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Novel Splice Variants of Type I Pituitary Adenylate Cyclase-Activating Polypeptide Receptor in Frog Exhibit Altered Adenylate Cyclase Stimulation and Differential Relative Abundance

DAVID ALEXANDRE, HUBERT VAUDRY, LUCA GRUMOLATO, VALÉRIE TURQUIER, ALAIN FOURNIER, SYLVIE JÉGOU, AND YOUSSEF ANOUAR

European Institute for Peptide Research (IFRMP 23), Laboratory of Cellular and Molecular Neuroendocrinology, Institut National de la Santé et de la Recherche Médicale, U-413, Unité Affiliée Centre National de la Recherche Scientifique, University of Rouen (D.A., H.V., L.G., V.T., S.J., Y.A.), 76821 Mont Saint Aignan, France; Institut National de la Recherche Scientifique/Institut Armand Frappier, University of Quebec (A.F.), H9R 1GG Montréal, Canada

Pituitary adenylate cyclase-activating polypeptide (PACAP) exerts its various effects through activation of two types of G protein-coupled receptors, a receptor with high affinity for PACAP named PAC1-R and two receptors exhibiting similar affinity for both PACAP and vasoactive intestinal polypeptide named VPAC1-R and VPAC2-R. Here, we report the characterization of PAC1-R and novel splice variants in the frog *Rana ridibunda*. The frog PAC1-R has 78% homology with human PAC1-R and is highly expressed in the central nervous system. Two splice variants of the frog receptor that display additional amino acid cassettes in the third intracellular loop were characterized. PAC1-R25 carries a 25-amino acid insertion that matches the hop cassette of the mammalian receptor, whereas PAC1-R41 carries a cassette with no homology to any mammalian PAC1-R variant. A third splice variant of PAC1-R, exhibiting a completely different intracellular C-terminal domain, named PAC1-Rmc has also been identified. Determination of cAMP formation in cells transfected with the cloned receptors showed that PACAP activated PAC1-R, PAC1-R25, and PAC1-R41 with similar potency. In contrast, PACAP failed to stimulate adenylate cyclase in cells transfected with PAC1-Rmc. Fusion of PAC1-R or PAC1-Rmc with the green fluorescent protein revealed that both receptors are expressed and targeted to the plasma membrane in transfected cells. The different PAC1-R variants are highly expressed in the frog brain and spinal cord and to a lesser extent in peripheral tissues, where only certain isoforms could be detected. The present data indicate that in frog, PACAP may act through different PAC1-R splice variants that differ in their G_s protein coupling and their abundance in various tissues. (*Endocrinology* 143: 2680–2692, 2002)

PITUITARY ADENYLATE cyclase-activating polypeptide (PACAP) has been initially isolated from the ovine hypothalamus, where it occurs in two molecular forms of 38 and 27 amino acids, named PACAP38 and PACAP27, respectively (1, 2). PACAP shares high sequence homology with members of the vasoactive intestinal polypeptide (VIP)/secretin/GHRH peptide family. PACAP exerts a wide variety of biological effects throughout the body, particularly in the endocrine and nervous systems, where the peptide acts as a neurohormone, a neurotransmitter/neuromodulator, and a trophic factor (3).

Shortly after the discovery of the peptide, specific PACAP receptors were identified (4, 5) and subsequently cloned. Two types of PACAP receptors have been characterized that belong to the superfamily of G protein-coupled, seven-transmembrane domain (TMD)-containing receptors. Type I receptors, or PAC1-R, exhibit a high affinity for PACAP and a much lower affinity for VIP (6). Type II receptors, in contrast, recognize both PACAP and VIP with high affinity and include two receptor subtypes, named VPAC1-R and VPAC2-R (7, 8). All three receptors are positively coupled to adenylate cyclase (AC), whereas only PAC1-R is coupled to phospholipase C (PLC). Alternative splicing of the gene encoding PAC1-R generates different variants with distinct pharmacological properties. Thus, four different splicing events in the region encoding the third intracellular loop give rise to the molecular variants hip, hop1, hop2, and hip-hop1 of PAC1-R (6). These splice variants exhibit differences in the efficacy of AC or PLC activation by PACAP (6, 9). Alternative splicing also generates PAC1-R variants in the extracellular N-terminal domain that lack 21 or 57 amino acids or, on the contrary, that exhibit an insertion of 24 amino acids and show increased potency of PACAP27 to stimulate PLC activity (10), increased affinity for VIP (11), and altered PACAP binding and signaling transduction pathways, respectively (12). Finally, a variant with discrete amino acid modifications in TMD II and IV is no longer coupled to AC or PLC, but mediates stimulation of calcium influx through L-type channels (13).

The neuropeptide PACAP and its receptors have been also extensively studied in an amphibian species model, the European green frog *Rana ridibunda* (see Ref. 14 for review). Soon after the discovery of PACAP in mammals, the sequence of frog PACAP38 has been characterized, and the peptide has been shown to stimulate AC in the pituitary (15).

Abbreviations: AC, Adenylate cyclase; GFP, green fluorescent protein; PACAP, pituitary adenylate cyclase-activating polypeptide; PLC, phospholipase C; poly(A^+), polyadenlated; SSC, standard saline citrate; TMD, transmembrane domain; VIP, vasoactive intestinal polypeptide.

Further studies revealed that in frog, PACAP increases the cytosolic calcium concentration in different pituitary cells (16) and stimulates the release of several pituitary hormones (17). The hypophysiotropic effect of PACAP is supported by the abundance of PACAP-encoding mRNA and PACAP immunoreactivity in the frog hypothalamus as well as the existence of a dense network of PACAP-containing fibers in the median eminence (18, 19). We recently cloned a frog PACAP/VIP receptor that displays a broad distribution in the central nervous system and peripheral tissues (20). Concurrently, a PAC1-R homolog has been recently characterized in *Xenopus laevis*, and this receptor has been found to be highly expressed in the brain (21). However, although several PAC1-R variants have been found in mammals, no such variants have been described in submammalian species. In the present study we characterized PAC1-R in the frog *R. ridibunda* and identified novel splice variants for this receptor that exhibit differential coupling to adenylate cyclase. The distribution of the mRNAs encoding PAC1-R and its variants in the brain and peripheral tissues of the frog *R. ridibunda* has also been determined.

Materials and Methods

Animals

Adult male frogs (*R. ridibunda*) of about 30 g body weight were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). The animals were maintained in glass tanks with free access to water at constant temperature (8 ± 0.5 C) under an established photoperiod of light (lights on from 0600–1800 h) for at least 1 wk before use. Animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

Cloning and sequencing

PCR was performed in 50-μl volume containing 500 ng reverse transcribed RNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Promega Corp., Charbonnières, France), and 50 pmol sense and antisense primers in the buffer (pH 9.0) supplied with the enzyme for 30 cycles (1 min at 94 C, 1 min at 44 C, and 1 min at 72 C) in a Robocycler Gradient 40 (Stratagene, La Jolla, CA). To amplify a frog PAC1-R cDNA fragment, two successive PCR were performed on reverse transcribed RNA isolated from pituitary neurointermediate lobe tissue, using oligonucleotides deduced from conserved regions of PACAP receptors in mammals. In the first PCR amplification, the sense primer 3P2 (5′-TGGYTITTYATYGARGGIYTITAYCT-3′; sequence in the third TMD) and the antisense primer HOPR (5′-GCICGYTGIGGYTTRCARTARCA-3′; sequence in the hop cassette of PAC1-R) were employed. The products obtained were used in a second PCR performed with the 3P2 primer described above and the antisense primer 45R (5′-TCDATIGTRTC-CCARCANCC-3′; sequence in the second extracellular loop) to amplify a 164-bp cDNA fragment encoding a portion of the frog PAC1-R sequence. Based on the sequence of this cDNA fragment, a homologous 44-mer oligonucleotide SFPVR1 (5′-GTGAAGTCGTAGCACAGC-CCATATAGTGACACATATAAGTGGGG-3′) was synthesized and used to screen a frog brain cDNA library (22) to isolate a full-length cDNA encoding the frog PAC1-R.

Approximately 750,000 recombinant phages of the library were screened with the ^{32}P-labeled SFPVR1 oligonucleotide in 50% formamide, 4× SSC (1× SSC = 0.15 M NaCl and 15 mM sodium citrate), 1× Denhardt's (0.1% BSA, 0.1% Ficoll, and 0.1% polyvinylpyrrolidone), 50 mM phosphate buffer (pH 6.5), 0.1% SDS, 200 μg/ml salmon sperm DNA, and 100 μg/ml tRNA at 42 C. Filters were washed twice at 42 C with 0.2× SSC/0.1% SDS for 15 min. Positive clones were isolated by plaque purification, zapped in pBluescript SK (Stratagene), and sequenced on both strands on a Li-Cor 4000L DNA sequencer (ScienceTec, Les Ulis, France) using fluorescent T7 and T3 primers (MWG-Biotech, Courtaboeuf, France) and the Thermosequenase kit (Amersham Pharmacia Biotech, Les Ulis, France). DNA sequences were analyzed using DNASIS V2.1 software (Hitachi, Olivet, France).

The frog PAC1-R variants were amplified by a primary PCR on reverse transcribed RNA isolated from the brain, testis, spleen, or distal lobe of the pituitary, using the primers S3F (5′-GAAATGAACAACAAT-GTTGCC-3′; sequence in the second extracellular loop) and S2R (5′-AGTGACATTTGACTGTTCTC-3′; sequence in the 3′-noncoding region), followed by a secondary PCR with the S2R primer combined to the S2F primer (5′-TGCAGTCTCCAGACATAGG-3′; sequence in the third intracellular loop). The PCR products were subcloned into the pGEMT vector and sequenced on both strands.

Construction of expression plasmids

The full-length frog PAC1-R cDNA in pBluescript SK was cut by *Sac*I, polished with T4 DNA polymerase, and digested by *Xho*I. The insert was subcloned into the *Eco*RV/*Xho*I sites of the pcDNA1 expression vector (Invitrogen, Leek, The Netherlands). The resulting PAC1-R-pcDNA1 plasmid was used to make expression vectors for the PAC1-R splice variants. The PAC1-R25 and PAC1-R41 cDNAs subcloned in the pGEMT vector were digested with *Pst*I/*Acc*III enzymes, and the digested inserts were introduced in the *Pst*I/*Acc*III-digested PAC1-R-pcDNA1 vector described above. The splice variant PAC1-Rmc was digested with *Acc*III/*Hin*cII enzymes, and the digested insert was ligated to the *Acc*III/*Hin*cII-cut PAC1-R-pcDNA1 vector.

To study the expression of PAC1-R and PAC1-Rmc in transfected cells, fusion proteins with the green fluorescent protein (GFP) were constructed. To this end, cDNA fragments encoding the C-terminal region of these two receptor variants were amplified using a common sense S3F primer and a specific antisense primer for each receptor, in which the stop codon had been mutated and replaced by an *Xho*I restriction site. These primers correspond to GFP1R (5′-TGCACTCGAGT-GTCACCAGGTTCTCCGC-3′) in the case of PAC1-R and GFP1Rmc (5′-TGCACTCGAGGGCAGTTTGGGAACTCAT-3′) in the case of PAC1Rmc. The *Xho*I site introduced (*underlined* sequence) is in-frame with the GFP sequence. The PCR fragments generated were digested by *Xho*I at the C terminus and by an internal *Hin*dIII site at the N terminus, and subcloned into the GFP-encoding vector phrGFP-C (Stratagene). The sequence encoding the N-terminal part to the *Hin*dIII site of these receptors was cut from PAC1-R-pcDNA1 and spliced in the phrGFP-C vector constructs to produce the fusion proteins of PAC1-R or PAC1-Rmc and GFP. All constructs were confirmed by sequencing.

Cell transfection

For pharmacological studies, expression plasmids were transfected by electroporation into LLC-PK1 cells, a pig kidney proximal tubular epithelial cell line (a gift from Dr. L. Journot, Centre National de la Recherche Scientifique-UPR 9023, Montpellier, France). Cells were grown in DMEM (Sigma-Aldrich Corp., Saint-Quentin Fallavier, France) containing 10% FCS (Bioproducts, Gagny, France) at 37 C in 5% CO_2. About 2.5×10^6 cells in 500 μl culture medium were transfected with 20 μg plasmid DNA at 1050 μF and 210 V using the EasyJect One electroporation system (EquiBio, Angleur, Belgium).

For the analysis of receptor-GFP fusions, plasmids were transfected into LLC-PK1, CHO-K1, and PC12 cells (ECACC, Salisbury, UK) using the Lipofectamine reagent (Life Technologies, Inc., Cergy-Pontoise, France). Approximately 5×10^5 cells/well were plated on glass coverslips (diameter, 25 mm) in six-well plates and allowed to grow for 24 h before transfection. Cells were washed twice with 1 ml DMEM and transfected with 2 μg GFP fusion plasmids using 8 μl Lipofectamine in 1 ml DMEM during 3.5 h. The medium was then replaced by 2 ml DMEM supplemented with 10% FCS.

Peptides

Frog PACAP38 was synthesized by the solid phase methodology as previously described (18). Frog/chicken VIP (23) was purchased from Peninsula Laboratories, Inc. (Merseyside, UK). Peptides were dissolved in the incubation medium immediately before the experiment.

cAMP measurement

Transfected LLC-PK1 cells were plated in 24-well culture dishes at a density of 10^5 cells/well and cultured for 72 h. Cells were preincubated for 15 min at 37 C in 500 μl Krebs buffer (11.8 mM NaCl, 0.25 mM $CaCl_2$, 0.12 mM KH_2PO_4, 0.12 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.47 mM KCl, 1 mM HEPES, and 0.2 g/liter glucose, pH 7.3) containing 1 mg/ml BSA and 0.2 mM 3-isobutyl-1-methylxanthine (Sigma). Peptides were added at the appropriate concentrations, and the cells were incubated for an additional 30 min at 37 C. The medium was removed, and intracellular cAMP was extracted by ice-cold ethanol. Alcohol was then evaporated in a Speed-Vac concentrator (AES 2000, Savant Instruments, Hicksville, NY), and the cAMP content was determined using a cAMP RIA kit (Amersham Pharmacia Biotech). Proteins were quantitated by the Bradford protein assay (Bio-Rad Laboratories, Inc., Marnes la Coquette, France).

Confocal laser scanning microscopy

Approximately 48–72 h after transient transfection of PAC1-Rs-GFP plasmids into LLC-PK1, CHO-K1, and PC12 cells, GFP was imaged in living cells using a CLSM NORAN OZ (Noran Instruments, Middleton, WI) and a 488-nm excitation line of an argon/krypton laser. The emission fluorescence was detected with a 525- to 552-nm bandpass filter. Images were acquired using a ×60 water immersion objective (Nikon, Melville, NY) and Intervision software (Noran Instruments).

Northern blot analysis

Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (24) using Tri-Reagent (Sigma-Aldrich Corp.). Polyadenylated [poly(A^+)] RNA was extracted with the Poly(A^+) tract mRNA Isolation System (Promega Corp.), separated on a formaldehyde-agarose denaturing gel, and transferred onto a nylon membrane Hybond NX (Amersham Pharmacia Biotech). The blot was hybridized with the ^{32}P-labeled fragment (position 1713–1938) of the frog PAC1-R cDNA in 50% formamide, 5× SSC, 5× Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 0.1% SDS, and 200 μg/ml salmon sperm DNA at 42 C and washed twice at 50 C with 0.1× SSC and 0.1% SDS for 15 min.

RT-PCR and Southern blot

Approximately 5 μg total RNA from various tissues were reverse transcribed using an oligo(deoxythymidine)$_{12–18}$ primer and the SuperScript II reverse transcriptase ribonuclease H^- (Life Technologies, Inc.) in the buffer supplied with the enzyme. One tenth of this reaction mixture was used for PCR with the oligonucleotides S3F and S2R described above for PAC1-R mRNA amplification. PCR was performed for 25 cycles at an annealing temperature of 55 C with a primer specific for each PAC1-R isoform and a primer common to all isoforms. The primer pair S2R/25F (5′-CAGTACTCTGTCAAGATGTC-3′) was used to amplify PAC1-R25. The primer pair S2R/41F (5′-GAATCACAACGGACTGACTG-3′) was used to amplify PAC1-R41. The primer pair S3F/TER (5′-TGCACCTTTAGGGAGAAA-3′) was used to amplify PAC1-Rmc. The PCR products were transferred to nylon filters and hybridized with the PAC1-R cDNA fragment 1388–1937 to confirm their identity.

In situ hybridization histochemistry

Adult male frogs were anesthetized and perfused transcardially with an ice-cold solution of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were postfixed overnight in the same solution, transferred into 0.1 M phosphate buffer containing 15% saccharose for 12 h, and frozen in isopentane at −30 C. Frontal sections (12-μm thick) were cut in a cryostat (2800 Frigocut, Leica Corp., Rueil-Malmaison, France) and used for *in situ* hybridization as described previously (19). Sense and antisense riboprobes were generated using T3 and T7 RNA polymerases in the presence of [^{35}S]UTP (Riboprobe Combination Systems, Promega Corp.) and a 695-bp fragment of the frog PAC1-R cDNA (position 1–695) subcloned into pBluescript II KS (Stratagene) between the *Pst*I and *Sma*I sites. Hybridization was performed overnight at 60 C using 1.5×10^7 cpm/ml heat-denatured RNA probes. Brain slices were washed in 2× SSC at 60 C and treated with ribonuclease A (50 μg/ml) for 60 min at 37 C. Five final washes were performed in 0.1× SSC containing 14 mM 2-mercaptoethanol and 0.05% sodium pyrophosphate. The slices were dehydrated in ethanol and exposed to Hyperfilm β-max (Amersham Pharmacia Biotech) for 5 d. Tissue slices were subsequently dipped into Kodak NTB2 liquid emulsion at 40 C, exposed for 30 d, and developed. To identify anatomical structures, sections were stained with hematoxylin and eosin. Nomenclature of frog brain structures was based on the atlas of Neary and Northcutt (25).

Results

Isolation of a frog PAC1 receptor cDNA

A cDNA encoding PAC1-R in the frog *R. ridibunda* was cloned from a brain cDNA library. This cDNA contains an open reading frame of 1365 bp encoding a protein of 455 amino acids with a predicted molecular mass of 52.4 kDa. A hydropathy plot of this deduced protein identified eight hydrophobic regions including a signal peptide encompassing the 16 N-terminal amino acid residues with a potential signal cleavage site between residues Ser^{16} and Leu^{17} (26) and seven putative TMDs (Fig. 1). Comparison with the PACAP/VIP receptor sequences revealed that the frog receptor had the greatest sequence identity with PAC1-R of *X. laevis* (88%), human PAC1-R (78%), and goldfish PAC1-R (71%; Fig. 1). *R. ridibunda* PAC1-R exhibited 47% amino acid identity with the previously characterized frog VPAC receptor (20). The PAC1-R domain encompassing the transmembrane segments and the C-terminal tail is strongly conserved (>90%), whereas the N-terminal region shows less identity (50–70%) in the different species (Fig. 1). Within the N-terminal domain, a short 21-amino acid sequence that is present in mammalian PAC1-Rs and not in other receptors of the VIP/secretin/glucagon family is only partially conserved (Fig. 1). Ten extracellular cysteine residues in the PAC1-R sequence, *i.e.* seven in the N terminus and three in the first and second extracellular loops, are strictly conserved across the different vertebrate species. Finally, the *R. ridibunda* PAC1-R sequence possesses five consensus *N*-glycosylation sites, two of which are conserved in all species studied, and the others are found in one or the other species (Fig. 1).

Tissue distribution of frog PAC1-R mRNA

Northern blot analysis of poly(A^+) RNA showed high levels of PAC1-R mRNA in the frog brain, whereas no hybridization signal was detected in the spinal cord or testis (Fig. 2A). RT-PCR analysis of different tissues confirmed the intense expression level of PAC1-R mRNA in the brain and revealed moderate expression levels in the spinal cord, the distal lobe of the pituitary, and the spleen (Fig. 2B). In other tissues, very low or no mRNA signal was detected. Note that more than one PCR product was amplified, indicating the existence of molecular variants in frog tissues. When reverse transcriptase was omitted in the RT-PCR, no amplification product was observed (Fig. 2B).

Distribution of PAC1-R mRNA in the brain of R. ridibunda

Localization of PAC1-R mRNA in the brain was studied by *in situ* hybridization histochemistry. The distribution and relative density of the hybridization signal are summarized in Table 1.

In the telencephalon, a strong hybridization signal was observed in the internal granular layer and the extragranular

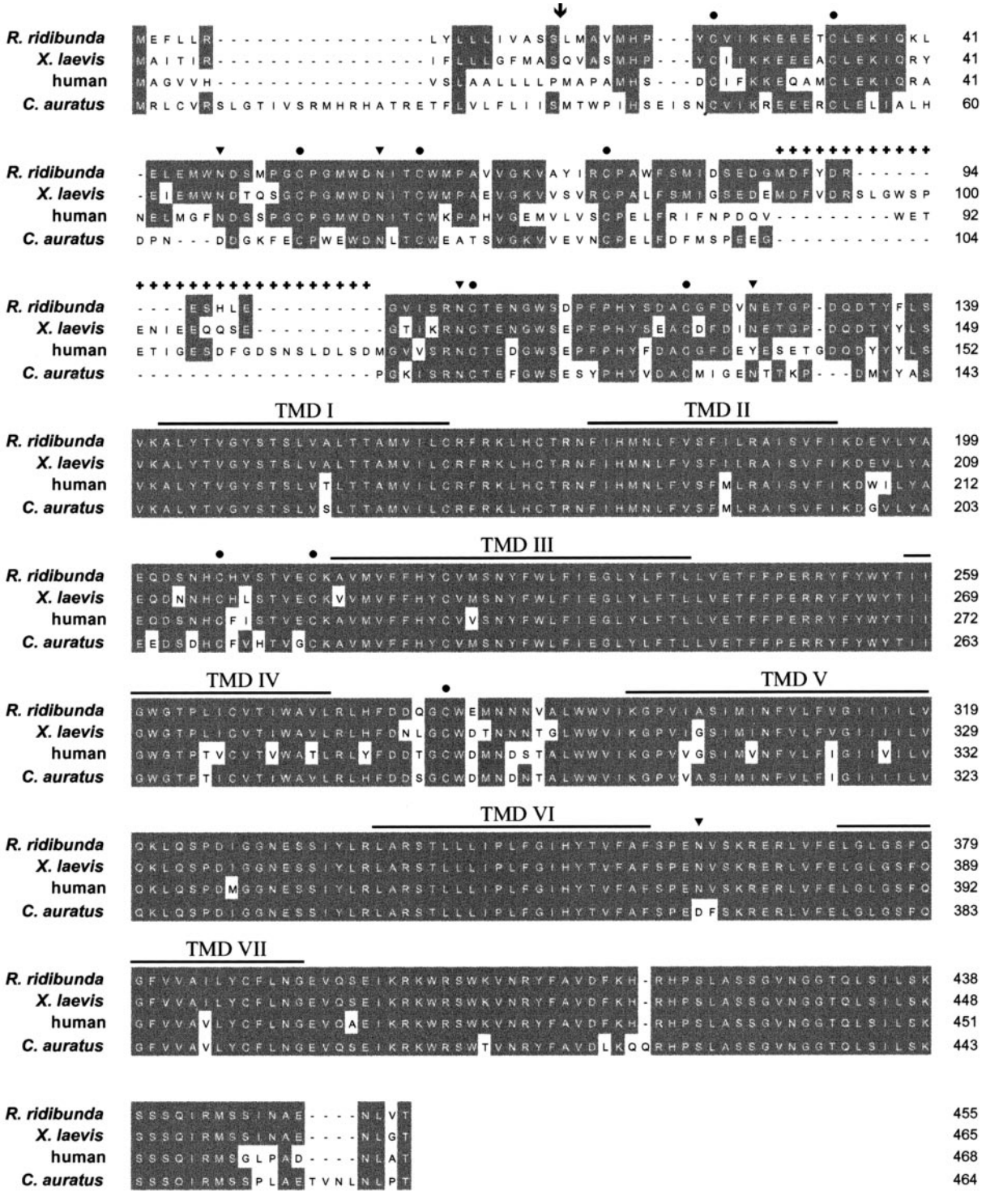

FIG. 1. Comparison of the primary structure of PAC1-R in *R. ridibunda*, *X. laevis* (21), human (44), and goldfish (45). Conserved amino acids in the sequences of PAC1-R in *R. ridibunda* and the other species are *shaded*. The symbols above the amino acid sequences indicate potential *N*-glycosylation sites (▼), conserved cysteine residues (●), and the short amino acid insertion present only in the N-terminal domain of the PAC1-R (+). Putative transmembrane regions are labeled TMD I–VII. The *arrow* shows the putative cleavage site of the signal peptide. Amino acid alignment was performed using DNASIS version 2.1 software. GenBank accession no. AF312682.

plexiform layer of the olfactory bulb, whereas the different subdivisions of the pallium exhibited only a weak signal (Fig. 3A). Low to moderate PAC1-R mRNA levels were detected in the striatum and the septum nuclei (Fig. 3B).

In the diencephalon, an intense hybridization signal was found in the suprachiasmatic nucleus, and a less intense signal was observed in the magnocellular preoptic nucleus (Fig. 3C). Caudally, the central thalamic nucleus and the

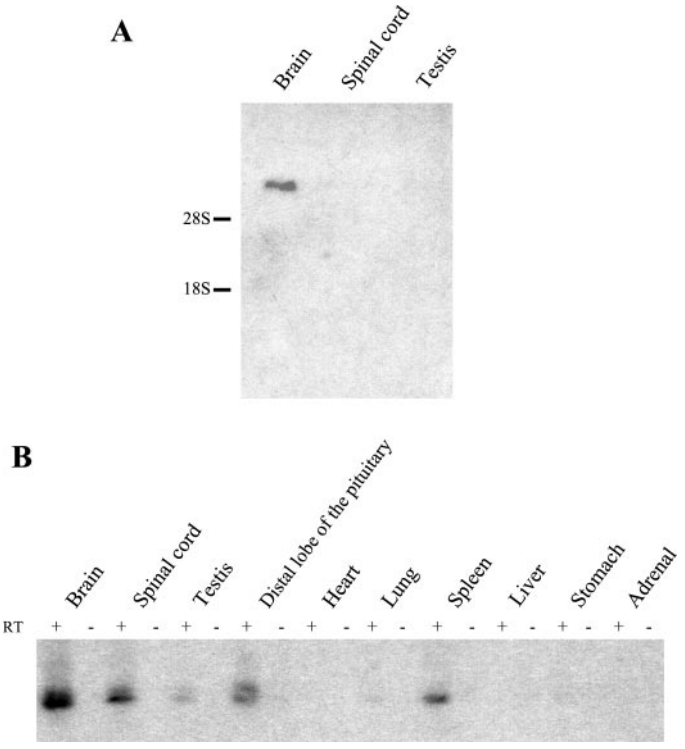

FIG. 2. Tissue distribution of frog PAC1-R mRNA. A, Northern blot analysis of frog PAC1-R mRNA. Equivalent amounts of poly(A^+) RNA purified from 1 mg brain and testis total RNA and an amount purified from 300 μg spinal cord total RNA were electrophoresed on a formaldehyde-agarose gel, hybridized to the random-primed ^{32}P-labeled frog PAC1-R cDNA probe, and exposed to Kodak X-OMAT films for 4 d. The positions of the 28S and 18S rRNA are indicated. B, Analysis of PAC1-R mRNA expression in various frog tissues by RT-PCR. RNA from the tissues indicated was incubated with an RT mixture in the presence (RT+) or absence (RT−) of reverse transcriptase. PCR was performed with specific frog PAC1-R primers. The PCR DNA products were analyzed by Southern blot using the ^{32}P-labeled frog PAC1-R cDNA probe.

ventral hypothalamus were moderately labeled, whereas the lateral thalamic nucleus and the lateral hypothalamic nucleus exhibited only a weak signal (Fig. 3D). More caudally, an intense mRNA signal was observed in the nucleus of the periventricular organ and in the posterior thalamic nucleus (Fig. 3E).

In the mesencephalon, a positive signal was observed in the optic tectum and in the dorsal and the ventral parts of the tegmentum (Fig. 3F). No hybridization signal could be detected in the pituitary gland (Fig. 3F).

Control sections, taken at different levels of the brain and treated with the sense PAC1-R probe, did not show any hybridization signal, as illustrated in Fig. 3G.

In all of the autoradiograms shown in Fig. 3, a robust signal was observed in the periventricular regions. Microscopic examination of emulsion-coated slices showed strong accumulation of silver grains over the ependymal cells bordering the ventricles (Fig. 4). For instance, in the telencephalon, intense labeling was seen around the ventricle, whereas neighboring cells of the distal pallium only displayed a weak signal (Fig. 4A). In the diencephalon, a high density of silver grains was observed in the ependymal cell layer and in the ventral hypothalamus (Fig. 4B), whereas the posterior thalamic nucleus was only moderately labeled (Fig. 4C). Incubation of consecutive sections with the sense probe only produced weak background staining (Fig. 4, D–F).

Isolation of molecular splice variants of the frog PAC1-R

PCR amplification of PAC1-R cDNA from different frog tissues revealed the existence of molecular variants of this receptor. Three isoforms characterized by the insertion of nucleotide cassettes either in the third intracellular loop (PAC1-R25 and PAC1-R41) or in the C terminus (PAC1-Rmc, modified C terminus; Fig. 5A) have been found in the brain, the distal lobe of the pituitary, the testis, or the spleen. The frog PAC1-R25 presents a 25-amino acid insertion after the Leu^{336} residue. The inserted sequence is highly homologous (68% amino acid identity) to the human hop cassette (6) (Fig. 5B). The frog PAC1-R41 contains an insertion of 41 residues at the same location as in the PAC1-R25 variant; however, the sequence of this cassette is completely different from those of the hip and hop cassettes of mammalian PAC1-Rs (Fig. 5C). The third variant, PAC1-Rmc, contains an insertion of 13 nucleotides located at the C-terminal end of the seventh TMD sequence, at the level of residue Glu^{393}. This insertion alters the reading frame leading to a completely different C-terminal sequence (Fig. 5D).

Pharmacological characterization of the frog PAC1-R variants

To determine the pharmacological profile of the cloned receptors, expression plasmids were transfected in LLC-PK1 cells, and the effects of PACAP and VIP on cAMP formation were studied (Fig. 6). PACAP induced a dose-dependent stimulation of cAMP accumulation in cells transfected with frog PAC1-R with an EC_{50} of approximately 10^{-8} M, whereas VIP was far less potent (Fig. 6). In cells transfected with PAC1-R, PAC1-R25, and PAC1-R41, PACAP also stimulated cAMP formation with an EC_{50} of approximately 10^{-8} M and similar efficacy (Fig. 7), although slightly greater cAMP production was systematically observed with PAC1-R25 (Fig. 7). Conversely, in PAC1-Rmc-transfected cells, PACAP and VIP were totally unable to stimulate cAMP formation (Fig. 7 and data not shown), suggesting that the sequence of the C-terminal tail of PAC1-R is crucial for its coupling to G_s proteins.

Expression of PAC1-Rs-GFP fusions in transfected cells

To study the expression and subcellular localization of frog PAC1-Rs, chimeric cDNAs encoding PAC1-R or PAC1-Rmc with GFP at the C terminus were constructed. Confocal laser scanning microscopy was used to determine the localization of the fluorescent signal resulting from the expression of PAC1-R-GFP and PAC1-Rmc-GFP fusion proteins in different cell lines. This analysis revealed that in transfected LLC-PK1 cells, GFP-labeled PAC1-Rs were both addressed to the plasma membrane (Fig. 8, A and B). In contrast, transfection of the plasmid encoding GFP alone resulted in evenly distributed fluorescence labeling (Fig. 8C). The PAC1-Rs-GFP fusion proteins were also targeted to the cell surface in

TABLE 1. Comparative distribution and relative abundance of PAC1-R mRNA and VPAC mRNA in the brain of the frog *R. ridibunda*

Structure	PAC1-R mRNA	VPAC mRNA
Telencephalon		
Olfactory bulb, internal granular layer (IGL)	+++	+++
Olfactory bulb, extragranular plexiform layer (EPL)	+++	−
Olfactory bulb, mitral cellular layer (ML)	+/−	+
Olfactory bulb, glomerular layer (GL)	−	−
Accessory olfactory bulb (AOB)	+/−	
Vomeronasal nerve (VN)	−	−
Postolfactory eminence (PE)	+++	+++
Lateral pallium (LP) dorsal	+	++
Lateral pallium (LP) ventral	+	++
Dorsal pallium (DP)	+	++
Medial pallium (MP)	+	−
Lateral septum (LS)	++	+++
Dorsal striatum (DS)	++	+
Medial septum (MS)	+	+
Ventral striatum (VS)	++	+
Nucleus of the diagonal band of Broca (NDB)	+/−	+/−
Nucleus accumbens (NA)	++	++
Medial amygdala (MA)	+	
Lateral amygdala (LA)	+	+
Anterior entopeduncular nucleus (Ea)	−	−
Posterior entopeduncular nucleus (Ep)	+	−
Bed nucleus of the pallial commissure (BN)	−	+++
Diencephalon		
Anterior preoptic area (Poa)	+++	+++
Magnocellular preoptic nucleus, dorsal part (Mgd)	+/−	
Magnocellular preoptic nucleus, ventral part (Mgv)	++	
Magnocellular preoptic nucleus (Mg)	+	+
Organum vasculosum (OV)	++	+
Suprachiasmatic nucleus (SC)	+++	++
Dorsal hypothalamic nucleus (DH)	+	
Ventral hypothalamic nucleus (VH)	++	+++
Lateral hypothalamic nucleus (LH)	+	−
Posterior tuberculum (TP)	+	++
Nucleus of the periventricular organ (NPv)	+++	+++
Thalamic eminence (TE)	−	
Habenular commissure (Hc)	−	
Dorsal habenular nucleus (Hd)	−	+/−
Ventral habenular nucleus (Hv)	+/−	+
Neuropil of Bellonci (B)	−	
Nucleus of Bellonci (NB)	−	
Ventrolateral thalamic nucleus, dorsal part (Vld)	+	++
Ventrolateral thalamic nucleus, ventral part (Vlv)	+	++
Ventromedial thalamic nucleus (VM)	++	++
Anterior thalamic nucleus (A)	+/−	+
Corpus geniculatum thalamicum (CP)	−	
Lateral thalamic nucleus, posterodorsal division (Lpd)	−	−
Lateral thalamic nucleus, anterior division (La)	+	+
Central thalamic nucleus (CNT)	++	++
Posterior thalamic nucleus (P)	+	++
Optic chiasma (OC)	−	
Optic nerve (ON)	−	−
Median eminence (ME)	−	−
Mesencephalon		
Optic tectum (OT)	+++	+++
Tectal lamina six (6)	++	+++
Nucleus of the posterior commissure (NPC)	−	
Nucleus lentiformis mesencephali (NLM)	−	
Nucleus of the medial longitudinal fasciculus (NMLF)	+	+
Basic optic nucleus (BON)		−
Nucleus profundus mesencephali (NPM)	−	−
Pretectal gray (PtG)	++	+
Pretoral gray (PtrG)	++	++
Torus semicircularis (TS)	+	
Anterodorsal tegmental nucleus (AD)	++	++
Anteroventral tegmental nucleus (AV)	++	++
Posterodorsal tegmental nucleus (PD)	++	
Posteroventral tegmental nucleus (PV)	++	
Nucleus reticularis isthmi (RIS)	−	
Nucleus interpeduncularis (NIP)	+	+
Nucleus isthmi (NI)	+/−	
Nucleus cerebelli (Cer)	+/−	
Metencephalon		
Molecular cell layer of the cerebellum (MC)	−	
Purkinje cell layer of the cerebellum (PC)		
granular cell layer of the cerebellum (GC)	−	
Auricular lobe of the cerebellum (CAL)	−	
Rhombencephalon		
Nucleus of the trigeminal nerve (V)		
Griseum central rhombencephali (Gc)		
Nucleus of the abducent nerve (VI)	++	
Spinal cord		
Dorsal horn (HD)	+	
Ventral horn (HV)	+	
Lateral cord (LC)	−	
Pituitary		
Pars nervosa (PN)	−	−
Pars intermedia (PI)	−	−
Pars distalis (Pdis)	−	+

The distribution of frog VPAC mRNA is from the report by Alexandre *et al.* (19). −, No hybridization signal; +/−, very low density; +, low density; ++, moderate density; +++, high density of mRNA. Abbreviations according to Neary and Northcutt (24).

transfected CHO-K1 and PC12 cells (Fig. 8, D–F). These results indicate that the frog PAC1-R variants are expressed properly and targeted to the plasma membrane, and that the loss of G_s coupling observed with the PAC1-Rmc variant is not due to a deficiency in the expression or the compartmentalization of this receptor.

Tissue distribution of frog PAC1-R mRNA splice variants

The distribution of the mRNAs encoding the splicing variant forms of PAC1-R was determined by RT-PCR analysis. All four variants were intensely expressed in the brain and spinal cord (Fig. 9). PAC1-R and PAC1-R25 mRNAs exhibited similar distribution patterns, and both messengers were detected in peripheral tissues such as the testis, spleen, and heart (Fig. 9, A and B). In contrast, PAC1-R41 mRNA was not detected in these peripheral tissues (Fig. 9C). Finally, PAC1-Rmc mRNA was detected in the testis, but not in the spleen (Fig. 9D). When reverse transcriptase was omitted in the RT-PCR, no amplification product was observed (Fig. 9).

Discussion

The European green frog *R. ridibunda* has been widely used as a model for studying neuroendocrine regulations exerted by PACAP in nonmammalian vertebrates and for characterizing the molecular mechanisms underlying the effects of this neuropeptide (14). The present study now describes the structural and pharmacological characterization of PAC1-R and three molecular variants in *R. ridibunda*, and their distribution in the central nervous system and peripheral tissues. The cloned frog PAC1-R is a 455-amino acid protein that includes a signal peptide and seven putative TMD. Se-

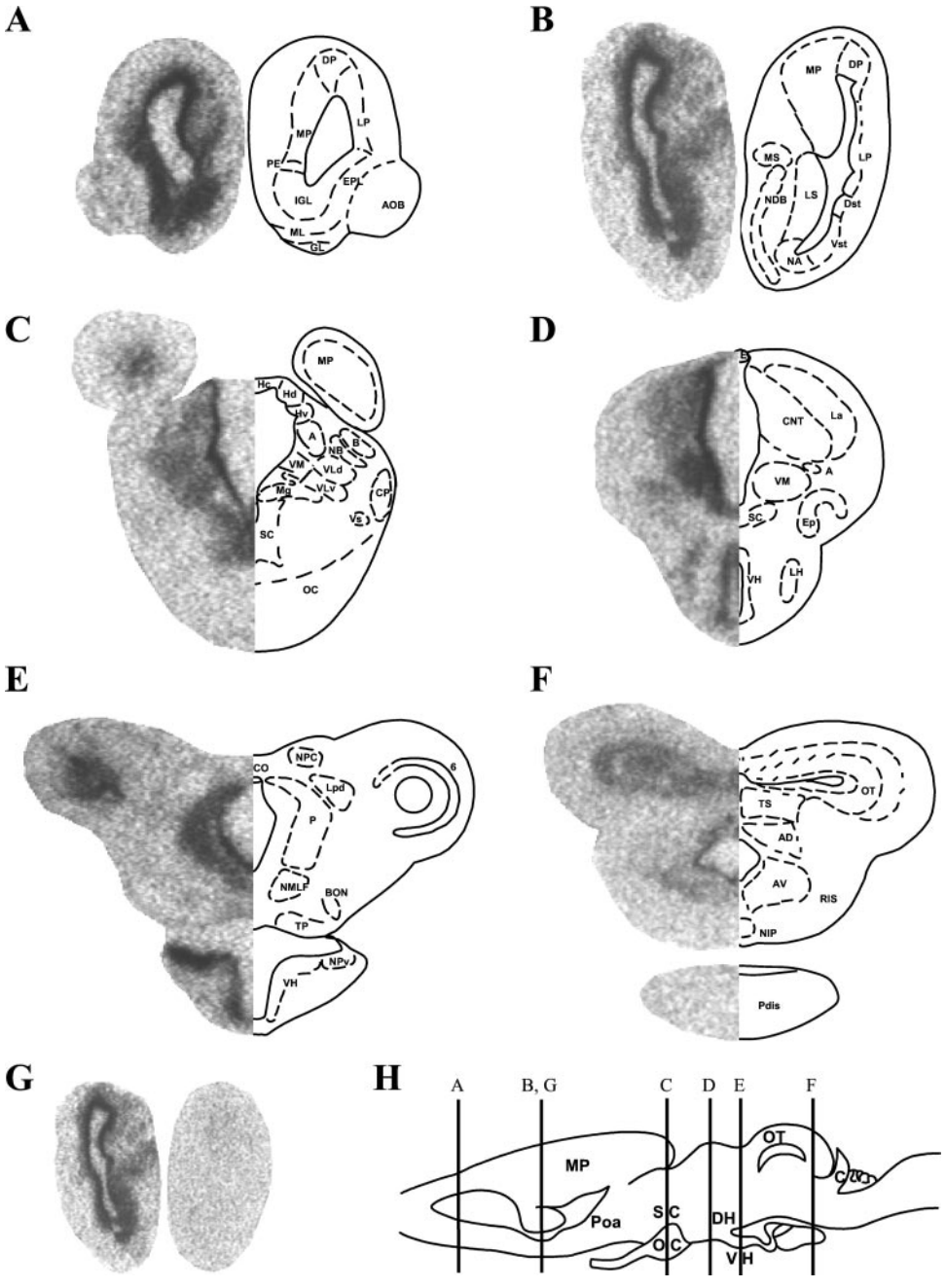

FIG. 3. *In situ* hybridization analysis of PAC1-R mRNA in the frog brain. A–F, Frontal brain sections were hybridized with an antisense ^{35}S-labeled PAC1-R riboprobe and exposed to Hyperfilm β-max for 5 d. The anatomical structures, identified by microscopic analysis, are presented on the *right* hemisections. G, A control section hybridized with a sense ^{35}S-labeled riboprobe (*right*) is compared with a consecutive section hybridized with the antisense probe (*left*). The rostrocaudal levels of the sections are indicated on the schematic sagittal section of the frog brain (H). Abbreviations are explained in Table 1.

quence comparison revealed a high degree of amino acid identity with the cloned *Xenopus* PAC1-R (88%), human PAC1-R (78%), and goldfish PAC1-R (71%). The transmembrane regions and the C terminus of this receptor are particularly well conserved (>90% amino acid identity), whereas the N terminus is less conserved (50–70% amino acid identity). Of note is the full conservation of seven cysteine residues in the N-terminal domain of PAC1-R in all species studied to date. The strict preservation of these residues across the vertebrate phylum along with previous mutagenesis studies of equivalent cysteines in the VPAC1-R (27, 28) strongly suggest that these cysteine residues and the disulfide bridges that they form are important for the tridimensional conformation of PAC1-R and thereby for its function. The PAC1-R of *R. ridibunda* shows five potential glycosylation sites, four in the N terminus and one in the third extracellular loop. Although the relevance of *N*-glycosylation to the function of PAC1-R has not yet been addressed, it has been shown that alteration of *N*-glycosylation sites in VPAC1-R prevents its expression at the plasma membrane of transfected cells (29). Further studies should be conducted to assess the contribution of glycosylation to the function of PAC1-R, and particularly at the Asn^{59} and Asn^{105} sites that are conserved in all species.

In mammals, a 21-amino acid domain is present in the N terminus of PAC1-R, but not at the equivalent position of other members of this receptor family, including VPAC-Rs (9). A similar 21-amino acid insertion is present

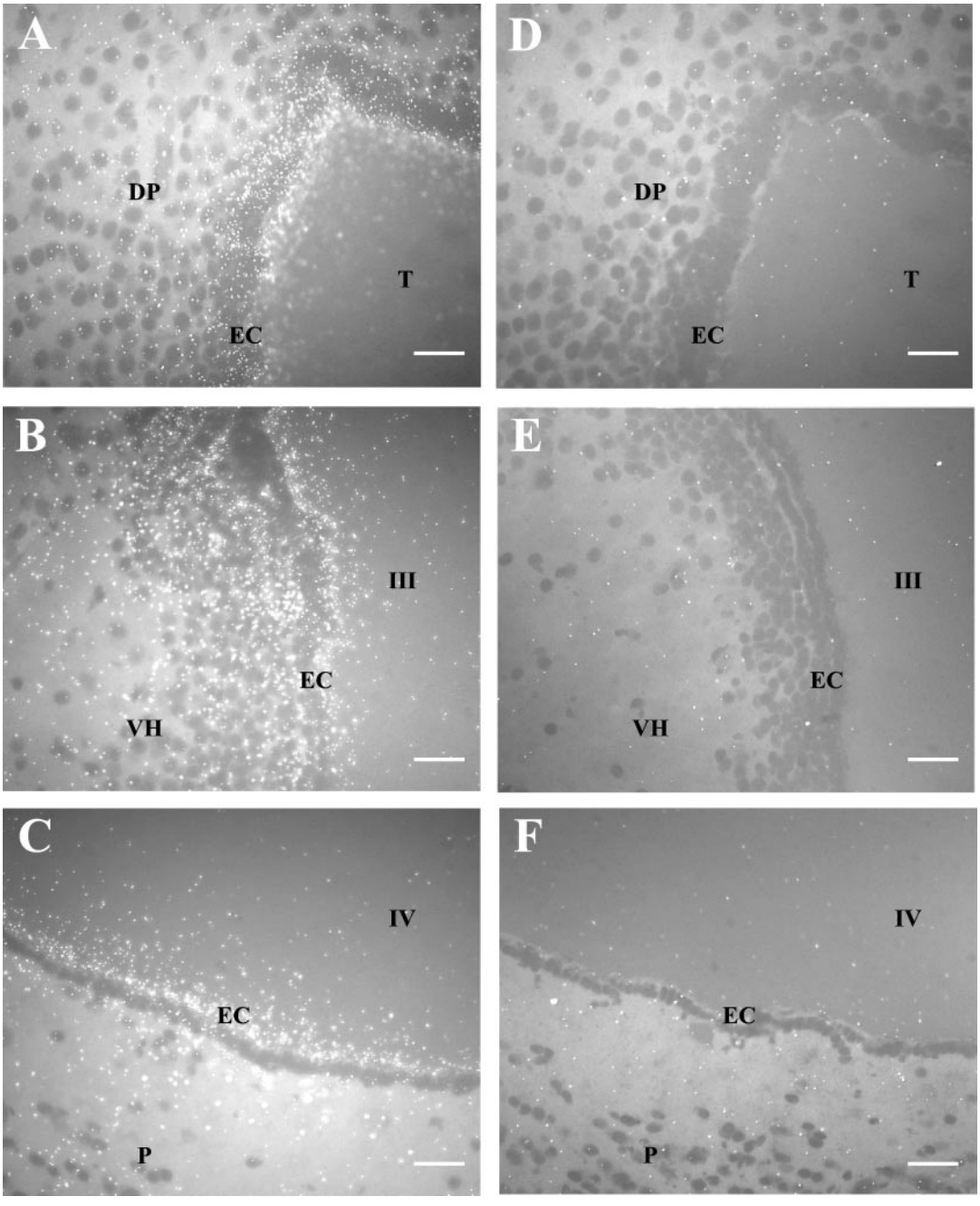

FIG. 4. Photomicrographs illustrating the cellular distribution of PAC1-R mRNA in selected regions of the frog brain. A, A high density of silver grains was observed in the ependymal cell layer (EC), and a moderate density was seen in the dorsal pallium (DP). T, Telencephalic ventricle. B, Intense labeling occurred in the EC and in the periventricular region of the ventral hypothalamic nucleus (VH). III, Third ventricle. C, Ependymal cells were strongly labeled compared with the posterior thalamic nucleus (P). IV, Fourth ventricle. D–F, Consecutive sections to A–C were hybridized with a sense probe as negative controls. *Scale bars*, 30 μm.

in the *Xenopus* PAC1-R (21). In the frog *R. ridibunda*, sequence alignment showed the occurrence of an insertion at the same position (Fig. 1). However, the sequence of frog PAC1-R encompasses only 12 residues (amino acids 88–99) at this position. In fact, it has been shown that this region in the human PAC1-R gene could be alternatively spliced to give rise to 2 receptor variants lacking either the 21-amino acid cassette (10) or a 57-amino acid cassette that includes the previous one (11). The insertion of these cassettes in the N-terminal domain of PAC1-R leads to a decrease in the ability of PACAP27 to stimulate PLC activity (10) and in the affinity for VIP (11) in transfected cells. *R. ridibunda* PAC1-R, which has a 12-amino acid cassette, exhibited a low affinity for VIP, as revealed by cAMP accumulation data (Fig. 6). Although the coupling efficacy of frog PAC1-R to PLC remains to be determined, the fact that the length of this cassette seems to impinge on the pharmacological properties of PAC1-R in mammals

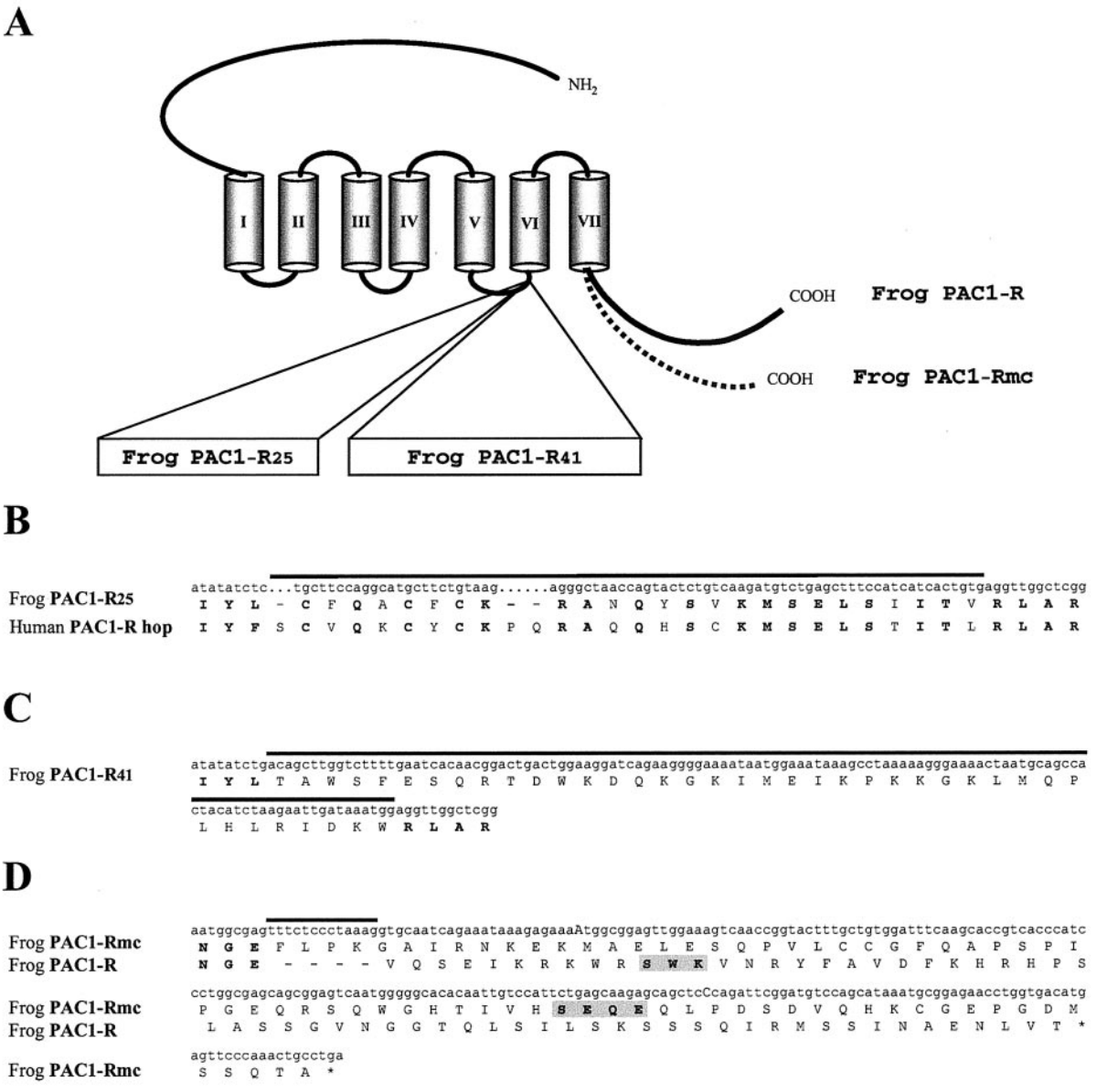

FIG. 5. Molecular variants of PAC1-R in the frog *R. ridibunda*. A, Schematic illustration of the four PAC1-R variants. PAC1-R25 and PAC1-R41 contain inserted cassettes in the third intracellular loop, whereas PAC1-Rmc exhibits a novel C-terminal tail compared with PAC1-R. B, Alignment of the deduced amino acid sequence of the cassette present in PAC1-R25, delineated by the *solid line*, with the hop cassette of the human PAC1-R. Homologous amino acids are shown in *bold*. C, Nucleotide and deduced amino acid sequences of the 41-residue cassette of PAC1-R41. D, Alignment of deduced amino acid sequences in PAC1-Rmc and PAC1-R showing that PAC1-Rmc contains an insertion of 13 bp delineated by the *solid line*, leading to a C terminus different from that of PAC1-R. Note that PAC1-R displays a consensus site for protein kinase C (S-W-K, shaded), which is replaced by a casein kinase II phosphorylation site (S-E-Q-E, *shaded*) in PAC1-Rmc. The *asterisks* denote the stop codon. GenBank accession nos. for the PAC1-R25, PAC1-R41, and PAC1-Rmc splice variants: AF312685, AF312684, and AF312683, respectively.

suggests that the frog receptor may exhibit some particular functional features.

The third intracellular loop is another site of alternative splicing in the mammalian PAC1-R. This region of the heptahelical receptors is involved in the coupling to and activation of G proteins (30). In *R. ridibunda*, two variants of the receptor also occur as the result of cassette insertion in the third intracellular loop, at the very position where the hip and hop cassettes occur in mammals. One of the frog isoforms, PAC1-R25, contains a cassette that is homologous to the hop cassette in mammals and exhibits a pharmacological profile similar to PAC1-R, in agreement with previous data reported in mammals (6). In contrast, the second variant of the third intracellular loop of the frog receptor, PAC1-R41, contains a cassette whose sequence is completely different from those of the mammalian hip and hop cassettes. The efficacy of PACAP to stimulate cAMP formation through PAC1-R41 in transfected cells was comparable to that observed with the two previous variants. It remains to be established whether these frog PAC1-R isoforms display any differential coupling efficacy to PLC or calcium mobilization, as has been shown for PAC1-R variants in mammals (6, 13).

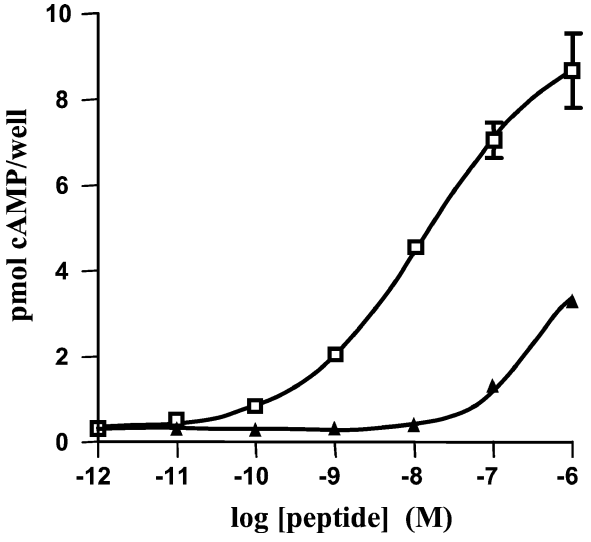

FIG. 6. Effect of PACAP and VIP on cAMP accumulation in LLC-PK1 cells transiently expressing the frog PAC1-R. Transfected cells were incubated with graded concentrations of synthetic frog PACAP38 (□) or frog VIP (▲), and cAMP contained in the cell extracts was measured by RIA. Data are from one representative experiment and are expressed as the mean ± SEM of three determinations (PACAP) or two determinations (VIP). Three independent experiments were conducted, and similar results were obtained.

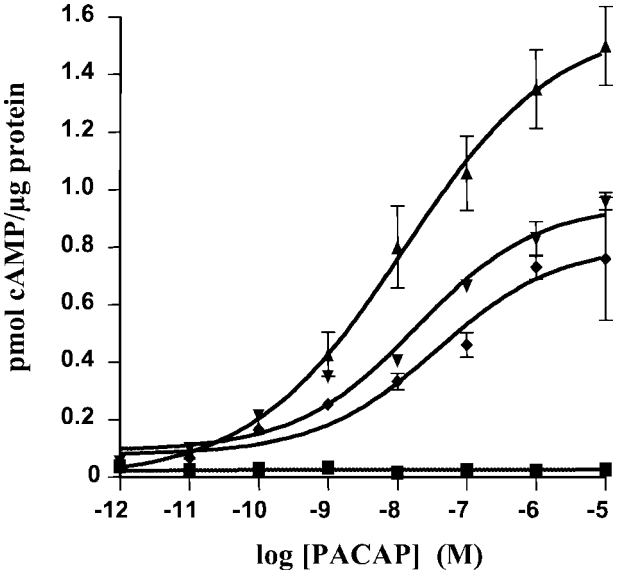

FIG. 7. Effect of PACAP on cAMP accumulation in LLC-PK1 cells transiently expressing the frog PAC1-R isoforms. Cells were transfected with PAC1-R (♦), PAC1-R25 (▲), PAC1-R41 (■), and PAC1-Rmc (■) and incubated with graded concentrations of synthetic frog PACAP38. cAMP contained in the cell extracts was measured by RIA and was normalized to the protein content. Data are from one representative experiment and are expressed as the mean ± SEM of three determinations. Three independent experiments were conducted, and similar results were obtained.

The third variant of frog PAC1-R that we have identified, PAC1-Rmc, is unique in that it carries a cytoplasmic C-terminal tail with a sequence completely different from that in PAC1-R. This modification is the consequence of a 13-nucleotide insertion at the cytoplasmic extremity of the seventh TMD that shifts the open reading frame of the receptor and thus alters the sequence and the size (10 amino acids longer than in PAC1-R) of the intracellular C-terminal domain. The variations at the C terminus of G protein-coupled receptors that have been described to date correspond to extended or shortened C-terminal tails, as in the somatostatin-2 receptor (31), the neurokinin-1 receptor (32), or the GnRH receptor (33). To our knowledge this is the first report describing a receptor variant with a totally different C-terminal tail in the superfamily of G protein-coupled receptors.

The modification of the C-terminal tail of the frog PAC1-R abolished the stimulatory effect of PACAP on adenylate cyclase in transfected cells, suggesting that PAC1-Rmc was either not expressed at the plasma membrane or had lost its coupling efficacy to a G_s protein. Given that PAC1-Rmc retains all its hydrophobic domains and its potential post-translational modifications, it seemed unlikely that this receptor was not addressed properly. Indeed, the fusion of this receptor variant to GFP showed that PAC1-Rmc is targeted to the cell surface in LLC-PK1 and CHO cells, as is the PAC1-R, and in PC12 cells, a neuroendocrine cell line that naturally expresses PAC1-R, confirming that PAC1-Rmc is actually expressed in transfected cells and correctly targeted to the plasma membrane. These findings, which show that the C-terminal domain is instrumental in the coupling of PAC1-R to adenylate cyclase, are consistent with a recent report demonstrating the critical role of the proximal C-terminal region of the human PAC1-R for signal transduction (34). In addition, it has been shown that mutation or deletion of amino acids within the C terminus or even complete truncation of this part of PAC1-R does not alter the ligand binding to the receptor (34), suggesting that the C-terminus modification in PAC1-Rmc should not substantially modify its affinity for the ligand. In agreement with these observations, there are multiple examples showing that alteration of the intracellular C-terminal domain of seven TMD receptors mainly impairs coupling efficiency to G proteins (35, 36).

It is noteworthy that within the mammalian PAC1-R gene an intron is located exactly at the position where the insertion occurs in the frog PAC1-Rmc. Indeed, the 14th intron in the rat and mouse PAC1-R genes occurs after the Glu residue located at the C-terminal end of the seventh TMD (37, 38), *i.e.* at the site where the modification of PAC1-Rmc is found. This structural feature of the gene supports the existence of an isoform of PAC1-R that is alternatively spliced in this region.

The transduction pathways associated with activation of PAC1-Rmc are currently unknown. Whether this receptor may stimulate PLC and/or MAPK or provoke calcium mobilization remains to be investigated. It should be mentioned that the modification of the C terminus in this receptor causes the loss of a putative protein kinase C phosphorylation site and the appearance of a potential casein kinase II phosphorylation site. This molecular switch may have important functional consequences, because the phosphorylation status of the C-terminal domain plays a crucial role in the activation, desensitization, and internalization of G protein-coupled receptors (39).

The expression of PAC1-R and its variants in the frog *R. ridibunda* was studied in the brain as well as in peripheral

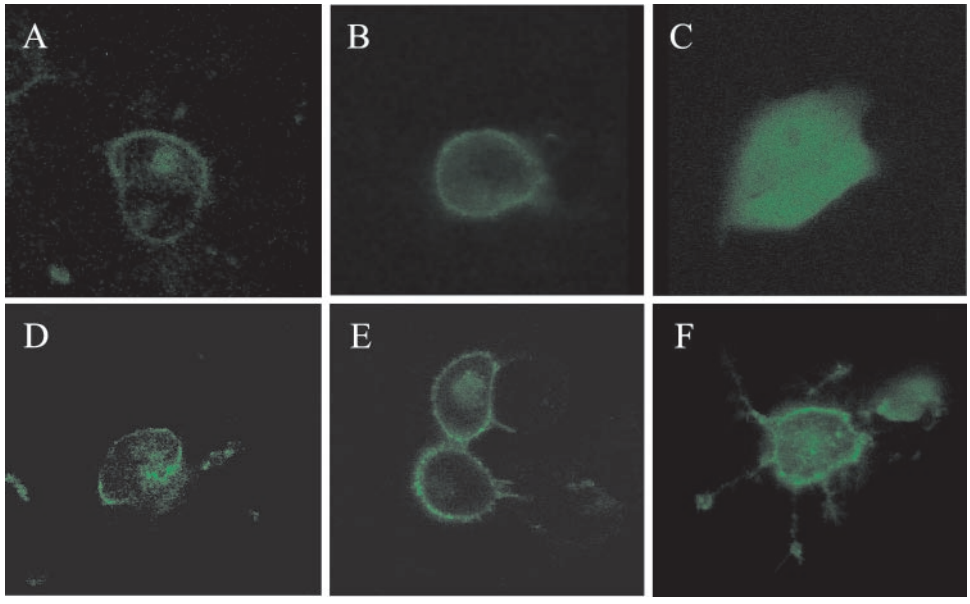

FIG. 8. Confocal laser scanning microscopy analysis of cell lines expressing frog PAC1-R-GFP fusion proteins. LLC-PK1 cells were transiently transfected with the phrGFP-C vector encoding the fusion protein PAC1-R-GFP (A), the PAC1-Rmc–GFP (B), and the phrGFP-C vector encoding only GFP (C). The PAC1-R-GFP and PAC1-Rmc-GFP fusion proteins were also expressed in CHO cells (D and E, respectively), and the PAC1-Rmc-GFP was also expressed in PC12 cells (F). Images were acquired and analyzed as described in *Materials and Methods*.

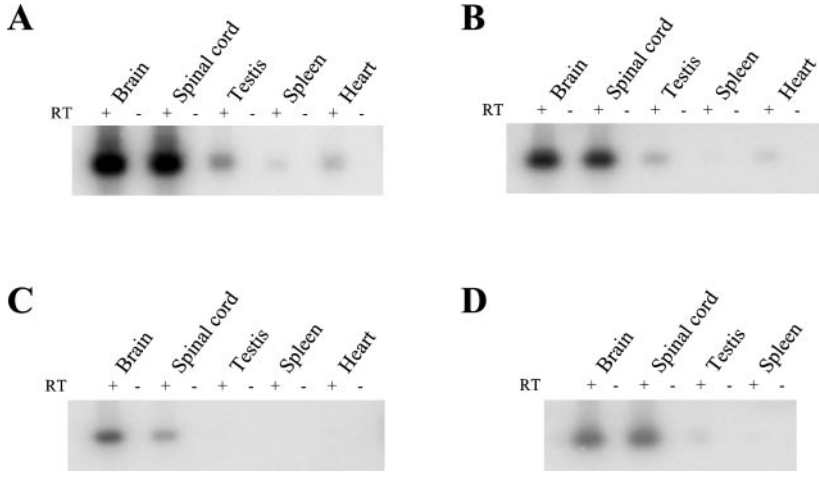

FIG. 9. Analysis of the expression of PAC1-R mRNA splice variants in different frog tissues by RT-PCR. RNA from the tissues indicated was incubated with an RT mixture in the presence (RT+) or absence (RT−) of reverse transcriptase. PCR was performed with specific primers for PAC1-R (A), PAC1-R25 (B), PAC1-R41 (C), and PAC1-Rmc (D). The PCR DNA products were analyzed by Southern blot using the ^{32}P-labeled frog PAC1-R cDNA probe.

tissues by Northern blot, RT-PCR, and *in situ* hybridization. The PAC1-R mRNA and the different isoforms were predominantly expressed in the central nervous system. Moderate to low levels of PAC1-R mRNA were observed in the distal lobe of the pituitary, the spleen, the testis, and the lung as revealed by RT-PCR. The other tissues examined were virtually devoid of PAC1-R mRNA. This expression pattern substantially differs from that of the frog VPAC receptor that we have characterized previously and which exhibits a widespread expression in brain and peripheral tissues (20). The distribution of frog PAC1-R mRNA is reminiscent of that of the frog PACAP precursor mRNA, which is also primarily expressed in the central nervous system (19), strongly suggesting that PACAP plays important regulatory functions in the brain and spinal cord in amphibians.

In situ hybridization analysis showed that in the brain of the frog *R. ridibunda*, intense expression of PAC1-R mRNA occurs in the olfactory bulb, the anterior preoptic area, the suprachiasmatic nucleus, the nucleus of the periventricular organ of the hypothalamus, and the optic tectum. These observations suggest that in the frog, PACAP may be involved in the regulation of the olfactory system, the neuroendocrine control of the hypothalamic-pituitary complex, and the processing of visual information. A remarkable feature regarding the distribution of PAC1-R mRNA in the frog brain is the high expression in the ependymal cells bordering the ventricles. This is noticeable because the frog VPAC receptor is not expressed in these cells (20), nor is PACAP precursor mRNA (19). The expression of PAC1-R mRNA in the ependymal cell layers suggests that PACAP acting on these receptors may originate from the cerebrospinal fluid and thus may participate in volume transmission (40).

Besides their occurrence in the central nervous system, PAC1-R and its variants are also expressed in peripheral tissues. Both PAC1-R and PAC1-R25 mRNA occur in the frog testis, spleen, and heart. In contrast, PAC1-Rmc mRNA was observed in the testis, but not in the spleen, whereas PAC1-R41 expression could not be detected in any of the peripheral tissues examined. The predominant expression of the PAC1-R variants in the central nervous system or in certain peripheral tissues suggests that they may allow PACAP to exert specific effects in particular cells that possess these

isoforms. It has previously been shown that a particular variant of PAC1-R that is expressed in β-cells of the pancreas mediates the effect of PACAP on insulin release through calcium influx only (13). In seminiferous tubules, high expression of another variant has been observed, and this variant has been postulated to play a specific role during spermatogenesis (12). Finally, it has been shown that PAC1-R isoform expression may determine the opposing mitogenic effects of PACAP observed in different neuroblasts (41).

In conclusion, we have characterized a type I PACAP receptor and three splice variants in the frog *R. ridibunda*, and we have shown that one of these receptor isoforms fails to stimulate adenylate cyclase upon PACAP treatment. In addition, the PAC1-R variants displayed differential relative abundance in various frog tissues. The novel variants described herein extend the family of PAC1-R isoforms, making this receptor one of the most alternatively spliced receptors among G protein-coupled receptors. The characterization of these variants will contribute to elucidation of the structure-function relationships of PAC1-R. The fact that several variants of this receptor exist in different vertebrate taxa indicates that these isoforms are involved in the physiological effects of PACAP. We have also shown that PAC1-R and its variants are intensely expressed in the frog central nervous system. Along with previous studies showing that high concentrations of PACAP mRNA and PACAP-immunoreactive elements are found in the brain of adult frogs and tadpoles (19, 42, 43), these data strongly suggest that PACAP plays a major role in the activity and development of the central nervous system of amphibians.

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Address all correspondence and requests for reprints to: Dr. Youssef Anouar, European Institute for Peptide Research (IFRMP 23), Laboratory of Cellular and Molecular Neuroendocrinology, Institut National de la Santé et de la Recherche Médicale, U-413, Unité Affiliée Centre National de la Recherche, University of Rouen, 76821 Mont Saint Aignan, France. E-mail: youssef.anouar@univ-rouen.fr.

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