Structural and functional identification of the pituitary adenylate cyclase-activating polypeptide receptor VPAC₂ from the frog *Rana tigrina rugulosa*

R L C Hoo, D Alexandre¹, S M Chan, Y Anouar¹, R T K Pang, H Vaudry¹ and B K C Chow

Department of Zoology, University of Hong Kong, Pokfulam Road, Hong Kong, China

¹European Institute for Peptide Research, Laboratory of Cellular and Molecular Neuroendocrinology, INSERM U-413, UA CNRS, University of Rouen, Mont-Saint-Aignan, France

(Requests for offprints should be addressed to B K C Chow, Department of Zoology, University of Hong Kong, Pokfulam Road, Hong Kong, SAR, China; Email: bkcc@hkusua.hku.hk)

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 VPAC₁R and VPAC₂R. In order to investigate the receptors responsible for mediating the actions of VIP and PACAP in amphibians, in this report, a frog VPAC₂ receptor (fVPAC₂R) cDNA was isolated. fVPAC₂R shares 47.7, 46.9 and 62.5% amino acid sequence identity with fPVR, human VPAC₁R and human VPAC₂R respectively. Functionally, fVPAC₂R, when expressed in CHO cells, was responsive to both frog peptides including VIP, PACAP38 and PACAP27 where the EC₅₀ 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 VPAC₂Rs were compared, and it was found that these peptides could only activate the frog receptor at micromolar concentrations. fVPAC₂R 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.

Journal of Molecular Endocrinology (2001) **27**, 229–238

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 insulintropic 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 (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 (PAC₁) 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.

VPAC₁R 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, Krempels *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 (fPVR) (Alexandre *et al.* 1999) which is structurally more related to the mammalian VPAC₁R was

isolated. Both the pharmacological properties and tissue distribution of this fPVR 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 fPVR, 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 fPVR 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,

pBK-CMV-fVPAC₂R1. The clone was sequenced and analyzed by DNasis v2.4 (Hitachi, San Bruno, CA, USA).

Functional studies of fVPAC₂R

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 1×10^6 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 500 µg/ml G418 in a humidified atmosphere of 95% air 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' CTCAATCTT GCCCTTTAGTGCTACG 3' and fVIP-reverse: 5' GGATGTAGTTCCTGGTACAATGCAG 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

FIGURE 1. Amino acid sequence alignment of fVPAC₂R with fPVR, hVPAC₁R, hVPAC₂R and hPAC₁R by GeneWorks. The arrows represent the seven putative TMDs. Identical and conservative sequences are boxed. Conserved N-linked glycosylation sites and Cys residues among these receptors are labeled '#' and '*' respectively.

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 EC₅₀ values of fVIP, fPACAP38 and fPACAP27 were 0.15, 0.18 and 0.16 μ M respectively. The pharmacological profiles (Fig. 4) of the frog receptor were compared with those of hVPAC₂R 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 EC₅₀ values of these two peptides toward fVPAC₂R were much higher than those for hVPAC₂R. 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 μ M. The distribution of fVPAC₂R 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 PAC₁R recently identified in frog (Hu *et al.* 2000), a VIP/PACAP type II receptor

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).

(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

FIGURE 3. Measurement of cAMP accumulation in CHO cells permanently expressing fVPAC₂R (see Materials and Methods). Transfected cells were incubated with increasing concentrations of frog peptides: VIP (■), PACAP38 (▲) and PACAP27 (▼). Data are expressed as the means $\pm$ s.e.m. of three independent experiments each in triplicate.

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

FIGURE 4. Measurement of cAMP accumulation in CHO cells permanently expressing (A) fVPAC₂R or (B) hVPAC₂R. Transfected cells were incubated with graded concentrations of human peptides, VIP (■), PACAP38 (▲), PHM (▼) and secretin (◆). Data are expressed as the means $\pm$ s.e.m. of three independent experiments each in triplicate.

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

FIGURE 5. Tissue distribution study of fVPAC₂R by RT-PCR analysis. (A) Agarose gel analysis of the 273 bp PCR products amplified from various frog tissues using fVPAC₂R specific primers (see Materials and Methods) and (B) Southern blots analysis of the agarose gel using a partial fVPAC₂R cDNA as probe.

forming disulfide linkages. It is interesting to note that a Cys residue preceding the 'IIRIL' 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.

FIGURE 6. Brain distribution study of fVPAC₂R by RT-PCR analysis. (A) Agarose gel analysis of the 273 bp PCR products amplified from various frog brain regions using fVPAC₂R specific primers (see Materials and Methods) and (B) Southern blots analysis of the agarose gel using a partial fVPAC₂R cDNA as a probe.

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.

ACKNOWLEDGEMENTS

We would like to thank Mr C M Yeung, Mr Dicky L Y Tse and Mr Raymond K H Hui for their excellent technical assistance. This work was supported by Research Grants Council HKU7181/99M and Committee on Research and Conference Grants 10203410 (to B K C C).

REFERENCES

- Alexandre D, Anouar Y, Jegou S, Fournier A & Vaudry H 1999 A cloned frog vasoactive intestinal polypeptide/pituitary adenylate cyclase activating polypeptide receptor exhibits pharmacological and tissue distribution characteristics of both VPAC₁ and VPAC₂ receptors in mammals. *Endocrinology* **143** 1285–1293.
- Besson J, Sarrieau A, Vial M, Marie J-C, Rosselin G & Rostene W 1986 Characterization and radiographic distribution of vasoactive intestinal peptide binding sites in rat central nervous system. *Brain Research* **398** 329–336.
- Chartrel N, Tonon MC, Vaudry H & Conlon JM 1991 Primary structure of frog pituitary adenylate cyclase-activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. *Endocrinology* **129** 3367–3371.
- Chartrel N, Wang Y, Fournier A, Vaudry H & Conlon JM 1995 Frog vasoactive intestinal polypeptide and galanin: primary structures and effects on pituitary adenylate cyclase. *Endocrinology* **136** 3079–3086.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Chow BKC, Yuen TTH & Chan KW 1997 Molecular evolution of vertebrate VIP receptors and functional characterization of a VIP receptor from goldfish *Carassius auratus*. *General and Comparative Endocrinology* **105** 176–185.
- Christophe J 1993 Type I receptors for PACAP (a neuropeptide even more important than VIP?). *Biochimica et Biophysica Acta* **1154** 183–199.

- Couvineau A, Gaudin P, Maoret JJ, Rouyer-Fessard C, Nicole P & Laburthe M 1995 Highly conserved aspartate 68, tryptophan 73 and glycine 109 in the N-terminal extracellular domain of the human VIP receptor are essential for its ability to bind VIP. *Biochemical and Biophysical Research Communications* **206** 246–252.
- Couvineau A, Fabre C, Gaudin P, Maoret JJ & Laburthe M 1996 Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry* **35** 1745–1752.
- Dickinson T & Fleetwood-Walker SM 1999 VIP and PACAP: very important in pain? *Trends in Pharmacological Sciences* **20** 324–329.
- Ekbald K 1999 Pharmacological evidence for both neuronal and smooth muscular PAC₁ receptors and a VIP-specific receptor in rat colon. *Regulatory Peptides* **85** 87–92.
- El-Gehani F, Tena-Sempere M & Huhtaniemi I 1998 Vasoactive intestinal peptide is an important endocrine regulatory factor of fetal rat testicular steroidogenesis. *Endocrinology* **139** 1474–1480.
- Gozes I & Brenneman DE 1989 VIP: molecular biology and neurobiological function. *Molecular Neurobiology* **3** 201–236.
- Gozes I, Fridkinb M, Hill JM & Brenneman DE 1999 Pharmaceutical VIP: prospects and problems. *Current Medical Chemistry* **6** 1019–1034.
- Hu Z, Lelievre V, Chao A, Zhou X & Waschek JA 2000 Characterization and messenger ribonucleic acid distribution of a cloned pituitary adenylate cyclase-activating polypeptide type I receptor in the frog *Xenopus laevis* brain. *Endocrinology* **141** 657–665.
- Ishihara T, Nakamura S, Kazino Y, Takahashi T, Takahashi K & Nagata S 1991 Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO Journal* **7** 1635–1641.
- Ishihara T, Shigemoto R, Mori K, Takahashi K & Nagata S 1992 Functional expression and tissue distribution of a novel receptor for vasoactive intestinal peptide. *Neuron* **8** 811–819.
- Ishioka C, Yoshida A, Kimata H & Mikawa H 1992 Vasoactive intestinal peptide stimulates immunoglobulin production and growth of human B cells. *Clinical and Experimental Immunology* **87** 504–508.
- Kaltreider HB, Ichikawa S, Byrd PK, Ingram DA, Kishiyama JL, Sreedharan SP, Warnock ML, Beck JM & Goetzl EJ 1997 Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. *American Journal of Respiratory Cell and Molecular Biology* **16** 133–144.
- Krepels K, Usdin TB, Harta G & Mezey E 1995 PACAP acts through VIP type 2 receptors in the rat testis. *Neuropeptides* **29** 315–320.
- Laburthe M, Couvineau A & Voisin T 1999 Receptors for peptides of the VIP/PACAP and PYY/NPY/PP families. In *Gastrointestinal Endocrinology*, pp 125–157. Ed. GH Greenly. Totowa, NJ, Humana Press Inc.
- Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G & Harmar AJ 1993 The VIP2 receptor: molecular characterization of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Letters* **334** 3–8.
- Martin JL, Dietl MM, Hof PR, Palacios JM & Magistretti PJ 1987 Autoradiographic mapping of [mono¹²⁵I]iodo-Tyr¹⁰,Met⁰¹⁷] vasoactive intestinal peptide binding sites in the rat. *Neuroscience* **23** 539–565.
- Miyata A, Arimura A, Dahi RR, Minamino N, Uehara A, Jiang L, Culler MD & Coy DH 1989 Isolation of a novel 38 residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochemical and Biophysical Research Communications* **164** 567–574.
- Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N & Arimura A 1990 Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38-residues (PACAP 38). *Biochemical and Biophysical Research Communications* **171** 643–648.
- Ngan ES, Chow LS, Tse DL, Du X, Wei Y, Mojsov S & Chow BK 1999 Functional studies of a glucagon receptor isolated from frog *Rana tigrina rugulosa*: implications on the molecular evolution of glucagon receptors in vertebrates. *FEBS Letters* **457** 499–504.
- Nicole P, Du K, Couvineau A & Laburthe M 1998 Site-directed mutagenesis of human VIP1 versus VIP2 receptors. *Annals of the New York Academy of Sciences* **865** 378–381.
- Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, Thomas A, Couvineau A, Martinez J, Brasseur R & Laburthe M 2000 Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 receptor agonist. Alanine scanning and molecular modeling of the peptide. *Journal of Biological Chemistry* **275** 24003–24012.
- Ogi K, Miyamoto Y, Masuda Y, Habata Y, Hosoya M, Ohtaki T, Masuo Y, Onda H & Fujino M 1993 Molecular cloning and functional expression of a cDNA encoding a human pituitary adenylate cyclase activating polypeptide receptor. *Biochemical and Biophysical Research Communications* **196** 1511–1521.
- Ottaway CA 1987 Selective effects of vasoactive intestinal peptide on the mitogenic response of murine T cells. *Immunology* **62** 291–297.
- Piggins HD, Antle MC & Rusak B 1995 Neuropeptides phase shift the mammalian circadian pacemaker. *Journal of Neuroscience* **15** 5612–5622.
- Pisegna JR & Wank SA 1993 Molecular cloning and functional expression of pituitary adenylate cyclase-activating polypeptide type I receptor. *PNAS* **90** 6345–6349.
- Robberecht P, Gourlet P, Vertongen P & Svoboda M 1996 Characterization of the VIP receptor from SUP T1. *Advanced Neuroimmunology* **135** 2569–2573.
- Said SI 1982 In *Advances in Peptide Hormones Research*, vol 1. Ed. SI Said. New York: Raven Press.
- Said SI 1991 Vasoactive intestinal polypeptide (VIP) in lung function and disease. *Nihon Kyobu Shikkan Gakkai Zasshi* **29** 1525–1531.
- Said SI 1995 Airways smooth muscle: peptide receptors, ion channels and signal transduction. In *Vasoactive Intestinal Polypeptide*, pp 87–113. Eds D Raebum & MA Giembycz. Basel, Switzerland: Birkhauser Verlag.
- Said SI 1998 Anti-inflammatory actions of VIP in lungs and airways. In *Pro-inflammatory and Anti-inflammatory Peptides*, Vol. 112, pp 345–361. Ed. Said SI. In the series *Lung Biology in Health and Disease*. Series Ed. Lenfant C. New York: Marcel Dekker, Inc.
- Said SI & Mutt V 1970 Polypeptide with broad biological activity: isolation from small intestine. *Nature* **225** 863–864.
- Shen S, Spratt C, Sheward WJ, Kallo I, West K, Morrison CF, Coen CW, Marston HM & Harmar AJ 2000 Overexpression of the human VPAC₂ receptor in the suprachiasmatic nucleus alters the circadian phenotype of mice. *PNAS* **97** 11575–11580.
- Sheward WJ, Lutz EM & Harmar AJ 1995 The distribution of VIP2 receptor messenger RNA in the rat brain and pituitary gland assessed by *in situ* hybridization. *Neuroscience* **67** 409–418.

- Sreedharan SP, Robichon A, Peterson KE & Goetzl EJ 1991 Cloning and expression of the human vasoactive intestinal peptide receptor. *PNAS* **88** 4986–4990.
- Sreedharan SP, Patel DR, Huang JX & Goetzl EJ 1993 Cloning and functional expression of a human neuroendocrine vasoactive intestinal peptide receptor. *Biochemical and Biophysical Research Communications* **93** 546–553.
- Svoboda M, Tastenoy M, Rampelbergh JV, Goossens JF, Beef PD, Waelbroeck M & Robberecht P 1994 Molecular cloning and functional characterization of a human VIP receptor from SUP-T1 lymphoblasts. *Biochemical and Biophysical Research Communications* **205** 1617–1624.
- Usdin TB, Mezey E, Button DC, Brownstein MJ & Bonner TI 1993 Gastric inhibitory polypeptide receptor, a member of the secretin–vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain. *Endocrinology* **133** 2861–2870.
- Usdin TB, Bonner TI & Mezey E 1994 Two receptors for VIP with similar specificity and complementary distributions. *Endocrinology* **135** 2662–2680.
- Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A & Vaudry H 2000 Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological Review* **52** 269–324.
- Wei Y & Mojsov S 1996 Multiple human receptors for pituitary adenylyl cyclase-activating polypeptide and vasoactive intestinal peptide are expressed in a tissue-specific manner. *Annals of the New York Academy of Sciences* **805** 624–627.
- Wong AO, Leung MY, Shea WL, Tse LY, Chang JP & Chow BK 1998 Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. *Endocrinology* **139** 3465–3479.
- Yon L, Chartrel N, Feuilloley M, De Marchis S, Fournier A, De Rijk E, Pelletier G, Roubos E & Vaudry H 1994 Pituitary adenylate cyclase-activating polypeptide stimulates both adrenocortical cells and chromaffin cells in the frog adrenal gland. *Endocrinology* **135** 2749–2758.

RECEIVED IN FINAL FORM 14 May 2001

ACCEPTED 13 June 2001