Structural and functional identification of the pituitary adenylate cyclase-activating polypeptide receptor VPAC$_2$ from the frog Rana tigrina rugulosa

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ABSTRACT

Recently, a frog pituitary adenylate cyclase-activating polypeptide (PACAP)/vasoactive intestinal peptide (VIP) receptor (fPVR) has been characterized, and interestingly, this receptor exhibits characteristics of both mammalian PACAP type II receptors VPAC$_1$R and VPAC$_2$R. In order to investigate the receptors responsible for mediating the actions of VIP and PACAP in amphibians, in this report, a frog VPAC$_2$ receptor (fVPAC$_2$R) cDNA was isolated. fVPAC$_2$R shares 47.7, 46.9 and 62.5% amino acid sequence identity with fPVR, human VPAC$_1$R and human VPAC$_2$R respectively. Functionally, fVPAC$_2$R, when expressed in CHO cells, was responsive to both frog peptides including VIP, PACAP38 and PACAP27 where the EC$_{50}$ values of these peptides in intracellular cAMP production were 0.15, 0.18 and 0.16 µM respectively. The pharmacological profiles of human peptides (VIP, PACAP38 and peptide histidine methionine) to stimulate frog and human VPAC$_2$Rs were compared, and it was found that these peptides could only activate the frog receptor at micromolar concentrations. fVPAC$_2$R was found to be widely distributed in various peripheral tissues as well as several regions of the brain. The presence of the receptor transcripts suggests the functional roles of the receptor in mediating the actions of PACAP and/or VIP in these tissues. As VIP and particularly PACAP27 are highly conserved peptides in vertebrate evolution, comparative studies of these peptides and their receptors in non-mammalian vertebrates should provide clues to better understand the physiology of these important peptides in human and other vertebrates.

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INTRODUCTION

Vasoactive intestinal peptide (VIP) is an amidated 28 amino acid peptide that was first discovered in porcine duodenum (Said & Mutt 1970). It belongs to a brain–gut peptide family that includes peptide histidine isoleucine, pituitary adenylate cyclase-activating polypeptide (PACAP), growth hormone-releasing hormone, secretin, glucose-dependent insulinotropic polypeptide, glucagon, glucagon-like peptide-1, glucagon-like peptide-2 and peptide histidine methionine (PHM). VIP is widely expressed in both the peripheral and central nervous systems. It exerts a variety of physiological effects on the circulation (Gozes & Brenneman 1989), gastrointestinal function (Said 1982), the immune system (Ottaway 1987, Ishioka et al. 1992), reproduction (El-Gehani et al. 1998) and the central nervous system (Besson et al. 1986, Martin et al. 1987). There is recent evidence indicating that VIP also plays an important role in the perception of pain (Dickinson & Fleetwood-Walker 1999) and suppression of inflammation (Said 1998). Clinical applications of VIP have been suggested in impotence, asthma, lung injury, a variety of tumors, and neurodegenerative diseases (Said 1991, Gozes et al. 1999, Laburthe et al. 1999).

VIP and PACAP are two structurally and functionally similar polypeptides. PACAP occurs in two amidated forms: PACAP38 (Miyata et al. 1989) and PACAP27 (Gozes & Brenneman 1989),
and PACAP27 (Miyata et al. 1990). VIP shares 68% sequence homology with the N-terminal sequences (1–28 residues) of PACAP38 (Vaudry et al. 2000). In vertebrates, VIP, PACAP38 and particularly PACAP27 are highly conserved and there are only a few substitutions found in non-mammalian species. The functions of VIP and PACAP are mediated through their interactions with specific cell-surface receptors. The PACAP type I (PAC1R) receptors have high affinity for PACAP but low affinity for VIP. The PACAP type II receptors, VPAC1R and VPAC2R, display no marked selectivity for VIP and PACAP (Christophe 1993). All the VIP/PACAP receptors belong to the secretin/glucagon receptor family, which represents a novel family of G protein-coupled receptors that contain a large N-terminal extracellular domain essential for ligand interactions (Ishihara et al. 1991). This family of receptors is generally coupled to adenylate cyclase activation and hence intracellular cAMP production.

VPAC2R cDNAs were characterized from rat lung (Ishihara et al. 1992) and human colon carcinoma cells (Sreedharan et al. 1991, 1993) while VPAC2R cDNAs were isolated from the rat olfactory bulb (Lutz et al. 1993) and human SUP-T1 lymphocyte cells (Svoboda et al. 1994). Although VPAC1R and VPAC2R bind VIP and PACAP with equal affinities (Lutz et al. 1993), they are very different in amino acid sequences and tissue distributions. The homologies between VPAC1R and VPAC2R in human and rat are 49 and 50% respectively. VPAC1R is present in the lung, intestine, liver and spleen, while VPAC2R can be found in many tissues where VIP acts but VPAC1R is not present or is present in relatively low concentrations such as the stomach and testes (Ishihara et al. 1992, Usdin et al. 1993, 1994, Krempeles et al. 1995, Sheward et al. 1995). Moreover, VPAC2R is also present in the pancreatic islets and ovary (Usdin et al. 1994). In the central nervous system, the distributions of VPAC1R and VPAC2R are almost mutually exclusive to each other. VPAC1R is expressed almost exclusively in the cerebral cortex and hippocampus (Ishihara et al. 1992) while VPAC2R has a wider distribution and is expressed in the olfactory bulb, hypothalamus, hippocampus, brainstem, pituitary and cerebellum (Lutz et al. 1993, Usdin et al. 1994, Sheward et al. 1995). Interestingly, high levels of VPAC2R were detected in the suprachiasmatic nucleus and it is possible that this receptor is involved in the control of circadian rhythm (Piggins et al. 1995, Shen et al. 2000). Recently, a frog PACAP/VIP receptor (fPVVR) (Alexandre et al. 1999) which is structurally more related to the mammalian VPAC1R was isolated. Both the pharmacological properties and tissue distribution of this fPVVR were found to overlap with the mammalian VPAC1R and VPAC2R. For example, secretin, which is a pharmacological agent to distinguish VPAC1R from VPAC2R, was unable to stimulate fPVVR, suggesting that amphibians may have a different VIP/PACAP ligand–receptor model when compared with the mammalian systems. In this report, the first non-mammalian VPAC2R was isolated from an intestinal cDNA library of a local Hong Kong frog species, *Rana tigrina rugulosa*. The pharmacological profiles as well as the distribution of this frog VPAC2R (fVPAC2R) were compared with those of human VPAC2R (hVPAC2R). Together with the fPVVR previously isolated, our study should provide a more comprehensive picture of the functions of these important neuropeptides and receptors in amphibians.

**MATERIALS AND METHODS**

**Isolation of fVPAC2R cDNA**

A partial cDNA clone corresponding to the transmembrane domains (TMDs) 2–6 of the putative fVPAC2R was obtained by a two-step PCR method essentially following a protocol described earlier (Chow et al. 1997). In brief, PCR amplification was performed using 50 pmol G2 and G7 primers (Chow et al. 1997) and 150 ng frog intestinal ZAP-Express cDNA library, which was constructed using the ZAP-Express cDNA synthesis kit (Stratagene, La Jolla, CA, USA), was used as the template in the presence of 200 µM dNTPs, 1·5 mM MgCl2 and 1 U Taq DNA polymerase. The PCR products were re-amplified by G2 and G6 primers using similar PCR conditions (1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 30 cycles) in a Robocycler Gradient 40. The primers G2, G6 and G7 were designed based on the conserved regions of the 2nd, 6th and 7th TMDs of mammalian VIP/PACAP/secretin receptors. A putative partial fVPAC2R cDNA was sequenced on both strands by ALF Express (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) using the Cy5 Auto-read sequencing kit (Pharmacia LKB Biotechnology) and was identified by phylogenetic analysis using GeneWorks software (IntelliGenetics, Mountain View, CA, USA).

The 32P-labeled partial fVPAC2R cDNA fragment was used as a probe to screen the frog intestinal cDNA library essentially following the protocol described earlier (Chow et al. 1997). A full-length cDNA encoding the putative fVPAC2R was isolated and excised to the phagmid,
For efficient expression of the putative receptor in mammalian cells, a NheI/XhoI restriction fragment containing the bacterial lac gene promoter was released from the clone to generate pBK-CMV-fVPAC2R. For functional assays, the receptor (6 µg pBK-CMV-fVPAC2R) was permanently transfected into 1 x 10⁶ Chinese hamster ovary (CHO) cells using the lipofectamine reagent (GIBCO/BRL, Carlsbad, CA, USA) followed by G418 selection (500 µg/ml, GIBCO/BRL, Carlsbad, CA, USA) for 2 weeks. Similarly, hVPAC2R cDNA (a gift from Dr S Mojsov, Rockefeller University, NY, USA) (Wei & Mojsov 1996) was also permanently transfected into CHO cells. Transfected cells were grown in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% CO₂ at 37 °C. cAMP assays were performed as described earlier (Chow et al. 1997). cAMP levels were measured by an RIA using the NEN cAMP assay kit (Amersham, Arlington Heights, IL, USA). All peptides used in the study were purchased from Bachem Fine Chemicals, Inc. (Torrance, CA, USA). fVIP and fPACAP38 were synthesized as previously described (Yon et al. 1994).

### Tissue distribution of fVPAC2R mRNA

Distribution of fVPAC2R transcripts in various peripheral tissues and brain regions was studied by an RT-PCR approach coupled to Southern blot analysis. Poly (A)⁺ RNA (1 µg) was used for the preparation of first strand cDNAs (Chomczynski & Sacchi 1987). PCR was performed using sequence specific primers fVIP-forward: 5’ CTCAATCCTT GCCCTTTAGTGCTACG 3’ and fVIP-reverse: 5’ GGATGTAATTTCCTGGTACAATGCA 3’. PCR conditions were 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 30 cycles. The probe used for Southern blot analysis was a 273 bp partial fVPAC2R cDNA fragment (from nucleotide 294 to 566).

### RESULTS

#### Isolation of fVPAC2R cDNA

A partial cDNA clone corresponding to TMDs 2–6 of fVPAC2R was obtained by a two-step PCR approach (Chow et al. 1997). The amino acid sequences of the clone were deduced and the phylogenetic relationship of the clone was studied (data not shown) by the GeneWorks software. The identity of this partial receptor is further suggested by the partial VIP/PACAP receptor cDNA clone previously isolated from the same species (Chow et al. 1997) which shares 100% amino acid sequence identity with fPVR. The partial fVPAC2R cDNA clone was then used as a probe to screen a frog intestinal cDNA library (0·5 million primary clones). Ten positive clones were obtained and analyzed and one of them, 1673 bp in length, was found to contain the full-length fVPAC2R with an open reading frame of 1305 bp (from nucleotide 77 to 1381) encoding a protein of 435 amino acids with a predicted molecular mass of 50 kDa. Kyte–Doolittle hydrophobicity analysis (data not shown) indicated that the protein is a G protein-coupled receptor with seven segments of hydrophobic amino acids, presumably forming the putative transmembrane-spanning regions. In addition, there is a hydrophobic signal peptide and hydrophilic cytoplasmic domains at the N- and the C-terminus of the receptor. The amino acid sequence of the putative fVPAC2R shares the highest level of homology with hVPAC2R (62·5%) (Fig. 1) and followed by rat (61·5%) and mouse (61·5%) VPAC2Rs (data not shown). On the other hand, fVPAC2R and fPVR share only 47·7% amino acid sequence identity. Phylogenetic analysis by protein alignment also revealed that the putative fVPAC2R is more related to the mammalian VPAC2R while fPVR is more related to the mammalian VPAC1R when compared with other members in the same gene family (Fig. 2). Altogether, there are 13 conserved Cys residues, and among them, ten were located in various extracellular domains including the N-terminal ectodomain (seven, positions 25, 38, 53, 62, 76, 94 and 110), the first exoloop (one, position 158) and the second exoloop (two, positions 192 and 199). In addition, three putative N-glycosylation sites (Asn-X-Ser/Thr) were identified within the N-terminal ectodomain. The motif ‘RLAK/R’, which is conserved in all VIP/PACAP receptors characterized in teleosts and mammals (Chow et al. 1997), located at the C-terminal junction of the 6th TMD, is also present in this receptor with a single amino acid substitution to ‘RLTK’.

#### Functional expression and tissue distribution of fVPAC2R

To demonstrate that the isolated cDNA clone encodes a functional fVPAC2R which is able to transduce a physiological signal, cAMP production
in response to frog peptide activation (VIP, PACAP38 and PACAP27) was measured (Fig. 3). All three peptides were able to stimulate cAMP accumulation in a dose-dependent manner and they exhibited nearly the same potencies and maximal responses. The EC$_{50}$ values of fVIP, fPACAP38 and fPACAP27 were 0.15, 0.18 and 0.16 µM respectively. The pharmacological profiles (Fig. 4) of the frog receptor were compared with those of hVPAC$_2$R using human peptides including VIP, PACAP38, PHM and secretin. It was found that hVIP and hPACAP38 (note: frog and human PACAP27s are identical) were able to stimulate both receptors but the EC$_{50}$ values of these two peptides toward fVPAC$_2$R were much higher than those for hVPAC$_2$R. PHM can also stimulate these receptors but is lower in potency and efficacy. On the other hand, secretin is unable to stimulate these receptors even at a concentration of 5 µM. The distribution of fVPAC$_2$R in various peripheral tissues and brain regions was studied by RT-PCR coupled to Southern blot analysis. Hybridization signals were observed in all the tissues examined with relatively stronger signals in the lung, stomach, small intestine and colon (Fig. 5). In the brain, strong hybridization signals were present in all the regions examined with a relatively weaker signal in the olfactory bulb (Fig. 6).

**DISCUSSION**

In addition to the PAC$_1$R recently identified in frog (Hu et al. 2000), a VIP/PACAP type II receptor.
(fPVR) has also been previously characterized. Interestingly, fPVR exhibited pharmacological characteristics and a tissue distribution pattern that overlap with both type II receptors, VPAC₁R and VPAC₂R, found in mammals (Alexandre et al. 1999). It was therefore of great interest to investigate if a second form of VIP/PACAP type II receptor is also present in amphibians. If it is, the

![Phylogenetic tree](image)

Figure 2. Phylogenetic analysis (unweighted pair group method with arithmetic mean tree) of fVPAC₂R with other members in the secretin/glucagon receptor family. The tree was generated using the default setting of GeneWorks protein alignment program: the cost to open a gap was 5, the cost to lengthen a gap was 25, the minimum diagonal length was 4 and the maximum diagonal offset was 10. The alignment program scores mismatches rather than matches; in other words, the lower the score, the better the alignment. The score between two protein sequences, which is a measure of the relative phylogenetic relationships between the two receptors, is represented by horizontal distance in this tree. References: receptor sequences for hVPAC₁R (Sreedham et al. 1993), rat VPAC₁R (rVPAC₁R) (Ishihara et al. 1992), fPVR (Alexandre et al. 1999), goldfish VPAC₁R (gfVPAC₁R) (Chow et al. 1997), hVPAC₂R (Svoboda et al. 1994), rVPAC₂R (Lutz et al. 1993), hPAC₁R (Ogi et al. 1993), rPAC₁R (Pisegna & Wank 1993), fPAC₁R (Hu et al. 2000), gfPAC₁R (Wong et al. 1998) and frog glucagon R (fGLUR) (Ngan et al. 1999).
The next question that we can ask is if the second form of VIP/PACAP type II receptor possesses characteristics similar to one of the type II receptors found in mammals or if it possesses distinctive properties as indicated in fPVR. The answers to these questions are of importance to our future understanding of the functional evolution of VIP and PACAP in vertebrates.

In this study, the first non-mammalian VPAC2R was characterized from the frog *R. tigrina rugulosa*. There are several lines of evidence suggesting that the receptor characterized is indeed a species variant of the mammalian VPAC2R. The most direct one is based on sequence comparison and phylogenetic studies of these receptors. The predicted amino acid sequence of fVPAC2R shares high levels of homology (>60%) with human, rat and mouse VPAC2Rs. fVPAC2R, on the other hand, shares only 47.7% sequence identity with fPVR. Phylogenetic studies (Fig. 2) also grouped fVPAC2R with other VPAC2Rs to form a sub-branch, again indicating that the identified receptor is structurally closely related to mammalian VPAC2Rs.

The putative TMDs of the two frog VIP receptors are the most conserved (59-7%) while their N-terminal sequences are less similar (27-9%). The N-terminal ectodomain of this receptor family is largely responsible for ligand specificity. Previous studies (Couvineau *et al.* 1995, Nicole *et al.* 1998) indicated that the amino acid residues that are responsible for VIP binding in hVPAC1R and hVPAC2R are different (Glu36, Asp68, Trp73 and Gly109 for hVPAC1R and Glu24 and Ile31 for hVPAC2R). fPVR contains all four VIP-binding amino acids that are found in hVPAC1R while fVPAC2R possesses only Glu24 but not Ile31 present in hVPAC2R. This observation suggested that: (i) the key amino acid residues responsible for VIP interaction in these two types of receptors are conserved during evolution; (ii) hVPAC1R and fPVR share similar mechanisms for VIP interaction; and (iii) fVPAC2R may not be able to bind mammalian VIP.

Thirteen Cys residues within fVPAC2R were found to be conserved with other VIP/PACAP receptors including fPVR. Some of these Cys residues are probably instrumental in proper folding and hence function of these receptors by...
forming disulfide linkages. It is interesting to note that a Cys residue preceding the ‘IRIL’ motif within TMD 5 is unique to VPAC₁R and is not present in other members within the same family. This Cys residue is also found in fPVR, again suggesting that fPVR may have the same origin as other VPAC₁Rs.

Three N-linked glycosylation consensus sites (Asn-X-Ser/Thr) are located at the N-terminal ectodomain of fVPAC₂R, and among them two (Asn59, Asn89) are conserved in human (PAC₁, VPAC₁ and VPAC₂) and frog PVRs. Asn59 has been shown to be essential for the correct delivery of the receptor to plasma membrane (Couvineau et al. 1996). The third glycosylation site (Asn113) is unique to fVPAC₂R; it is possible that this putative site may contribute to different functional and/or structural properties of this receptor. The motif ‘RLAK/R’ is located in front of the 6th TMD of all members in the secretin/glucagon receptor family from teleosts to mammals. However, in fVPAC₂R, the alanine (non-polar) residue in the ‘RLAK’ motif is replaced by a threonine (polar) residue. The functional importance of this substitution remains to be studied.

As structural and phylogenetic analyses indicated that the newly identified receptor is a fVPAC₂R, we next sought to investigate the functional properties of this receptor. Initially, frog peptides including VIP, PACAP38 and PACAP27 (Chartrel et al. 1991, 1995) were used to stimulate the putative fVPAC₂R permanently expressed in CHO cells. All these peptides showed almost the same potency and efficacy in stimulating intracellular cAMP accumulation. The EC₅₀ values of these peptides are about 0.15 µM, which is about two orders of magnitude higher than those of hVPAC₁R (Sreedharan et al. 1993). In fPVR (Alexandre et al. 1999), these peptides also showed high EC₅₀ values (~30 nM). The reason for the relatively higher EC₅₀ values in frog receptors is unclear. It is possible that the frog receptors are less compatible with the mammalian G protein-coupling mechanisms present in mammalian cells. Further experimentation is needed to confirm this hypothesis.

Human peptides, including VIP, PACAP38, PHM and secretin, were employed to functionally compare the frog and human VPAC₂Rs. In general, these peptides were a lot less potent in stimulating the frog receptor. Nevertheless, they showed a similar order of potency; VIP and PACAP had higher affinities than PHM. It is likely that the lower potency of hVIP to fVPAC₂R is due to structural changes of the ligand. There are four amino acid substitutions among human and frog VIPs (Thr11 Ser, Leu13 Phe, Ile26 Val and Asn28 Thr) (Chartrel et al. 1995). Moreover, Thr11 and Asn28 in hVIP are found to be the key residues for its interaction with hVPAC₂R (Nicole et al. 2000). In addition, as discussed earlier, one of the key residues (Ile31) in hVPAC₂R responsible for VIP interaction has been changed to Leu, and this may further account for the lack of binding of hVIP to fVPAC₂R. This observation further indicates the importance of using homologous peptides for studying the pharmacological profiles of receptors isolated in different species.

Secretin was used in the study as secretin is known to be a weak agonist of VPAC₁R (Robberecht et al. 1996). Consistently, human secretin was unable to stimulate both human and frog VPAC₂Rs. Together with the fact that this peptide was unable to activate fPVR, it is possible that (i) the structure of frog and human secretin are very different, hence human secretin is unable to pharmacologically distinguish the two frog receptors; or (ii) neither form of frog VIP/PACAP type II receptors can bind secretin.
As the tissue distributions of VPAC₁R and VPAC₂R were found to be quite different in rat (Ishihara et al. 1992, Usdin et al. 1994, Sheward et al. 1995), in this study, the distribution of fVPAC₂R was investigated. Strong hybridization signals were detected in the lung and gastrointestinal tissues such as the colon, small intestine and stomach. In rat, both VPAC₁R and VPAC₂R are expressed in the lung; VIP is one of the most important non-adrenergic-non-cholinergic inhibitory transmitters in this tissue. VIP controls airway relaxation, water-electrolyte and mucus secretion as well as the pulmonary microvascular system (Said 1995). It is also important to the defense mechanisms in the lung by promoting migration and proliferation of lymphocytes (Kaltreider et al. 1997). The function of VIP in the small intestine is to stimulate water and electrolyte secretion from the mucosa (Said 1982). In the colon, VIP acts to relax longitudinal muscle (Ekbald 1999). Consistent with previous studies in the rat, fVPAC₂R is expressed in the stomach.

In order to compare the distribution of fVPAC₁R and fVPAC₂R transcripts in various regions of the brain, we performed an RT-PCR study. Strong hybridization signals were detected in all the brain regions examined. Our result is consistent with previous studies in mammals (Usdin et al. 1994, Sheward et al. 1995); the rat VPAC₂R is expressed throughout the central nervous system including the pituitary, olfactory bulb, telencephalon (including cerebral cortex), hypothalamus, thalamus, diencephalons (including optic lobe), cerebellum and spinal cord. Recently, both VPAC₁R and VPAC₂R were found in the olfactory bulb but were expressed at different layers: VPAC₁R is present in the external plexiform layer while VPAC₂R is located in the internal granular layer (Sheward et al. 1995). Their presence in this region of the brain is believed to influence the olfactory bulb output by modulating the activities of the internal granule cells. The expression of these receptors in the cerebral cortex had been controversial. Although some reports (Lutz et al. 1993, Sheward et al. 1995) claimed that VPAC₂R is not present in the cerebral cortex, Usdin et al. (1994) showed that VPAC₁R is abundant in layer III and V, while VPAC₂R is present exclusively in layer VI of the cortex. It was proposed that these receptors in the cerebral cortex are involved in the regulation of energy metabolism. In the hypothalamus, VPAC₁R is not expressed while VPAC₂R is present at high levels in the suprachiasmatic nucleus and there was evidence suggesting the involvement of this receptor in regulating circadian rhythm (Piggins et al. 1995, Shen et al. 2000). In summary, our results are consistent with previous reports; the fVPAC₂R homologue is widely distributed in both peripheral tissues and various regions of the brain.

As the cloned receptor exhibits structural and pharmacological characteristics as well as tissue distribution pattern that are similar to those of the mammalian VPAC₂Rs, our data strongly indicated that the frog receptor isolated in the present study is a novel VPAC₂R in amphibians. We believe that amphibians have both forms of VIP/PACAP type II receptors. By structural and functional studies, fPVR previously characterized is probably an amphibian VPAC₁R, although paradoxically, the distribution of fPVR in the brain is more similar to that of mammalian VPAC₂R than VPAC₁R. Some of the unique features possessed by fPVR or fVPAC₂R may contribute to the functional differences of VIP and PACAP in various species in vertebrates.

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