Molecular Cloning and Characterization of Two Pig Vasoactive Intestinal Polypeptide Receptors (VPAC₁-R and VPAC₂-R)

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We here report the cloning, tissue expression, and functional analyses of the two pig vasoactive intestinal polypeptide (VIP) receptors (pVPAC₁-R and pVPAC₂-R). The cloned full-length pVPAC₁-R and pVPAC₂-R share high structural similarity with their mammalian counterparts. Functional assay revealed that the full-length pVPAC₁-R and pVPAC₂-R-expressed Chinese hamster ovary (CHO) cells could be activated by pVIP and pPACAP₃₈ potently, indicating that pVPAC₁-R and pVPAC₂-R are capable of binding VIP and pituitary adenylate cyclase-activating polypeptide (PACAP). In addition to the identification of the transcripts encoding the two full-length receptors, multiple splice transcript variants were isolated. Comparison with the pig genome database revealed that pVPAC₁-R and pVPAC₂-R share a unique gene structure with 14 exons different from other vertebrates. Reverse transcription and polymerase chain reaction (RT-PCR) assays further showed that the transcript encoding the full-length pVPAC₂-R is widely expressed in all adult tissues whereas the splice variants of pVPAC₁-R are predominantly expressed in all tissues instead of the transcript encoding the full-length receptor, hinting that pVPAC₂-R may play more important roles than pVPAC₁-R in mediating VIP and PACAP actions. Our present findings help to elucidate the important role of VIP and PACAP and promote to rethink of their species-specific physiological roles including their actions in regulation of phenotypic traits in pigs.

Introduction

VASOACTIVE INTESTINAL PEPTIDE (VIP) is a 28-amino acid peptide, which was first isolated from porcine intestine and has been implicated in a broad range of biological processes, such as induction of vasodilation in the canine femoral artery, and its roles as a neuroendocrine hormone and putative neurotransmitter in vertebrates (Said and Mutt, 1970, 1972; Harmar *et al.*, 1998). Another structurally related peptide, pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that was first isolated from ovine hypothalamic extracts and exerts pleiotropic physiological functions, including gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) release in the anterior pituitary (Hart *et al.*, 1992; Osuga *et al.*, 1992; Propato-Mussafiri *et al.*, 1992), neurotransmitter release in the central nervous system (CNS) (Huang *et al.*, 1996), and steroid hormone secretion in adrenal glands (Watanabe *et al.*, 1990, 1992). Belonging to the same VIP/glucagon/GHRH/secretin superfamily, both VIP and PACAP share high similarity in structure and function (Sherwood *et al.*, 2000).

It is reported that, in mammals, the biological actions of VIP and PACAP are mediated by three receptors, namely PAC₁-R, VPAC₁-R, and VPAC₂-R (Harmar *et al.*, 1998). PAC₁-R binds PACAP with a 100-fold higher affinity than VIP and is hence viewed as a PACAP-specific receptor. In contrast, VPAC₁-R and VPAC₂-R bind VIP and PACAP with equally high affinity, and thus they are believed to function as receptors common for VIP and PACAP (Harmar *et al.*, 1998; Dickson and Finlayson, 2009). VPAC₁-R and VPAC₂-R belong to G protein-coupled receptor (GPCR) family B and share its characteristic structures including seven-transmembrane domains and a large N-terminal domain proposed for ligand binding (Ulrich *et al.*, 1998). In general, the two receptors function through adenylate cyclase (AC)-mediated signaling pathway upon ligand binding (Dickson and Finlayson, 2009).

The VPAC₁-R gene was first cloned from rat lung cDNA library (Ishihara *et al.*, 1992). Later, the VPAC₁-R cDNA was further identified in other vertebrate species including humans, chicken, frogs, and zebrafish (Sreedharan *et al.*, 1993, 1995; Alexandre *et al.*, 2000; Cardoso *et al.*, 2004; Fradinger *et al.*, 2005). The human VPAC₁-R gene encodes 457 amino

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acids with the deduced molecular weight of 52 kDa. Mapped on the chromosome 3p23-21, it spans across a whole length of 22 kb and contains 13 exons (from 42 to 1479 bp) and 12 introns (from 0.3 to 6.1 kb) (Sreedharan *et al.*, 1995). In humans, VPAC₁-R is expressed in the lungs in high abundance and detected in other tissues including prostate, peripheral blood leukocytes, liver, brain, small intestine, colon, heart, spleen, placenta, kidneys, thymus, and testes (Sreedharan *et al.*, 1993). In addition, it is also expressed in T lymphocytes and peripheral blood monocytes (Delgado *et al.*, 1996; Ganea, 1996; Lara-Marquez *et al.*, 2001).

The VPAC₂-R was initially cloned from a rat pituitary cDNA library and then from a human placenta cDNA library (Lutz *et al.*, 1993; Adamou *et al.*, 1995). The full-length cDNA of human VPAC₂-R is 1317 bp in length encoding a protein of 438 amino acids. In humans, this gene is localized on the chromosome 7q36.3 spanning a region of 117 kb. In line with the gene structure of VPAC₁-R gene, the human VPAC₂-R gene also contains 13 exons. In comparison with the expression profile of VPAC₁-R, the distribution of VPAC₂-R is relatively selective. VPAC₂-R is expressed in human skeletal muscle, intestine, and brain, especially in the thalamus and cerebral cortex (Usdin *et al.*, 1994; Vertongen *et al.*, 1995).

Although VPAC₁-R and VPAC₂-R genes have been reported in several mammalian and nonmammalian species (Ishihara *et al.*, 1992; Lutz *et al.*, 1993; Sreedharan *et al.*, 1993, 1995; Adamou *et al.*, 1995; Alexandre *et al.*, 2000; Cardoso *et al.*, 2004), little is known about their structures and functions in pigs. Our present study aims to clone the full-length cDNA of the two genes, investigate their tissue distribution, and perform functional analyses to examine their potency in ligand binding. As an important medical drug model and a breeding animal of great economic importance, characterization of the two receptors helps to provide comparative insights into their conserved roles across vertebrates.

Materials and Methods

Chemicals and hormones

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), and restriction enzymes were obtained from TaKaRa Biotechnology (Dalian) Co. Ltd (Dalian, China) unless stated otherwise. Pig VIP (pVIP), pig PACAP₃₈ (pPACAP₃₈), pig SCT (pSCT), and pig PHI (pPHI) were synthesized by solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). These hormones were first dissolved and then diluted to various concentrations with culture medium immediately before use.

Total RNA extraction

Adult pigs of landrace strain were purchased from a local farm. Pig tissues (including brain, pituitary, lungs, heart, liver, kidneys, intestine, muscle, spleen, ovary, and testes) were isolated and then quickly frozen in liquid nitrogen. Total RNA was extracted with TRIzol-reagent (Invitrogen Life Technologies Shanghai Corporation, Shanghai, China) according to the manufacturer's instructions. All experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

Reverse transcription and polymerase chain reaction

Reverse transcription (RT) was performed at 42°C for 1.5 h in a total volume of 10 µL consisting of 2 µg total RNA, 5 × MMLV buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), 0.5 µg oligo-deoxythymide, and 100 U MMLV reverse transcriptase (TaKaRa). All negative controls were carried out under the same condition without reverse transcriptase in the 10-µL reaction mix.

The RT products were then diluted to a final volume of 60 µL with MilliQ-water before use. Polymerase chain reaction (PCR) was carried out in a total volume of 10 µL consisting of 1 × PCR buffer, 0.2 mM each dNTP, 0.2 M each primer, and 0.5 U of Taq DNA polymerase (Invitrogen) on the Thermal Cycler (Bio-Rad, Hercules, CA). RT-PCR assays were performed to examine the relative mRNA levels of pVPAC₁-R and pVPAC₂-R in adult pig tissues according to our previously established methods (Wang *et al.*, 2007). For the GAPDH gene, 25 cycles of 2 min at 94°C, 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, followed by 5 min of extension at 72°C. For the pVPAC₁-R and pVPAC₂-R genes, 38 cycles of 2 min at 94°C, 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C were used, followed by 5 min of extension at 72°C. The primers used are listed in Table 1. The PCR products were visualized on a UV-transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA). To confirm the PCR specificity, the identity of each PCR product was verified by sequencing.

Cloning of the cDNAs of pVPAC₁-R and VPAC₂-R genes

Gene-specific primers were designed based on the VPAC₂-R cDNA sequences of *Homo Sapiens* (Accession No.: NM_003382), *Rattus norvegicus* (Accession No.: NM_017238), *Mus musculus* (Accession No.: NM_009511.2), and the reference sequence pVPAC₁-R (Accession No.: U49434) to amplify the full-length cDNA of the two receptors from adult pig brain or liver (Table 1). The amplified PCR products were cloned into pTA2 vector (TOYOBO; Toyobo Co., Ltd., Osaka, Japan) and subjected for sequence analyses (ABI PRISM[®] 3100 Genetic Analyzer). The full-length cDNA of each receptor was finally determined by sequencing at least three independent clones.

Functional characterization of pVPAC₁-R and VPAC₂-R in Chinese hamster ovary cells

The gene-specific primers flanking the start and stop codons (with restriction enzyme recognition site added at their 5' ends) were employed to amplify the open reading frame of each receptor (pVPAC₁-R and pVPAC₂-R). The amplified PCR products were cloned into pTA2 vector. After sequence confirmation, the insert was subcloned into the pcDNA3.1 (+) expression vector (Invitrogen).

To test the functionality of pVPAC₁-R and pVPAC₂-R, the two receptors were transiently expressed in Chinese hamster ovary (CHO) cells and treated by VIP, PACAP, and other structurally related peptides. Receptor activation was then monitored by a pGL3-CRE-luciferase reporter system established in our previous studies (Wang *et al.*, 2007). In brief, the CHO cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (HyClone, Logan, UT), 100 U/mL

TABLE 1. PRIMERS USED

Designation	Sequence	Size (bp)	Orientation
Primers for cloning the full-length cDNAs ^a			
pVPAC ₁ -R-F1	5'-CGGGGTACCGCAGGCTCGCAGCAGACCAT-3'	29	Forward
pVPAC ₁ -R-R1	5'-CCGGAATTCTGCCCCTGGTGCCATGGA-3'	27	Reverse
pVPAC ₂ -R-F1	5'-GGTACCGGCAGCCAGGCCCATGGT-3'	24	Forward
pVPAC ₂ -R-F2	5'-GGTACCCATGGTGGTGCCGCTGAG-3'	24	Forward
pVPAC ₂ -R-R1	5'-GAATTCCATGGCTGGGATGAGCCCA-3'	25	Reverse
Primers for RT-PCR assay			
pVPAC ₁ -R-F2	5'-GCAGCGCAGCTCAGTCAGGCT-3'	21	Forward
pVPAC ₁ -R-R2	5'-TGTGGACCGTCGTCAGAAT-3'	19	Reverse
pVPAC ₂ -R-F3	5'-CTGCTGGGATACCAATGA-3'	18	Forward
pVPAC ₂ -R-R2	5'-CTACAGGCTGCACAGTTCATC-3'	21	Reverse
Primers for cloning, screening, and internal control detection			
pcDNA3.1-F1	5'-CACTGCTTACTGGCTTATCGA-3'	21	Forward
pcDNA3.1-R1	5'-GCACAGTCGAGGCTGATCA-3'	19	Reverse
pTA2-F1	5'-GTAATACGACTCACTATAGGGCG-3'	23	Forward
pTA2-R1	5'-AATTAACCCTCACTAAAGGGA-3'	21	Reverse
GAPDH-F1	5'-ACCACAGTCCATGCCATCAC-3'	20	Forward
GAPDH-R1	5'-TCCACCACCCTGTTGCTGTA-3'	20	Reverse

All primers were synthesized by Invitrogen.

^aThe restriction enzyme recognition sites added are underlined.

RT-PCR, reverse transcription and polymerase chain reaction.

penicillin, and 100 µg/mL streptomycin (Life Technologies, Inc., Grand Island, NY) in a 90-cm culture dish (Nunc, Rochester, NY) and incubated at 37°C with 5% CO₂. Cells were then plated in a six-well plate at a density of 3×10^5 cells/well 1 day before transfection. A mixture containing 700 ng of pGL3-CRE-luciferase reporter construct, 200 ng of pcDNA3.1 expression plasmid (or empty vector), and 6 µL of Lipofectamine-2000 (Invitrogen) was prepared in 50 µL of PBS solution. Transfection was performed according to the manufacturer's instructions. After 24 h culture, CHO cells were trypsinized and cultured in a 96-well plate at a density of 2×10^4 cells/well at 37°C for 24 h before peptide treatment. After the removal of culture medium from the plate, 100 µL of peptide-containing medium (or hormone-free medium) was added. The cells were incubated for additional 6 h at 37°C before being harvested for luciferase assay. After the removal of culture medium, CHO cells were lysed by adding 50 µL of $1 \times$ passive lysis buffer (Promega, Madison, WI) per well, and the luciferase activity of 15 µL of cellular lysates was determined using luciferase assay reagent (Promega).

Data analysis

The luciferase activities in each treatment group were expressed as relative fold increase in comparison with the control group (without hormone treatment). The data were analyzed by one-way analysis of variance followed by Dunnett's test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). To validate our results, all experiments were repeated at least twice.

Results

Cloning of the full-length cDNA of pVPAC₁-R gene and its transcript variants

In this study, the full-length cDNA encoding pVPAC₁-R was cloned from pig brain using RT-PCR based on the ref-

erence sequence of pVPAC₁-R deposited in GenBank (Accession No.: U49434). The cloned cDNA is 1471 bp in length encoding a precursor protein of 458 amino acids, which shares 99.6% sequence identity to the reference sequence. Sequence analyses further revealed that the putative pVPAC₁-R protein shows high amino acid sequence identity to that of human (hVPAC1R:NP_004615, 87.3%), rat (rVPAC1R:NP_036817, 82.8%), mouse (mVPAC1R:NP_035833, 83.3%), chicken (cVPAC1R:NP_001090992, 64.1%), and zebrafish (zfVPA-C1R:AAI62971, 54.3%) (Fig. 1). The putative pVPAC₁-R protein includes the typical hydrophobic signal peptide and seven-transmembrane domains (Ulrich *et al.*, 1998). As shown in Figure 1, a total of 13 conserved cysteine residues were identified, where 6 are located in the large N-terminal extracellular domain. Three N-linked glycosylation sites (Asn-X-Thr/Ser, where X represents any amino acid except proline) were found in its large N-terminal extracellular domain. In addition, the PDI/V motif is located in the third intracellular loop and the motif IIRIL was found with one single amino acid substitution (IIGIL motif, R → G). Other conserved motifs, such as WD and GWS/T motifs, were also noted in the predicted pVPAC₁-R protein.

In addition to the isolation of the full-length cDNA encoding pVPAC₁-R, multiple transcript variants were identified in this study. With reference to the pig genome database (www.ensembl.org), the pVPAC₁-R gene structure was obtained and schematically shown in Figure 2A. The whole gene of VPAC₁-R spans across 35 kb of chromosome 13 and contains 14 exons and 13 introns. The exons vary in length, ranging from 42 to 266 bp. Among them, exon 1a is a novel exon, which is 115 bp in length and located between exon 1 and exon 2.

In the present study, a total of nine alternatively spliced transcript variants, named as pVPAC₁-R V1 to V9 respectively, were identified from pig brain using the same pair of primers. In comparison with the full-length cDNA sequence of pVPAC₁-R, the exon organization of these transcript variants were schematically presented. As shown in

FIG. 1. Amino acid sequence alignment of VPAC₁-R gene from pigs and other species. The amino acid sequence of pVPAC₁-R was aligned with that of humans (hVPAC1R:NP_004615), rats (rVPAC1R:NP_036817), mice (mVPAC1R:NP_035833), chicken (cVPAC1R: NP_001090992), and zebrafish (zVPAC1R: AAI62971). Dashes indicate gaps introduced to facilitate alignment and dots indicate identical amino acids among all aligned receptors. The seven putative TMDs are shaded and labeled accordingly. Conserved motifs are in bold and underlined. The conserved cysteine residues critical for disulfide bonds are boxed. The conserved N-linked glycosylation sites (N-X-T/S, where X represents any amino acid residue except proline) are in bold and boxed. Conserved residues—Arg in TMD2, Asn in TMD3, and Gln in TMD7—are in bold and underlined.

Figure 2B, the transcript V1 (pVPAC₁-R V1) is 1365 bp in length and resulted from the loss of exon 2 and a 3-bp insertion in exon 5 (Accession No.: HM209403). Sequence analyses revealed that it encodes a N-terminally truncated receptor with intact seven-transmembrane domains (data not

shown). Among the transcript variants identified, pVPAC₁-R V2 is 1127 bp in length and resulted from the loss of exons 4, 5, and 6 (Accession No.: HM209404). As shown in Figure 2B, pVPAC₁-R V3 is 1255 bp in length and generated by the deletion of exons 2 and 4 (Accession No.: HM209405).

FIG. 2. (A) Gene structure of pVPAC₁-R gene. Fourteen exons are presented as numbered boxes, whereas the numbers in italics indicate their respective sizes (bp). The novel exon (exon 1a) identified in pVPAC₁-R gene is shaded. Thirteen introns (from i1a to i12) are labeled. (B) Exon organization of pVPAC₁-R cDNA and nine pVPAC₁-R transcript variants. [pVPAC₁-R-V1(V1), pVPAC₁-R-V2(V2), pVPAC₁-R-V3(V3), pVPAC₁-R-V4(V4), pVPAC₁-R-V5(V5), pVPAC₁-R-V6(V6), pVPAC₁-R-V7(V7), pVPAC₁-R-V8(V8), and pVPAC₁-R-V9(V9)]. “CAG insertion” indicates a 3-bp insertion of exon 5 at its 5′-end noted in pVPAC₁-R-V1. “20-bp deletion” indicates a 20-bp deletion at its 3′-end of exon 7 noted in pVPAC₁-R-V6 and pVPAC₁-R-V9, while “32-bp deletion” indicates a 32-bp deletion at its 3′-end of exon 7 noted in pVPAC₁-R-V7. Arrows indicate the location of the putative translation start codon (or stop codon), and dotted arrows denote the putative translation start codon for pVPAC₁-R transcript variants.

pVPAC₁-R V4 is generated by an insertion of the novel exon 1a, and is 1586 bp in length, encoding an N-terminally truncated receptor with 416 amino acids (HM209406). pVPAC₁-R V5 is 1364 bp in length, which resulted from the loss of exon 5 thus sharing the same open reading frame as transcript V3 (Accession No.: HM209407). The transcript variant V6 (pVPAC₁-R V6) (Accession No.: HM209408) is 1107 bp in length, generated by a partial deletion in exon 7 (20-bp deletion) and the loss of exons 4, 5, and 6. pVPAC₁-R V7 shows the similar exon deletion as pVPAC₁-R V6, where the exons 4, 5, and 6 were lost and an extra 32-bp deletion in exon 7, causing a reduction in its length (1095 bp) (Accession No.: HM209409). As shown in Figure 2B, transcript variants pVPAC₁-R V8 and V9 share the largest exon deletion where exons 2, 4, 5, and 6 were lost. pVPAC₁-R V8 (Accession No.: HM209410) and V9 are 1018 and 998 bp in length, respectively, while V9 harbors an extra 20-bp deletion in exon 7 (Accession No.: HM209411).

Cloning of the full-length cDNA of pVPAC₂-R and its transcript variant

According to cDNA sequences of VPAC₂-R from *H. sapiens* (Accession No.: NM_003382), *R. norvegicus* (Accession No.: NM_017238), and *M. musculus* (Accession No.: NM_009511.2), gene-specific primers were designed to amplify the full-length cDNA of VPAC₂-R from pig liver.

The cloned pVPAC₂-R cDNA is 1481 bp in length with an open reading frame of 1326 bp encoding a precursor protein of 442 amino acids (Accession No.: GU576975). Sequence analyses revealed that it shows high amino acid sequence identity to that of human (hVPAC₂-R:NP_003373, 87.3%), rat (rVPAC₂-R:NP_058934, 83.0%), mouse (mVPAC₂-R:NP_033537, 83.0%), chicken (cVPAC₂-R:NP_001014970, 72.5%), and zebrafish (zVPAC₂-R: also called zebrafish PHI-R, NP_571854, 56.4%) (Fig. 3). It shows the characteristic features of a GPCR B family member including the seven-transmembrane domains and the hydrophobic signal peptide (Ulrich *et al.*, 1998). In line with the structure of pVPAC₁-R, it also shows the conserved cysteine residues for disulfide formation, the N-linked glycosylation sites for intracellular signaling mediation (Mayo *et al.*, 2003). As shown in Figure 3, the RLAK/R motif with one single amino acid substitution (A→T) was found in putative pVPAC₂-R protein.

In addition to the identification of the cDNA encoding the full-length pVPAC₂-R, a novel alternatively spliced transcript variant (named pVPAC₂-R-V1) was identified in this study. Based on the comparison to the pig genome database, the whole gene was mapped on chromosome 18 and found to be 69 kb in length. Similar to pVPAC₁-R gene, the VPAC₂-R contains 14 exons with sizes ranging between 42 and 288 bp. A novel exon of 106 bp (exon 6a) was found between exons 6 and 7 (Fig. 4A).

The transcript variant pVPAC₂-R-V1 is 1587 bp in length and predicted to be resulted from the insertion of a novel

FIG. 3. Amino acid sequence alignment of the VPAC₂-R gene from pigs and other species. The amino acid sequence of pVPAC₂-R was aligned with that of humans (hVPAC2R:NP_003373), rats (rVPAC2R:NP_058934), mice (mVPAC2R:NP_033537), chicken (cVPAC2R:NP_001014970), and zebrafish (zVPAC2R:NP_571854). Dashes indicate gaps introduced to facilitate alignment, dots indicate identical amino acids among all aligned receptors. The seven putative TMDs are shaded and labeled accordingly. Conserved motifs are in bold and underlined. The conserved cysteine residues critical for disulfide bonds are boxed. The conserved N-linked glycosylation sites (N-X-T/S, where X represents any amino acid residue except proline) are in bold and boxed. Conserved residues—Arg in TMD2, Asn in TMD3, and Gln in TMD7 are in bold and underlined.

106-bp exon between exons 6 and 7 (Fig. 4B). Thus, it is predicted to encode a C-terminally truncated receptor of 214 amino acids (accession no.: KJ410050), which lacks transmembrane domains 3–7 and a cytoplasmic tail (data not shown).

Tissue expression of VPAC₁-R and VPAC₂-R gene in adult pig tissues

Using RT-PCR, the mRNA expression of VPAC₁-R and VPAC₂-R genes was examined in adult pig tissues including

FIG. 4. (A) Gene structure of pVPAC₂-R gene. Fourteen exons are presented as numbered boxes, whereas the numbers in italics indicate their respective sizes (bp). The novel exon (exon 6a) identified in pVPAC₂-R gene is shaded. Thirteen introns (from i to i12) are labeled. (B) Exon organization of pVPAC₂-R cDNA and its transcript variant (pVPAC₂-R-V1). Arrows indicate the location of the putative translation start codon (or stop codon), and arrowhead denotes the putative premature stop codon appeared in transcript variant.

brain, heart, pituitary, liver, spleen, kidneys, muscle, intestine, lungs, testes, and ovary.

The primer pair (F2/R2) was employed to examine the mRNA expression of pVPAC₁-R among pig tissues. As shown in Figure 5A, only two major bands corresponding to the two transcript variants—pVPAC₁-R V8 and pVPAC₁-R V1 (band sizes of 350 and 700 bp, respectively)—were easily detected in most tissues examined. In striking contrast, the PCR band corresponding to the full-length VPAC₁-R was undetectable, implying its extremely low mRNA level in pig tissues.

For pVPAC₂-R gene, the strong PCR signal was detected in brain, heart, spleen, muscle, lungs, and ovary. A moderate signal was detected in pituitary, kidneys, intestine, and testes, while no PCR signal was noted in liver (Fig. 5B).

FIG. 5. Expression of VPAC₁-R and VPAC₂-R gene in adult pig tissues. The brain (Br), heart (He), pituitary (Pi), liver (Li), spleen (Sp), kidneys (Ki), muscle (Mu), small intestine (In), lungs (Lu), testes (Te), and ovary (Ov) were used in tissue distribution analyses. GAPDH was amplified as an internal control. Numbers in parentheses indicate the polymerase chain reaction (PCR) cycles used. (A) Expression of VPAC₁-R gene in adult pig tissues. The primers pVPAC₁-R-F2 and pVPAC₁-R-R2 were employed for the amplification where two PCR bands (350 and 700 bp) were detected in most tissues. (B) Expression of VPAC₂-R gene in adult pig tissues. The primers pVPAC₂-R-F3 and pVPAC₂-R-R2 were employed for the amplification where the PCR band (400 bp) was detected in most tissues.

Although the primer pair (F3/R2) employed in the present study was able to detect the mRNA transcripts of both pVPAC₂-R-V1 and full-length VPAC₂-R, only a strong PCR band of 400 bp corresponding to the full-length receptor was detected.

Functional characterization of pVPAC₁-R and pVPAC₂-R in cultured CHO cells

VIP exerts its biological actions through its two receptors—VPAC₁-R and VPAC₂-R—for downstream signaling (Dickson and Finlayson, 2009). In an effort to determine whether the cloned pVPAC₁-R and pVPAC₂-R could be potentially and specifically activated by its ligands such as pPACAP₃₈, pVIP, and other secretin family peptides, including pPHI and pSCT, a pGL3-CRE-luciferase reporter system was employed to examine their potencies in receptor activation.

As shown in Figure 6A, both pPACAP₃₈ and pVIP activate pVPAC₁-R and pVPAC₂-R in dose-dependent manners. pVIP (EC₅₀: 1.65 nM) shows a higher potency than pPACAP₃₈ (EC₅₀: 2.5 nM) in pVPAC₁-R activation, while pPACAP₃₈ and pVIP are equally potent (EC₅₀: 0.36 nM for pPACAP₃₈; 0.38 nM for pVIP) (Fig. 6B) in pVPAC₂-R activation. Interestingly, pVPAC₁-R, but not pVPAC₂-R, could be activated by pPHI potentially (EC₅₀: 0.8 nM for pVPAC₁-R; 6.23 nM for pVPAC₂-R), indicating that pVPAC₁-R can function as a common receptor for PACAP, VIP, and PHI. In the present study, we also noted that only pVPAC₁-R could be activated by pSCT (EC₅₀: 13.9 nM), however, its potency is much lower than that of pPACAP₃₈ and pVIP.

Discussion

VIP and PACAP are two structurally related peptides playing important physiological roles in vertebrate immune, reproductive, circulatory, and endocrine systems (Harmar *et al.*, 1998; Dickson and Finlayson, 2009). These neuropeptides function through their three receptors: PAC₁-R, VPAC₁-R, and VPAC₂-R. Our present study is the first to clone the full-length cDNA of VPAC₁-R and VPAC₂-R genes from pigs, isolate their splice variants, examine their tissue expression, and investigate their functionality. These findings help to elucidate their physiological roles in pigs and hence provide comparative insights across vertebrates.

FIG. 6. Functional assays of pVPAC₁-R (A) and pVPAC₂-R (B). Activation of pVPAC₁-R (A) and pVPAC₂-R (B) upon peptide [pig PACAP38, VIP, PHI, and secretin (SCT): 10^{-12} – 10^{-6} M, 6 h] treatment was monitored by a system of cotransfection of pGL3-CRE-luciferase reporter construct and receptor expression plasmid in cultured Chinese hamster ovary (CHO) cells. Cotransfection of the empty pcDNA3.1 vector and pGL3-CRE-luciferase reporter construct was used as an internal control, and peptide treatment did not increase the luciferase activity of CHO cells at any concentration tested (data not shown). Each data point represent mean $\pm$ SEM of three replicas.

Cloning of pVPAC₁-R gene and its transcript variants

In the present study, the full-length cDNA encoding the VPAC₁-R was successfully cloned from pig brain. It is 1471 bp in length encoding a precursor protein of 458 amino acids. Sequence analyses revealed that pVPAC₁-R protein shows high amino acid sequence identity (54–87%) with its counterpart in humans, rats, mice, chickens, and zebrafish. As a GPCR family member, pVPAC₁-R includes the typical seven-transmembrane domains and the large N-terminal domain for ligand binding. In addition, the cysteine residues proposed for disulfide bond formation and the N-linked glycosylation sites in the large N-terminal domain are accurately positioned and conserved (Mayo *et al.*, 2003). The structural conservation helps in proper folding, cell surface targeting, and high-affinity ligand binding in the receptor (Brubaker and Drucker, 2002). The multiple structural motifs are also conserved in the putative pVPAC₁-R. The RLAK motif, which is known to be critical for Gs α protein coupling, is conserved therein (Chow *et al.*, 1997). The PDI motif, which is localized in the IL3 and characteristic for all VIP-binding receptors, is also found in the receptor. The structure motif “IIRIL” conserved in all VPAC receptors (Brubaker and Drucker, 2002) was found in pVPAC₁-R where the arginine (R) residue replaces the glycine (G) residue. However, further studies are needed to elucidate whether this amino acid residue substitution influences receptor function.

Nine transcript variants were isolated from pig brain in this study. Among the transcripts, only transcript V4 is resulted from the insertion of a novel exon 1a, which is located between exon 1 and exon 2. All the other eight splice variants (V1–V3 and V5–V9) resulted from the loss of exons (exon 2, exon 4, exon 5, and exon 6) or the partial deletion (20-bp deletion and 32-bp deletion) at its 3'-end of

exon 7. Our study reports, for the first time, the existence of multiple VPAC₁-R transcript variants in pigs. VPAC₁-R splice variants are rather rare in mammals (Harmar *et al.*, 1998; Dickson and Finlayson, 2009). To present, only one VPAC₁-R splice variant, which consists of only five transmembrane domains but lacks the third intracellular loop, the fourth extracellular loop, and the sixth and seventh transmembrane domains owing to a 246-bp deletion, has been reported in humans (Bokaei *et al.*, 2006). Functional study revealed that this receptor variant still keeps its G-protein-binding capability and shows differential ability for agonist stimulation and different biological implications. Among the isolated pVPAC₁-R transcript variants, transcripts V1 and V4 show truncation in their N-terminals while the seven-transmembrane domains are intact. The other pVPAC₁-R transcripts show the partial loss of transmembrane domains owing to exon deletions. Whether these transcript variants are functional in signal transduction needs further investigation.

In the present study, based on the pig genome database and the isolated multiple transcript variants, the whole gene structure of pVPAC₁-R gene was revealed. The gene spans 35 kb on the chromosome 13, consisting of 14 exons ranging from 42 to 266 bp in length (Fig. 2). To our knowledge, this is the first report that the VPAC₁-R gene contains 14 exons, in contrast with the 13 exons reported across vertebrates (Dickson and Finlayson, 2009). Interestingly, we also found another VIP receptor gene, pVPAC₂-R, which contains 14 exons with size ranging from 42 to 288 bp. Exon insertions and deletions give rise to the multitude of splice transcript variants. There are reports from different research models that the truncation of GPCR could influence ligand binding and hence allow differential signaling pathways (Alexandre *et al.*, 2002; Abu-Hamdan *et al.*, 2006; Lutz *et al.*, 2006). The unique gene structures of pVPAC₁-R and pVPAC₂-R

genes revealed in the present study suggest species-specific transcription profiles.

Cloning of pVPAC₂-R gene and its transcript variants

In the present study, we successfully cloned the full-length cDNA of pVPAC₂-R, which is 1481 bp in length encoding a precursor protein of 442 amino acids. Sequence analyses revealed that the deduced pVPAC₂-R amino acids show high degrees of amino acid sequence identity (72–87%) to its mammalian counterparts and comparatively lower identity (56%) to zebrafish VPAC₂-R (also called PHI-R). Structurally speaking, the cloned receptor shows the typical GPCR B family member features including seven-transmembrane domains, the hydrophobic signal peptide, and the large N-terminal domain (Mayo *et al.*, 2003). The important motifs, such as PDI and IIRIL, which are characteristic for VIP-binding receptors, are conserved in pVPAC₂-R (Brubaker and Drucker, 2002). The RLAK motif known to be critical for Gs α protein coupling is also noted therein (Chow *et al.*, 1997). However, the substitution that the alanine (A) residue is replaced by threonine (T) within the motif awaits further investigation concerning its functional importance.

A novel splice variant of pVPAC₂-R was isolated from pig brain. Sequence analyses revealed that it resulted from an insertion of a novel exon (exon 6a, 106bp) between exons 6 and 7. As shown in Figure 4, the inserted exon 6a causes a frame-shift mutation and thus introduces a premature stop codon within exon 6a. The putative variant receptor protein is a C-terminally truncated receptor of 214 amino acids (KJ410050). Our study provides support to the existence of VPAC₂-R variants in pig genome. In mice, a VPAC₂-R variant was isolated from lymphocytes (Grininger *et al.*, 2004). It is generated by a deletion of exon 12 (42 bp) therefore resulting in a 14-a.a. deletion (a.a. residues: 367–380) at the C-terminal end of the transmembrane domain 7. In humans, a VPAC₂-R variant has been identified from malignant T-cell cell line. This variant is believed to be generated by the deletion of exon 11 thus resulting in the loss of 114 a.a. (a.a. residues: 325–438) and an insertion of 10 new a.a. at position 325–334 (Miller *et al.*, 2006). Functional studies showed that, in comparison with the wild-type VPAC₂-R, VIP shows similar affinity for mice VPAC₂-R variant but lower affinity for human VPAC₂-R variant. However, both mouse and human VPAC₂-R variants failed to stimulate the intracellular signaling pathways after ligand treatment (Grininger *et al.*, 2004; Miller *et al.*, 2006).

In humans, the PAC₁-R and VPAC₂-R genes are localized on the same chromosome 3 while the gene VPAC₁-R is localized on the chromosome 7 (Cai *et al.*, 1995; Sreedharan *et al.*, 1995; Brabet *et al.*, 1996). The localization of PAC₁-R and VPAC₂-R gene on the same chromosome has been confirmed in rats where both genes are localized on rat chromosome 4, whereas the rat VPAC₁-R gene is located on chromosome 8 (Cai *et al.*, 1995; Mackay *et al.*, 1996; Vaudry *et al.*, 2000). Such kind of gene localization has been suggested to result from gene duplication events. A first gene duplication may give rise to the VPAC₁-R gene and a common ancestral VPAC₂-R/PAC₁-R gene. And then, a second duplication event occurred in which the ancestral

VPAC₂-R/PAC₁-R gene further duplicates into the two separate receptor genes. Thus, VPAC₂-R gene and PAC₁-R gene are located on the same chromosome, whereas VPAC₁-R gene is located on a different chromosome (Cardoso *et al.*, 2004). In the present study, the pVPAC₂-R gene was revealed to be located on chromosome 18 spanning 69 kb. Recently, we found that the pPAC₁-R gene was also localized on the same chromosome 18 (He *et al.*, unpublished data). The same chromosome localization from both genes are hence in line with the reports from humans (Cai *et al.*, 1995; Sreedharan *et al.*, 1995) and rats (Mackay *et al.*, 1996). Together with the information that pVPAC₁-R is localized on the chromosome 13, the findings revealed in the present study provide support for the early gene duplication events during the vertebrate evolution (Cardoso *et al.*, 2004).

Tissue expression of pVPAC₁-R and pVPAC₂-R genes

In the present study, RT-PCR assay revealed that pVPAC₁-R was expressed in all tissues examined including brain, spleen, kidneys, muscle, intestine, lungs, ovary, heart, pituitary, liver, and testes. This finding is in line with the reports that VPAC₁-R is widely expressed in the CNS in high abundance and peripheral tissues including liver, lungs, and intestine (Dickson and Finlayson, 2009). The primers employed for RT-PCR assay can detect both full-length pVPAC₁-R and its variants and are expected to amplify multiple PCR bands. However, in the present study, only two major PCR bands of 350 and 700 bp, corresponding to pVPAC₁ V8 and pVPAC₁ V1 respectively, were detected in nearly all tissues examined. In sharp contrast, the PCR band of 809 bp corresponding to the full-length VPAC₁-R was almost undetectable, suggesting that the mRNA coding for the full-length pVPAC₁-R have much lower expression level than its two splice variants. VPAC₁-R V1 is predicted to host an N-terminal truncation with intact seven-transmembrane domains while VPAC₁-R V8 is short of the intact seven-transmembrane domains. Given the truncated receptor structures, whether the two transcripts with tissue abundance are physiologically significant needs further investigation.

Like pVPAC₁-R, pVPAC₂-R was also detected in all tissues examined with the strong PCR signal noted in brain, heart, spleen, lungs, and ovary. This observation partially coincides with the findings in mice, where VPAC₂-R is widely expressed in peripheral and CNSs (Usdin *et al.*, 1994; Harmar *et al.*, 2004). Unlike pVPAC₁-R gene, the mRNA transcript coding for the full-length pVPAC₂-R was detected to be predominantly expressed in tissues where VPAC₂-R-V1 was hardly detected. This finding strongly suggests that full-length VPAC₂-R, not its variant, plays a substantial role in mediating the actions of VIP and PACAP in pig tissues.

Considering the low mRNA expression level of the full-length pVPAC₁-R in pig tissues and its relative low potency for PACAP and VIP binding, the overlapping mRNA expression of pVPAC₁-R and pVPAC₂-R in most pig tissues led us to hypothesize that pVPAC₂-R may play much more important role than pVPAC₁-R to mediate the actions of VIP and PACAP in pigs. pVPAC₂-R may partially supplement the function of pVPAC₁-R owing to its low full-length

receptor mRNA expression and low affinity to VIP/PACAP. More studies on the two receptors will undoubtedly help to elucidate their physiological roles in pigs.

Functional characterization of pVPAC₁-R and pVPAC₂-R

Previous studies demonstrated that the administration of VIP and its structurally related peptides including PACAP and PHI induce a dose-dependent increase in cAMP level in the cells expressing VPAC receptors (Chow *et al.*, 1997). In the present study, based on a pGL3-CRE-luciferase reporter system, the activation potency of the two receptors by multiple ligands such as pPACAP₃₈, pVIP, pPHI, and pSCT were evaluated. As shown in Figure 6, both pVPAC₁-R and pVPAC₂-R could be effectively activated by pPACAP₃₈ and pVIP in dose-dependent manners with similar potencies. This observation coincides with the concept that both VPAC₁-R and VPAC₂-R share an equal affinity for PACAP and VIP and thus may function as the two receptors common for both peptides in humans and rats (Lutz *et al.*, 1993; Svoboda *et al.*, 1994; Adamou *et al.*, 1995). Interestingly, we further noted that pPHI, pPACAP₃₈, and pVIP seem to be equipotent in activating pVPAC₁-R, whereas pPHI is 15-fold less potent than VIP and PACAP in activating pVPAC₂-R. This finding implies that VPAC₁-R, but not VPAC₂-R, may also function as a potential PHI receptor in pigs. In consistence with our findings, PHI has been reported to activate VPAC₁-R in rat liver and intestinal membranes, but not VPAC₂-R, with potency similar to VIP and PACAP (Huang *et al.*, 1989; Huang and Rorstad, 1990). Similarly, we also reported that chicken VPAC₁-R can be activated by turkey PHI potently and thus be viewed as a potential PHI receptor in chicken in one of our previous studies (Wang *et al.*, 2010). All these findings tend to support the idea that VPAC₁-R can function as a PHI receptor in mammals and birds. Although VPAC₂-R has been reported to act as a PHI-specific receptor (PHI-R) in fish or a potential PHI receptor in chicken, pPHI is 15-fold less potent than pVIP or pPACAP in activating pVPAC₂-R as reported in other mammalian species, suggesting that pVPAC₂-R may not be a receptor for PHI in pigs. Our findings support that VPAC₂-R may have undergone drastic changes in its pharmacological property during vertebrate evolution, as proposed in our recent study (Wang *et al.*, 2010). Similar to those findings in rats and humans, pSCT could also activate pVPAC₁-R with a lower potency (EC₅₀: 13.9 nM) than pPACAP₃₈ and pVIP, whereas pVPAC₂-R show no response to SCT either at the physiological or the pharmacological concentration tested (from 10⁻¹² to 10⁻⁶ M).

Conclusion

Our present study reports, for the first time, the pVPAC₁-R and VPAC₂-R gene structure, expression profile, and functionality. The cloned full-length pVPAC₁-R and pVPAC₂-R share high structural similarity with their mammalian counterparts. Both pVPAC₁-R and pVPAC₂-R are capable in VIP and PACAP binding, thus supporting their conserved roles across vertebrates. In addition, pVPAC₁-R and pVPAC₂-R share the unique gene structure with 14 exons, which is different from the 13-exon structure shared by other vertebrates. RT-PCR assays showed that the

mRNA transcript coding for the full-length pVPAC₂-R is widely expressed in all adult tissues, whereas the splice variants of pVPAC₁-R, not its full-length receptor, are predominantly expressed in nearly all tissues examined, hinting that pVPAC₂-R may play much more important roles than pVPAC₁-R in mediating VIP and PACAP actions. All these findings help to elucidate the important role of VIP and PACAP in pigs and promote us to rethink their species-specific physiological roles, including their actions in regulating phenotypic traits of pigs.

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Disclosure Statement

No competing financial interests exist.

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