procedure by Chirgwin et al. (1979; RNA STAT-60, Tel-Test B; Friendswood, Texas). One microgram of total RNA was reverse-transcribed using oligo d(T) (SuperScript preamplification system; Life Technologies). Double-stranded cDNA was amplified from 0.5 µl of transcription reaction in 10 mM TRIS-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 20-pmol primers, and 1 U Taq DNA polymerase. Amplification was preceded by an initial denaturation of 94°C for 5 min. The cycling parameters used were as follows: PACAP (94°C/45 s, 59°C/45 s, 72°C/45 s; 28 cycles); PAC1-R (61°C annealing for 28 cycles); VPAC1-R (58°C annealing for 35 cycles); VPAC2-R (55°C annealing for 35 cycles), followed by a final extension of 72°C for 5 min. The primers used were as follows: PACAP (F=5´ATGCCTCTCTGGTTGTGATTC3´; R=5´CGCTATTCGGCGTCCTTTGTT3´); PAC1-R (F=5´CTTGTACAGAAGCTGCAGTCCCCAGACATG3´; R=5´CCGGTGCTTGAAGTCCATAGTGAAGTAACGGTTCACCTT3´); VPAC1-R (F=5´GCCCCCATCCTCCTCTCCATC3´; R=5´TCCGCCTGCACCTCACCATTG3´); VPAC2-R (F=5´AAGCTAACTTCTCCAGATGTT3´; R=5´CAGCTAAATGACTGAGGTCTC3´). Ten microliters of amplified product were electrophoresed on 1.6% agarose gels and visualized with ethidium bromide. For sequence-specific hybridization of amplified products, the DNA was transferred to Zeta-Probe membrane (Bio-Rad; Hercules, Calif.) and hybridized in 1.5× SSPE containing 10% polyethylene glycol (PEG) and 7% sodium dodecyl sulfate (SDS) at 65°C for 48 h with an [^{32}P]ATP end-labeled internal oligonucleotide probe (HIP-specific: 5´GTCTGAGGGCACAGGCAGGGGGTCCTCTCGGGTTTTCTT3´; HOP-specific: 5´TGACATCTTGCAAGAGTGCTGCTGAGCCCGCTGTGGCTT3´).

Surgical procedure

The cortical stab injury was performed as previously described (Jaworski et al. 1999). Young adult (postnatal day 45) Sprague-Dawley rats were anesthetized by intraperitoneal injection of chloral hydrate (420 mg/kg weight). A craniostomy was performed over the right cerebral hemisphere. A 27-gauge needle was inserted through the cortex into the underlying thalamus. The needle was held in place for 1 min, then withdrawn slowly. The craniostomy hole was sealed with dental wax and the incision closed with surgical staples. Following surgery, the animals were placed on thermal pads, with recovery from anesthesia generally occurring within 3 h. As the animals displayed no obvious discomfort or neurologic abnormalities as a result of the surgery, postoperative analgesia was not administered. Animals were killed by decapitation at 6 h, 12 h, 24 h, 4 days, 7 days, and 14 days postsurgery. At least two animals were examined at each postoperative time-point.

In situ hybridization

In situ hybridization was performed as previously described (Jaworski et al. 1994). A 606-bp fragment of rat pro-PACAP cDNA representing nucleotides 486–1091 was synthesized as previously described (Brandenburg et al. 1997). To generate the antisense and sense riboprobes T3 (Life Technologies) and T7 (New England Biolabs, Beverly, Mass.) RNA polymerase were used, respectively. A 449-bp PCR product encompassing the coding region for the intracellular carboxy-terminal tail of the rat PAC1-R (GenBank accession No. Z23279; nucleotides 1185–1633) was cloned into pBluescript II KS^-. The construction was characterized by restriction analysis and direct sequencing; T3 and T7 RNA polymerase were used to generate, respectively, the sense and antisense riboprobes. Combined in situ hybridization and immunohistochemistry was performed as previously described (Jaworski et al. 1999). Following hybridization, sections were incubated in glial fibrillary acidic protein (GFAP) antibody at 1:2000 (Sigma, St. Louis, Mo.) for 48 h at 4°C, incubated with biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, Calif.) for 2 h, avidin-biotin-HRP complex for 90 min, and the HRP reaction product visualized with DAB. The slides were dipped in NTB-2 emulsion (Kodak), developed after 14 days, and counterstained with cresyl violet. Figures were prepared from scanned images using Adobe Photoshop.

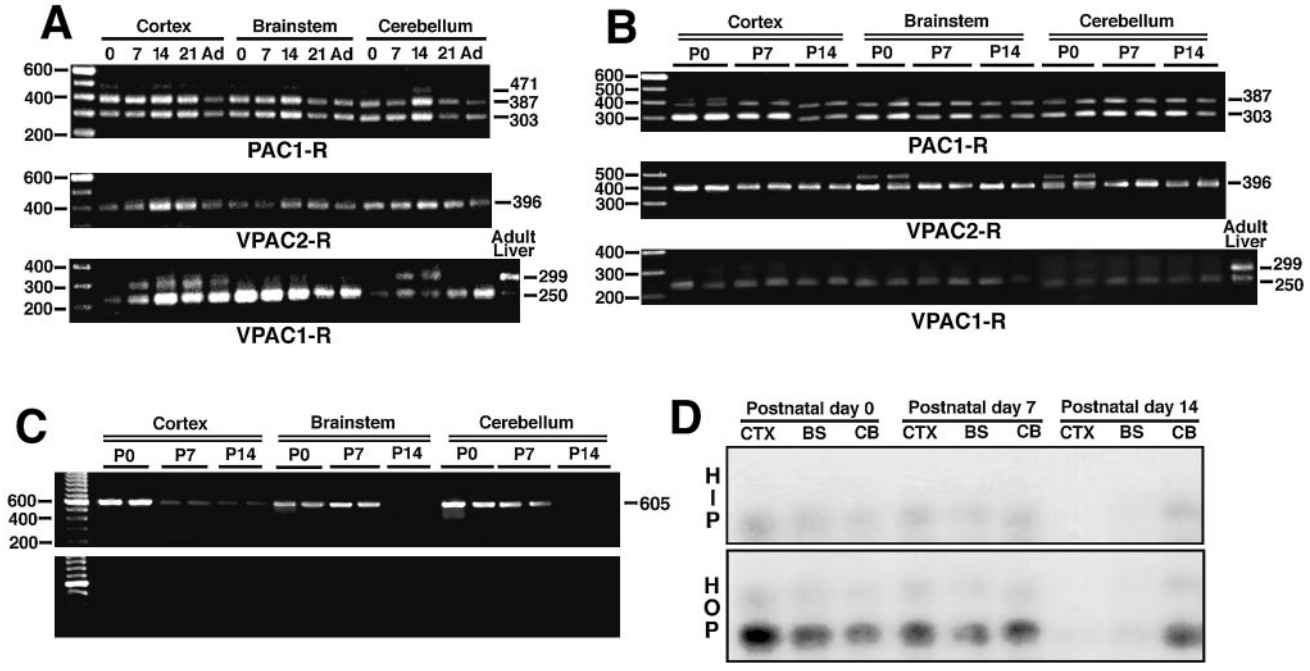

Fig. 1A–D Pituitary adenylate cyclase-activating polypeptide (PACAP) receptor isoforms are differentially regulated in vivo and in primary cultures of rat astrocytes. **A** The expression of PACAP receptor isoforms was examined by RT-PCR analysis in vivo from postnatal day 0 (P0, within 24 h of birth) to adulthood. While the expression of a 250-bp PACAP nonselective receptor (*VPAC1-R*) product predominates, the predicted product size of 299 bp, as demonstrated in adult liver, is only present at low levels. *VPAC2-R* expression is temporally regulated in that expression increases up to a peak at postnatal week 2. PACAP selective receptor (*PAC1-R*) expression is similar to that of VPAC2-R; expression increases to a peak at the 2nd (brainstem and cerebellum) to 3rd (cortex) postnatal week, then declines. In the brainstem and cerebellum, the expression of the short (303 bp) and one-cassette (387 bp) PAC1-R variants are equivalent. In contrast, expression of a one-cassette variant predominates until P14 in the cortex. **B** The expression pattern of PACAP receptor isoforms was examined in primary astrocyte cultures derived from *P0*, *P7*, and *P14* animals maintained in vitro for 6 days. Data are presented from two independently performed cultures and reverse transcriptions. The expression of only the 250-bp VPAC1-R product, which is neither spatially nor temporally regulated, is detected in vitro. VPAC2-R expression declines during cortex development, is not regulated in the brainstem, and parallels in vivo cerebellar expression. In contrast to the enriched expression of the one-cassette PAC1-R variant in the cortex in vivo, cortical astrocytes primarily express the short-splice variant until P14. The expression of the short isoform predominates at P0 and P7. At P14, expression declines and the level of the short and one-cassette isoform expression is equal. **C** PACAP expression in vitro is developmentally downregulated (*top panel*) and requires the presence of serum (*bottom panel*). **D** Sequence-specific hybridization reveals that the one-cassette PAC1-R variant expressed in vitro is predominantly the HOP isoform. Sequence analysis demonstrates the cassette is primarily the HOP2 variant

For chromogenic in situ hybridization of astrocyte cultures, 1 µg cDNA was transcribed in: 40 mM TRIS-HCl (pH 7.9); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM DTT; 100 µg/ml BSA; 1 mM each of ATP, CTP, and GTP; 0.65 mM UTP; 0.35 mM digoxigenin-UTP; 40 U RNase inhibitor (Promega, Madison, Wis.); and 100 U RNA polymerase. After treatment with DNase, the transcription product was purified by precipitation and transcription yield determined by dot blot relative to control RNA of known concentration. Astrocyte cultures were fixed with 2% paraformaldehyde containing 0.1% glutaraldehyde, rinsed twice with 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and twice with 2× SSC (0.30 M sodium chloride, 0.03 M sodium citrate), and acetylated with 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8.0). Cells were then rinsed in 2× SSC, 1× PBS, dehydrated in graded ethanols, and air dried. Cells were hybridized in 50% formamide, 600 mM NaCl, 1× Denhardt's (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 10 mM TRIS-HCl (pH 7.4), 1 mM EDTA, 10 mM DTT, 0.2 µg yeast tRNA, 10% dextran sulfate, and 100 ng/ml cRNA probe at 42°C for 24 h. Cells were washed twice in 2× SSC at room temperature for 20 min and treated with 20 µg/ml RNase A in 2× SSC at 37°C for 20 min. After washing with SSC as before, the cells were blocked with 2× SSC containing 0.5% Triton X-100 and 0.5% normal goat serum at room temperature for 1 h. Cells were washed in TRIS-buffered saline (TBS; 50 mM TRIS-HCl, pH 7.4, 150 mM NaCl) for 10 min and incubated in alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2,000; Boehringer Mannheim, Indianapolis, Ind.) at room temperature for at least 2 h. After washing twice in TBS, for 20 min, and equilibration in alkaline phosphatase buffer (100 mM TRIS pH 8.9, 100 mM NaCl, 5 mM $MgCl_2$), immunoreactive cells were visualized with 0.225 mg/ml nitroblue tetrazolium (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, St. Louis, Mo.).

Results

Cultured rat astrocytes express PACAP and PAC1-R mRNA

The expression pattern of PACAP and PAC1-R mRNA was investigated in vivo and in vitro (Fig. 1). To distinguish the PAC1-R isoforms expressed, the presence or absence of the HIP and/or HOP cassettes was examined using primers adjacent to the cassette insertion site. Since gliogenesis in vivo occurs along a caudal to rostral temporal gradient and astrocytes display regional morphological heterogeneity, in vitro expression was exam-

ined in three brain regions (neocortex, brainstem, and cerebellum) at three developmental stages (P0, P7, and P14).

The expression pattern of PACAP receptor isoforms in vivo is spatially and temporally distinct (Fig. 1A). Overall, the VPAC1-R displays the most significant level of expression. The predicted size of the VPAC1-R product is 299 bp, as demonstrated in adult liver. However, a prominent 250-bp product, also expressed in liver, is detected at all ages and in all regions examined. While the authenticity of the PAC1-R and VPAC2-R products have been verified by sequencing, experiments are required to determine whether the 250-bp product may arise via alternative splicing. The expression of both the 299-bp and 250-bp VPAC1-R products increases throughout neocortical development. In contrast, expression in the brainstem, where only the 250-bp product is detected, declines with development. In the cerebellum, the expression of the 250-bp product increases during cerebellar development, while the 299-bp product is only detected at P7 and P14, the period of maximal granule cell proliferation. The expression of the VPAC2-R is similar in the cortex, brainstem, and cerebellum. VPAC2-R expression increases to reach maximal levels at postnatal week 2; expression then declines. The temporal regulation of PAC1-R is similar to that of VPAC2-R. PAC1-R expression increases up to a peak at P14 in the brainstem and cerebellum and P21 in the cortex; expression then declines. PAC1-R expression is greater in the cortex than the brainstem or cerebellum. Six PAC1-R isoforms are generated by alternative splicing of cassettes containing: (1) neither HIP nor HOP (303 bp); (2) HOP2 alone (384 bp); (3) HOP1 alone (387 bp); (4) HIP alone (387 bp); (5) HIP and HOP2 (468 bp); and (6) HIP and HOP1 (471 bp). Throughout the development of the brainstem and cerebellum, an equivalent level of expression of PAC1-R containing neither the HIP nor HOP insert and of a one-cassette variant is observed. Gel resolution does not permit the determination of whether the cassette inserted is HIP, HOP1, or HOP2. A very low level of PAC1-R containing two cassettes is detected at P14. In contrast, the cortex predominantly expresses the one-cassette isoform at P0 and, as development proceeds, the relative ratio of the short (no cassette) isoform increases to P21, where expression of the two isoforms is equivalent.

The pattern of PACAP receptor expression in cultured astrocytes differs from that observed in vivo (Fig. 1B). Data are presented as duplicate samples from independently performed cultures and reverse transcriptions. The expression of VPAC2-R and PAC1-R predominates in vitro. As observed in vivo, the 250-bp VPAC1-R product predominates in vitro. Although the very low level of expression may preclude definitive conclusions, VPAC1-R expression does not appear to be spatially or temporally regulated in cultured astrocytes. In contrast to an increase in VPAC2-R expression in vivo, expression in cortical astrocytes declines during development. While the expression of VPAC2-R in the brainstem does not appear to be regulated, the temporal expression in the cerebellum mimics that in vivo. VPAC2-R expression in vivo peaks at P14 and in astrocytes derived from P7 animals maintained in vitro for 6 days (equivalent to P13). The predominant form of PAC1-R mRNA in primary cultured rat astrocytes derived from the cortex, brainstem, and cerebellum of P0 and P7 animals contains neither insert; very low expression of a one-cassette variant is also observed. Astrocyte cultures from P14 animals exhibit lower receptor mRNA levels and equivalent expression of the short and one-cassette isoforms. The developmental switch in PAC1-R isoform differs in the intact neocortex, where the one-cassette isoform initially predominates. To determine the cassette type present in cultured astrocytes, sequence-specific hybridization was performed (Fig. 1D). The expression of the HOP cassette in the single-cassette isoform predominates. Although the 471-bp isoform containing two cassettes is not observed on ethidium bromide-stained gels (Fig. 1B), a weak HOP hybridization signal is present. A very low HIP hybridization signal is also detected in the one-cassette variant. Of 12 clones (384–387-bp product) selected for sequence verification, all 12 contained the HOP2 cassette. Although a temporal regulation of PAC1-R isoform expression is observed, expression does not appear to be spatially regulated, since the cassette type expressed in vitro is similar in cortex, brainstem, and cerebellum.

The expression of PACAP in cultured astrocytes was also examined (Fig. 1C). PACAP expression in cortical astrocytes is rapidly downregulated. In contrast, PACAP expression in the brainstem increases from P0 to P7 and then is absent at P14. Cerebellar PACAP expression declines, although not as rapidly as that observed in the cortex. The decline is PACAP expression is not likely due to the presence of neurons in cultures prepared from younger animals. At all ages, more than 98% of the cells are immunoreactive for GFAP, a marker of differentiated astrocytes (Eng et al. 1971). The expression of PACAP in vitro requires serum-derived components, since PACAP mRNA is not detected in cells grown in serum-free media.

To verify that PACAP and PAC1-R mRNA are indeed expressed by astrocytes in vitro, in situ hybridization was performed (Fig. 2). Expression was examined in P0 cortical astrocytes, which display the highest level of PACAP and PAC1-R mRNA by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The specificity of hybridization was verified with sense PACAP (Fig. 2D) and PAC1-R (Fig. 2B) probes. Phase microscopy demonstrates the presence of numerous astrocytes, all of which are completely devoid of hybridization signal. Hybridization with digoxigenin-labeled antisense probes demonstrates that cortical astrocytes express both PACAP (Fig. 2C) and PAC1-R (Fig. 2A) mRNA in vitro. PAC1-R-positive astrocytes exhibit a more intense reaction product than PACAP-positive astrocytes. Due to its immunohistochemical nature, however, nonisotopic in situ hybridization is nonquantitative. Nevertheless, these

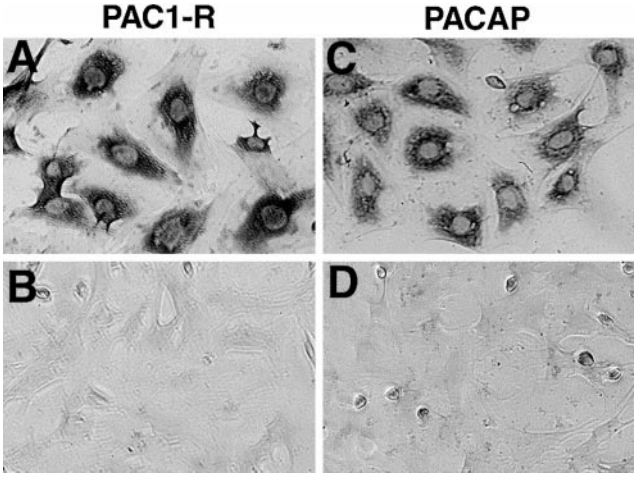

Fig. 2A–D PACAP and PAC1-R mRNA expression in astrocytes in vitro. In situ hybridization was performed with digoxigenin-labeled probes on P0 cortical astrocytes. Both PAC1-R (**A**) and PACAP (**C**) mRNA are expressed in astrocytes in vitro. Hybridization specificity is demonstrated by the lack of hybridization signal with sense PAC1-R (**B**) and PACAP (**D**) probes. ×50

results confirm the RT-PCR data of PACAP and PAC1-R mRNA expression by astrocytes in vitro.

PAC1-R, but not PACAP mRNA is expressed in surgical samples of human glioma

Given the significant expression level detected in primary rat astrocyte cultures, the expression of PAC1-R mRNA was examined in human brain tumors (Fig. 3A). Samples were obtained either at autopsy (normal brain) or as fresh-frozen surgical samples (glial and nonglial tumors). Relative to the expression of PAC1-R mRNA in samples of adult human cortex from individuals who died without neurological complications (Table 1, Fig. 3, lanes 1–3), PAC1-R expression was significantly increased in surgical samples of human glioma (Table 1, Fig. 3, lanes 12–27). Of 17 samples examined, PAC1-R expression was not upregulated in one grade III astrocytoma (lane 12) and one grade IV astrocytoma (lane 19), and not detected in one grade IV astrocytoma (lane 22). PAC1-R expression was particularly enriched in oligodendroglioma (lanes 23–26) and gliosarcoma (lane 28). The upregulation of PAC1-R in glioma was simply not due to the proliferative state, since expression was not detected in rapidly proliferating primary adenocarcinoma of the breast (lane 9), colon (lane 10), or lung (lane 11). The expression of PAC1-R in a central nervous system (CNS) metastasis of breast adenocarcinoma (lane 8) and CNS lymphoma (lanes 6, 7) were comparable with normal brain. PAC1-R expression was also upregulated in neurocytoma (lane 5) and a surgical sample of an epileptic focus (lane 27). A low level of PAC1-R expression was also detected in rodent glioma (lane 29) and gliosarcoma (lane 30) cell lines maintained under standard cell culture conditions.

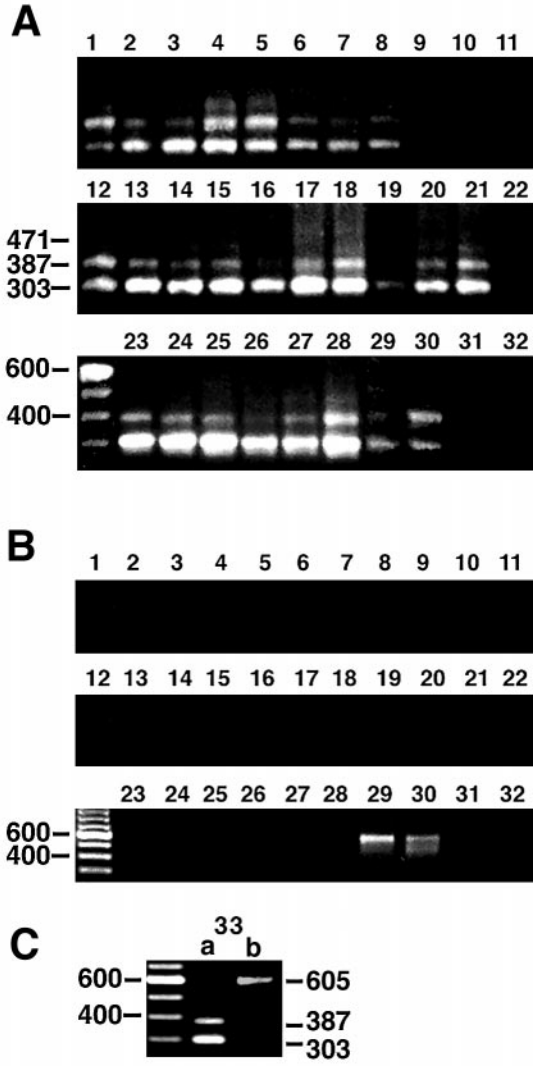

Fig. 3A–C Expression of PAC1-R mRNA is upregulated in glioma. PAC1-R (**A**) and PACAP (**B**) expression was examined by RT-PCR in samples of normal brain (*lanes 1–3*), nonglial tumors (*lanes 5–11*), and glioma (*lanes 12–26, 4, 28*). Samples were obtained either at autopsy (normal brain) or as fresh-frozen surgical samples (glial and nonglial tumors). Patient history and pathology are shown in Table 1. Relative to normal brain, PAC1-R expression is significantly upregulated in all but three glioma samples (*lanes 12–22* in **A**). PAC1-R expression is particularly enriched in oligodendroglioma (*lanes 23–26* in **A**). In sharp contrast to PAC1-R expression, where 10 µl of product (amplified for 28 cycles) was examined, PACAP expression (**B**) is not detected in 20 µl of product (amplified for 35 cycles). However, expression is present in cultured rodent glioma (*lane 29* in **B**) and gliosarcoma (*lane 30* in **B**) cell lines (10-µl product, 28 cycles). To determine whether the lack of PACAP expression may be due to RNA degradation, a recently obtained fresh-frozen grade IV astrocytoma surgical sample was examined by RT-PCR (**C**) and in situ hybridization (Fig. 4). PCR of PAC1-R was performed with 0.5 µl transcription reaction, amplified for 28 cycles, and 5 µl of PCR product electrophoresed (*lane 33a* in **C**), while PACAP was amplified from 1.0 µl transcription reaction, amplified for 35 cycles, and 20 µl of PCR product electrophoresed (*lane 33* in **C**). Although both PACAP and PAC1-R expression are detected, the relative abundance of PACAP in glioma is extremely low, especially given the differences in amplification parameters

Table 1 Patient profile and tissue pathology

Lane[a]	Patients		Pathology/other details
	Age (years)	Sex	
1	76	M	Myocardial infarction
2	72	M	Myocardial infarction
3	56	F	Normal brain
4	51	F	Brain adjacent to GBM
5	40	M	Neurocytoma
6	67	F	CNS lymphoma
7	75	M	CNS lymphoma
8	47	F	CNS metastasis of breast adenocarcinoma
9	46	F	Breast adenocarcinoma
10	66	M	Colon adenocarcinoma
11	62	F	Lung adenocarcinoma
12	25	F	Grade III astrocytoma
13	40	M	Gemisticytic astrocytoma
14	5	M	Cerebellar astrocytoma
15	59	F	Grade IV astrocytoma
16	50	F	Grade IV astrocytoma
17	22	M	Grade IV astrocytoma
18	23	F	Grade IV astrocytoma
19	13	F	Grade IV astrocytoma
20	64	F	Grade IV astrocytoma
21	50	F	Grade IV astrocytoma
22	58	F	Grade IV astrocytoma
23	35	M	Anaplastic oligodendroglioma
24	22	F	Mixed oligodendroglioma
25	36	F	Oligodendroglioma
26	54	F	Oligodendroglioma
27	51	F	Epileptic focus
28	66	F	Gliosarcoma
29	n.a.	n.a.	Cultured CNS-1 glioma cell line
30	n.a.	n.a.	Cultured 9L gliosarcoma cell line
31	n.a.	n.a.	Control transcription lacking RNA
32	n.a.	n.a.	Control PCR lacking cDNA
33	66	F	Grade IV astrocytoma

[a] Lanes in Fig. 2

Surprisingly, PACAP expression was not detected in any of the samples tested, including normal brain (Fig. 3B). The lack of a detectable amplification product was unlikely to be due to RNA degradation, since PAC1-R amplified from these samples generated products of the predicted size. In addition, the reaction cocktail used for the human samples successfully amplified a product of the appropriate size from cDNA of glioma cell lines (lanes 29, 30). The RNA from many of the tumor specimens had been stored for protracted periods (more than 4 years). Even when properly stored, human RNA is notoriously susceptible to degradation. While there is no reason to suspect that PACAP RNA is more labile than that of the PAC1-R, additional experiments were undertaken to examine expression in a recently obtained fresh-frozen surgical specimen (Fig. 3C). The conditions that failed to amplify a PACAP product from stored specimens (0.5 µl transcription reaction amplified 28 cycles, with 10 µl PCR reaction electrophoresed) similarly failed to amplify a product from the freshly obtained specimen. A very weak signal was ultimately obtained after increasing the target cDNA concentration (1.0 µl of transcription reaction), the rounds of amplification (35 cycles), and the amount of PCR reaction electrophoresed (20 µl; Fig. 3C, lane 33b). In contrast, PAC1-R was detected when as little as 5 µl of PCR product (from 0.5 µl transcription reaction amplified 28 cycles) was examined (Fig. 3C, lane 33a).

To more closely examine PACAP and PAC1-R mRNA expression in glioma, in situ hybridization with ^{35}S-labeled cRNA probes was performed (Fig. 4). Following hybridization, sections were counterstained with cresyl violet to reveal the histopathological details of cellular profiles (Fig. 4C,E,H,J). A portion of the surgical sample utilized for PCR analysis (Fig. 3C) was examined to clarify the reason for the low level of PACAP expression. Examination of the tumor specimen at low magnification (Fig. 4A) confirmed the results observed in PCR analyses. With the exception of the intense nonspecific hybridization signal present at the edge of the tumor, which was also observed in the sense control, a distinct PACAP signal was not readily detectable. Upon closer examination (Fig. 4B,D), the extremely limited expression of PACAP in glioma was clearly evident. The distribution of PACAP mRNA, which was present in very few cells, does not appear to correlate with a particular cellular profile (Fig. 4C,E). In sharp contrast to the sparse distribution of PACAP, PAC1-R hybridization signal was evident even when observed at low magnification (Fig. 4F). Examination at higher magnification (Fig.

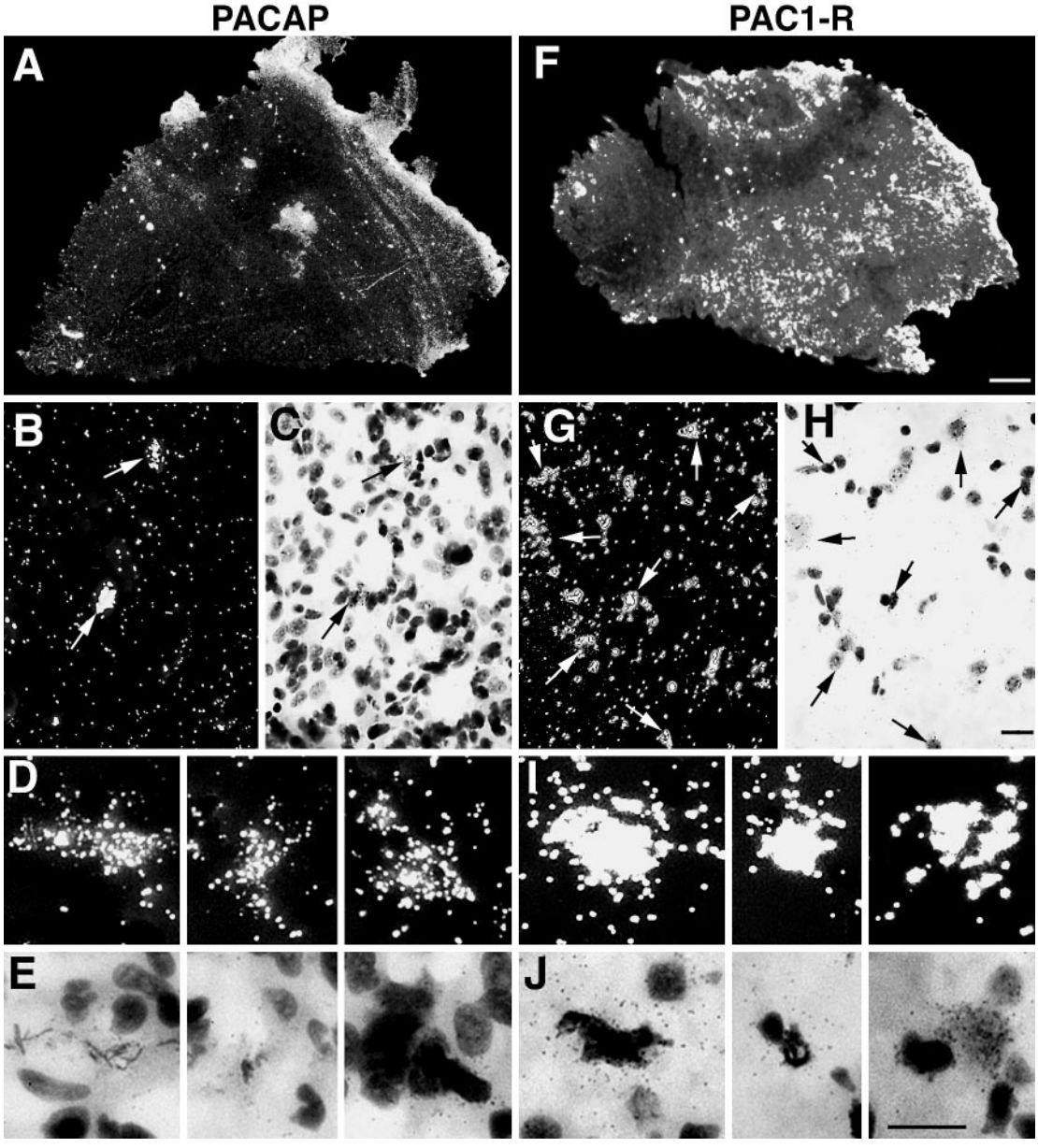

Fig. 4A–J Spatial distribution of PACAP and PAC1-R mRNA in glioma. The localization of PACAP (**A–E**) and PAC1-R (**F–J**) mRNA was determined by in situ hybridization with ^{35}S-labeled cRNA probes in the same surgical glioma sample used for RT-PCR analysis (Fig. 3**C**). To reveal cellular profiles, sections were counterstained with cresyl violet (**C,E,H,J**) following hybridization. **A** With the exception of the nonspecific signal present at the tissue's edge, a hybridization signal for PACAP is barely detectable when examined at low magnification. **B–D** Upon closer examination, the limited number of cells that express PACAP (*arrows*), above background hybridization, is revealed. **F** In sharp contrast to the level of PACAP expression, an intense PAC1-R hybridization signal is detectable even when observed at low magnification. **G–I** Examination at higher magnification demonstrates the diffuse distribution of PAC1-R throughout the specimen. *Bars* **A,F** 1 mm; **B–E,G–J** 20 µm

4G,I) revealed the diffuse distribution of PAC1-R mRNA throughout the glioma specimen. Similar to PACAP expression, PAC1-R mRNA was present in cells containing condensed heterochromatin, as was well as uncondensed heterochromatin (Fig. 4H,J). Since glioma surgical samples contain a mixture of tumor and reactive, nontumorous glial cells, the regulation of PACAP and PAC1-R expression during reactive gliosis was examined.

Neither PACAP nor PAC1-R mRNA is upregulated consequent to intracranial injury

In situ hybridization was performed with ^{35}S-labeled cRNA probes 6 h, 12 h, 24 h, 4 days, and 7 days after a stab wound was made to the adult rat brain. Following hybridization, sections were counterstained with cresyl violet to demonstrate the distribution of cells and the site

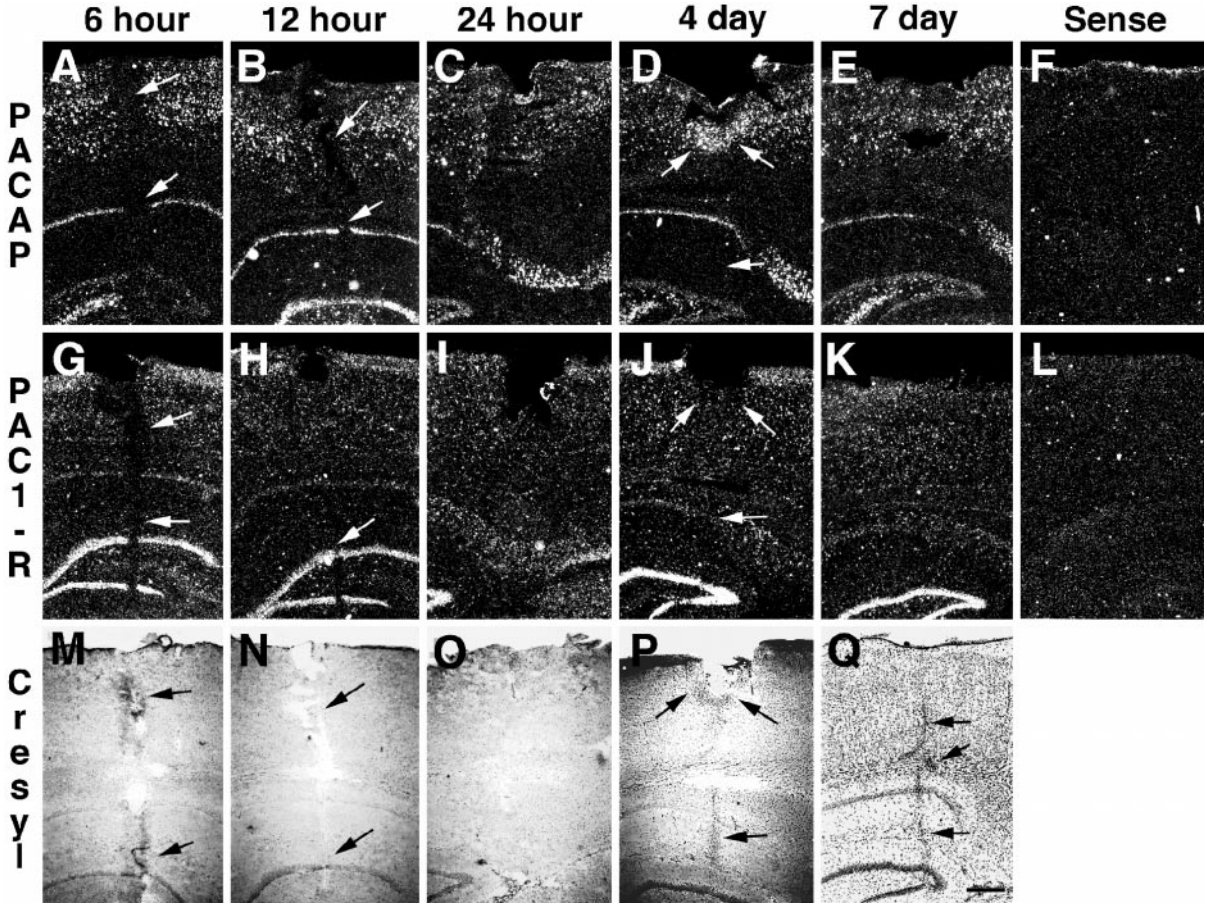

Fig. 5A–Q PACAP and PAC1-R expression in response to intracranial injury. The expression of PACAP (**A–F**) and PAC1-R (**G–L**) expression was examined by in situ hybridization with ^{35}S-labeled cRNA probes following a penetrating stab wound to the mature rat brain. Hybridization specificity is demonstrated by the lack of detectable signal at 7 days postinjury with sense probes (**F,L**). Following hybridization, sections were counterstained with cresyl violet (**M–Q**). Within the first 12 h after injury, PACAP (**A**,**B**) and PAC1-R (**G,H**) hybridization signal is absent along the lesion tract (*arrows*) in a region devoid of cells consequent to injury (**M**,N). Expression in the injury penumbra is no greater than that present in the contralateral uninjured brain. At 24 h after injury, as the extent of tissue damage is diminished, PACAP (**C**) and PAC1-R (**I**) expression is now present at the lesion site (**O**). Four days after injury, while PAC1-R expression returns to control levels (**J**), PACAP expression appears upregulated (*upper arrows* in **D**). However, this expression pattern is largely due to structural tissue damage (**P**) and not a result of hyperplasia (*lower arrow* in **D,P**). At 7 days after injury PACAP (**E**) and PAC1-R (**K**) expression have returned to control levels even in the presence of significant hypertrophy and hyperplasia (**Q**) (cf. Fig. 6). *Bar* 500 μm

of the lesion (Fig. 5M–Q). To determine hybridization specificity, near-adjacent sections were hybridized with sense probes, which produced no detectable hybridization signal (Fig. 5F,L). The expression of neither PACAP nor PAC1-R mRNA was upregulated in response to the hypertrophy and hyperplasia associated with reactive gliosis (Fig. 5).

Increased PACAP mRNA expression in response to a penetrating stab wound was not detected at any postinjury time-point examined (Fig. 5A–F). At short recovery time-points (6 h and 12 h), the absence of PACAP expression at the lesion site (Fig. 5A,B) occurred in a region devoid of cells (Fig. 5M,N). PACAP expression in the area immediately adjacent to the lesion, which contains the neuronal soma whose axons were severed, was no greater than that observed in the contralateral uninjured cortex. At 24 h postinjury, a low level of PACAP expression was detected along the lesion tract (Fig. 5C). Four days after injury, increased PACAP expression was observed in the cortex (Fig. 5D); however, this region corresponded to an area of tissue damage (Fig. 5P). This PACAP expression pattern was not observed in the other hyperplastic region of this tissue section or in another animal examined at 4 days postinjury (see Fig. 6). By 7 days after injury, the level of PACAP expression had reached control levels (Fig. 5E), yet the normal cortical laminar expression pattern was not attained until 14 days after injury, the latest time-point examined (data not shown).

In contrast to the increased expression in glioma, PAC1-R expression was not upregulated during reactive gliosis (Fig. 5G–L). At 6 h after injury, PAC1-R hybridization signal was lacking in both the cortex and dentate gyrus (Fig. 5G). By 12 h postinjury, PAC1-R expression in the cortex was increased, while expression in the dentate gyrus was still diminished (Fig. 5H). With the exception of the superficial cortical damage, the normal

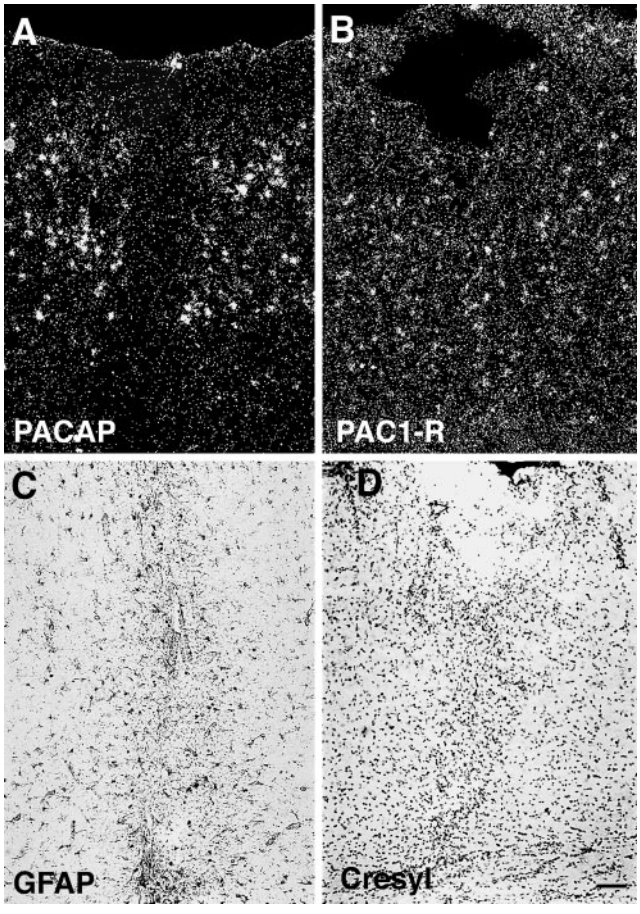

Fig. 6A–D Neither PACAP nor PAC1-R expression is correlated with the hypertrophy and hyperplasia induced by intracranial injury. To more closely examine PACAP and PAC1-R expression in response to injury, combined in situ hybridization and immunohistochemistry was performed 4 days after injury. Following hybridization for PACAP (**A**), the intermediate filament glial fibrillary acidic protein (*GFAP*) was immunohistochemically localized (**C**). PACAP expression is excluded from the region of hypertrophied GFAP-positive reactive astrocytes. Similarly, PAC1-R expression (**B**) does not correspond to the region of hyperplasia revealed by cresyl violet staining (**D**). *Bar* 100 μm

level and spatial distribution of PAC1-R expression following injury was attained 24 h after injury (Fig. 5I).

To more closely examine PACAP and PAC1-R expression, combined in situ hybridization and immunohistochemistry was performed at 4 days after injury, the peak of the gliotic response to injury (Fig. 6). Following hybridization with PACAP (Fig. 6A), immunohistochemical localization of the intermediate filament protein GFAP was performed (Fig. 6C). Although GFAP is normally expressed by glial cells in the adult brain, the hallmark of the gliotic response is the significant upregulation of GFAP at the injury site (Eng and Ghirnikar 1994; Bignami and Dahl 1976). While GFAP-positive reactive astrocytes are primarily restricted to the lesion tract (Fig. 6C), cell bodies displaying PACAP hybridization signal are located immediately adjacent to the injury site (Fig. 6A). Preliminary analyses of the number of cellular profiles displaying PACAP mRNA and the level of PACAP expressed per cell (based on silver grain counts) indicated that PACAP was not upregulated following a penetrating stab wound. Similarly, PAC1-R mRNA (Fig. 6B) was not present in GFAP-immunoreactive cells (data not shown) or in regions of increased cellular densities at the lesion site (Fig. 6D). Thus, neither PACAP nor PAC1-R expression was upregulated coincident with the hypertrophy and hyperplasia consequent to a stab wound to the rat brain.

Discussion

Astrocytes, the main interstitial cells in the brain, serve diverse roles in the development and maintenance of neuronal function (reviewed by Barres, 1991). While binding sites for both VIP (Gozes et al. 1991) and PACAP (Tatsuno et al. 1991) are present on astrocytes, the second-messenger signaling cascade initiated, and thus the functional outcome, is regulated based on the PACAP receptor bound. VPAC1-R and VPAC2-R are coupled solely to adenylate cyclase, while PAC1-R coupling to adenylate cyclase and PLC differs when bound to PACAP-38 and PACAP-27 (Spengler et al. 1993). The molecular complexity of receptor activation is further complicated by the presence of alternative splicing of the PAC1-R. Isoforms differing within the N-terminal extracellular domain (Pantaloni et al. 1996), transmembrane domains (Chatterjee et al. 1996), and five-splice variants differing in the third intracellular domain, the region involved in receptor/G protein-coupling (Spengler et al. 1993), exist.

As previously reported (Ashur-Fabian et al. 1997) and confirmed here, cultured rat astrocytes primarily express the VPAC1-R and the PAC1-R splice variant containing the HOP2 cassette. In addition, Grimaldi and Cavallaro (1999) recently demonstrated the presence of PAC1-R, VPAC1-R, and VPAC2-R in astrocytes. The present study expands the characterization of PAC1-R expression to demonstrate that PAC1-R isoform expression in cultured astrocytes is temporally regulated. In contrast to neuronal tissue that primarily expresses the PAC1-R splice variant containing one cassette, astrocytes derived from P0 and P7 brain (cortex, brainstem, and cerebellum) predominantly express the short variant containing neither HIP nor HOP cassette. At P14, however, the expression of both variants declines and reaches equivalent levels. This developmental switch in isoform expression is correlated with the significant downregulation in PACAP expression from P7 to P14. Sequence-specific hybridization suggests that astrocytes also express a HIPHOP variant. The expression of the HOP2 variant in astrocytes is unexpected, since the HOP1 isoform predominates in other cultured cells (Rawlings et al. 1995). Interestingly, it has been suggested that the neurotrophic properties of both VIP and PACAP may be mediated through the PAC1HOP2-R (Ashur-Fabian et al. 1997). Although the PAC1-R containing HOP1 or HOP2 can activate adenylate cyclase and PLC (Spengler et al.

1993), the functional significance of the short variant lacking both HIP and HOP is yet to be elucidated. In addition, studies need to be undertaken to determine whether the short variant expressed by astrocytes also lacks the 21-amino acid insert (Pantaloni et al. 1996) in the amino-terminal receptor domain involved in ligand binding (Cao et al. 1995; Couvineau et al. 1995), and whether it is similarly temporally regulated.

Although the physiological functions of PACAP in astrocytes have not yet been clearly established, studies suggest a role in the modulation of neurotrophic factor production and proliferation (Gottschall et al. 1994; Tatsuno et al. 1996). Since PACAP activates several intracellular signals, including cAMP, PLC, and intracellular calcium, PACAP may either directly or indirectly affect astrocyte proliferation. Recent studies suggest that PACAP can increase mitogen-activated protein kinase (MAPK) via a PKA- and PKC-independent mechanism (Moroo et al. 1998; Tatsuno et al. 1996). Hence, PACAP may directly stimulate the proliferation of cultured rat astrocytes as a mitogen. Alternatively, PACAP may mediate proliferation via intracellular signaling cascades. PACAP stimulates sympathetic neuroblast proliferation, which express the HOP PAC1-R isoform, by activation of the phosphoinositol signaling pathway and cAMP accumulation. In contrast, PACAP inhibits cortical precursor proliferation, which express the short PAC1-R lacking HIP and HOP cassettes, via increased cAMP. Similarly, while VIP and PACAP stimulate astrocyte proliferation in vitro (Brenneman et al. 1990; Tatsuno et al. 1996) and in vivo (Zupan et al. 1998), an antiproliferative effect is exerted in glial and neural tumor cell lines (Suzuki et al. 1994; Vertongen et al. 1996a). The opposing mitogenic activity in glial cells may be regulated by the differential expression of PACAP receptor isoforms as observed in neural precursors (Lu et al. 1998). The antiproliferative effect of VIP and PACAP in the human T98G glioblastoma cell line occurs via the VPAC2-R (Vertongen et al. 1996a). Perhaps the proliferative activity of PACAP in primary cultured rat astrocytes occurs via interaction with the PAC1-R. Astrocytes primarily express the short (noninsert) PAC1-R, which was demonstrated to inhibit cortical precursor proliferation. However, since the proliferative effect of PACAP and VIP appears to be cell type-specific, the activation of a distinct signal transduction cascade in a different cellular context may be associated with enhanced proliferation.

Given the role for PACAP in stimulating astrocyte proliferation, the presence of PACAP and PACAP receptors in human brain tumors of glial origin is not unexpected. PACAP has been shown to stimulate adenylate cyclase activity in human glioma, but specific PACAP-27 binding was detected in only 16 of 23 tumors examined (Robberecht et al. 1994). Further RT-PCR analysis demonstrated PACAP mRNA in 13 and PAC1-R mRNA in 15 of 16 gliomas studied (Vertongen et al. 1995). These reports did not, however, analyze the level of expression relative to normal human brain or represent negative controls (RT lacking enzyme and PCR lacking cDNA). The present study confirms and extends the previous reports on PAC1-R expression in glioma. PAC1-R mRNA is detected in 16 of 17 glioma samples and upregulated relative to control in 14. Expression is most significantly upregulated in oligodendroglioma. PAC1-R expression is also upregulated in neuroblastoma (Vertongen et al. 1996b,1997; the present report). The upregulation of PAC1-R expression is not simply due to the cells' mitotic state, since expression is not detected in primary adenocarcinoma of the breast, colon, and lung, and not upregulated in CNS lymphoma. In contrast to previous reports, PACAP mRNA expression was detected in only one sample. The PCR primers utilized in the present study correspond to rat PACAP and PAC1-R sequences and differ from human PACAP (Hosoya et al. 1992) and PAC1-R (Ogi et al. 1993) at three residues. However, amplification with primers specific to human PACAP similarly failed to produce a detectable signal (unpublished observation). Although PCR is a powerful technique capable of detecting low-level gene expression, the limited spatial distribution of PACAP mRNA demonstrated here by in situ hybridization partially explains the difficulties of detecting PACAP by PCR. The disparity in PACAP expression reported here compared with previous reports is, at present, unresolved. Additionally, the cellular source (tumor or reactive astrocytes) of the intense, diffuse PAC1-R mRNA in glioma is yet to be identified.

Injury to the CNS elicits a complex cellular response, referred to as reactive gliosis. Following injury, growth factors and mitogens are rapidly released to attenuate the extent of neuronal damage (Ridet et al. 1997). PACAP expression is upregulated in response to nerve injury (Larsen et al. 1997; Moller et al. 1997; Zhang et al. 1996), transient focal cerebral ischemia (Banks et al. 1996; Gillardon et al. 1998), and traumatic brain injury (Skoglösa et al. 1999). In contrast to these studies, this report demonstrates that neither PACAP nor PAC1-R mRNA expression is upregulated in response to the glial hypertrophy and hyperplasia associated with a penetrating stab wound to the mature rat brain. Altered PACAP or PAC1-R mRNA expression in local circuit neurons in the lesion penumbra, callosal neurons in the contralateral cortex, and thalamic afferents was not observed. Since PAC1-R expression is not always upregulated following injury (Skoglösa et al. 1999; Zhou et al. 1999), PACAP may exerts its activity through binding VIP receptors. While the present study only investigated the expression at the mRNA level, PACAP release may be increased in response to injury even in the absence of increased mRNA as reported for other neuropeptides (Ma and Bisby 1999).

The lack of PACAP response to injury reported here in comparison with that reported by Skoglösa and colleagues (1999) is probably due to differences in the model of traumatic brain injury investigated. In the compression contusion model, a 21-g free-falling weight is dropped from 35 cm onto a 4.5-mm piston resting on the exposed dura. In this model, PACAP expression in the

cortex steadily increased to reach maximal levels at 3 days, the latest time-point examined. The cortical upregulation was most pronounced circumferentially at the piston's edge. In the present report, a small (27-gauge) needle was inserted through the cortex, hippocampal formation, and underlying thalamus. While the stab physically severs rather than compresses axons, it probably does not induce as severe an inflammatory response as the contusion model. Since PACAP expression is associated with proinflammatory responses (Zhang et al. 1998), the upregulation of PACAP in the compression contusion model may reflect an inflammatory response to the severe trauma. In both models, serum-derived components are released as a result of vascular damage. In the stab model, which penetrates the lateral ventricles, cerebrospinal fluid-derived factors could attenuate PACAP expression. The differential regulation of PACAP in the compression contusion and penetrating injury models suggests a heterogeneity in nervous system response to injury.

While PACAP exhibits neurotrophic activity in vitro, the physiologic significance of increased PACAP expression in response to injury in vivo is yet to be determined. If PACAP is upregulated following injury to protect neurons from apoptosis (Ciani et al. 1999; Tanaka et al. 1997; Villalba et al. 1997), one would expect both injury models to be associated with increased PACAP expression. Inasmuch as VIP exerts neurotrophic actions (Waschek 1995) and astrocytes express VIP receptors (Grimaldi and Cavallaro 1999; present report), further studies need to be undertaken to investigate VIP and VIP receptor expression following a penetrating stab wound, to ascertain whether VIP may serve as the physiologically relevant neurotrophic factor during reactive gliosis.

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