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African lungfish, *Protopterus annectens*, possess an arginine vasotocin receptor homologous to the tetrapod V2-type receptor

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SUMMARY

In tetrapods, arginine vasopressin and its counterpart, arginine vasotocin (AVT), are involved in renal water conservation through vascular V1a-type and tubular V2-type receptors, and only the former has thus far been cloned in fish. We successfully cloned the V1a-type and V2-type AVT receptor from the kidney of the African lungfish, *Protopterus annectens*, and the deduced amino acid sequences exhibited high homology with amphibian V1a- and V2-type receptors, respectively. Functional analysis showed that AVT addition to CHO cells transfected with lungfish V1a-type receptor increased $[Ca^{2+}]_i$ in a concentration-dependent manner, whereas CHO cells transfected with lungfish V2-type receptor responded with cAMP accumulation after AVT stimulation. Lungfish V2-type receptor mRNA was strongly expressed in the heart and kidney, while V1a-type receptor mRNA was ubiquitously expressed in all the tissues examined. In the kidney, immunohistochemistry using a specific antibody to lungfish V2-type receptor showed localization in the basolateral area of the cells in the late part of the distal tubules. Artificial estivation (EST) for 90 days significantly increased plasma osmolality and sodium and urea concentrations. There was no significant difference in the V2-type receptor mRNA and protein expression levels in the kidney between the freshwater and EST lungfish, while the AVT precursor mRNA level in the hypothalamus was remarkably higher in the EST lungfish. Our results indicate that African lungfish possess a functional V2-type receptor similar to that in tetrapods, suggesting that elevated plasma AVT during estivation exerts a renal tubular antidiuretic effect through the V2-type receptor expressed in the distal segments of lungfish kidney.

Key words: arginine vasotocin (AVT), V2-type receptor, kidney, osmoregulation, lungfish.

INTRODUCTION

Water conservation by the kidney is essential in terrestrial vertebrates. Neurohypophysial hormones, arginine vasopressin (AVP) and its counterpart arginine vasotocin (AVT), play a pivotal role in an antidiuretic action crucial for water conservation in the kidney of terrestrial vertebrates (Bentley, 2002). In tetrapods, the antidiuretic action of AVP and AVT are mediated through two subtypes of AVP/AVT receptors, namely V1a- (Morel et al., 1992) and V2-type receptors (Lolait et al., 1992). The V1a-type receptors are linked intracellularly to the phospholipase C (PLC)/protein kinase C (PKC) signaling pathway and exert an antidiuretic action by regulating vascular smooth muscle contraction and thereby modifying the glomerular filtration rate (Bankir, 2001). By contrast, the responses through the V2-type receptor are mediated by the adenylyl cyclase (AC)/protein kinase A (PKA) signaling system (Thibonnier et al., 1998), and the V2-type receptor is involved in water reabsorption by promoting the expression and insertion of the AVP/AVT-dependent water channel aquaporin 2 (AQP2) in the luminal membrane of the distal convoluted tubule and the collecting tubule in the kidney (Lolait et al., 1992; Nielsen et al., 2002).

All tetrapods possess two types of renal water conservation mechanisms through the V1a- and V2-type receptors; however, only the V1a-type receptor and its vascular effects have been reported in fish (Mahlmann et al., 1994; Warne, 2001). Sawyer and colleagues reported that in the South American lungfish, *Lepidosiren paradoxa*, the effects of exogenous AVT appear to be primarily responsible for vasoconstriction and have no effect on tubular antidiuresis (Sawyer et al., 1982). Therefore, the tubular antidiuretic action through the V2-type receptor was thought to have emerged in the terrestrial tetrapods (Pang, 1983). However, Balment and colleagues suggested that the AVT receptor associated with the AC/cAMP signaling pathway might be present in teleosts (Perrott et al., 1993; Warne, 2002). However, molecular biological evidence of the V2-type receptor has not been reported thus far in fish.

Lungfish, recognized as living fossils, are an archaic group of fish belonging to the class of lobe-finned fish (Sarcopterygii), which differ from the class of ray-finned fish (Actinopterygii). African lungfish, including *Protopterus aethiopicus*, *Protopterus annectens* and *Protopterus dolloi*, spend an aquatic life in flooded swamplands in the rainy season but can estivate in subterranean mud cocoons without water during the dry season. Thus, during the switch from freshwater (FW) to estivating status, they experience opposite changes in body fluid balance. In the FW lungfish, the main physiological function of the kidney is to excrete excessive water. By contrast, during estivation (EST), the lungfish must retain water in the body, enabling it to survive for months without water. Thus, we suppose that African lungfish employ two types of water conservation mechanisms through vascular V1a-type and tubular V2-type receptors in the kidney.

It is interesting to identify AVT receptors expressed in the kidney of lungfish because lungfish might be a key species for the

investigation of the molecular and functional evolution of the neurohypophysial hormone system in vertebrates, in particular from aquatic fish to terrestrial tetrapods. Here, we report that the AVT receptor, homologous to the tetrapod V2-type receptor, is expressed in the kidney of the African lungfish, *P. annectens*. The functional significance of the lungfish renal V2-type receptor will be discussed in terms of the ecological characteristics, i.e. estivation in a dry environment.

MATERIALS AND METHODS

Animals and experimental protocols

African lungfish (*Protopterus annectens* Owen) were purchased from a commercial supplier in Japan. Lungfish with a mean body mass of 115 g (range, 79–163 g) were housed in individual containers containing dechlorinated tap water and were held at 25–28°C under a 12 h:12 h L:D photoperiod until use. Lungfish were induced to estivate individually at ~80% humidity in plastic bags (12 cm×12 cm×30 cm, L×W×H) containing a mass of damp cotton. It took 1 week for the lungfish to be encased in a brown dried mucus cocoon, and the lungfish (EST; *N*=9) were allowed to estivate for 90 days. Specimens in freshwater (FW; control; *N*=9) were placed in dechlorinated tap water for the same period of time. After 90 days, the FW specimens were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (Sigma, St Louis, MO, USA), while the EST specimens were killed directly by pithing. Blood samples were collected by cardiac puncture using heparinized 1-ml syringes and hematocrit capillaries for determination of the plasma components. The tissue samples were quickly dissected and frozen in liquid nitrogen. The experiments described in this manuscript were performed according to the Guidelines for Care and Use of Animals approved by the ethics committees of the University of Toyama and the University of Tokyo.

Analyses of plasma components

Plasma osmolality and plasma sodium concentrations were measured using an osmometer (Wescor 5520, Logan, UT, USA) and an atomic absorption spectrophotometer (Hitachi Z5300, Tokyo, Japan), respectively. Plasma urea concentration was measured by using the Wako Urea NB test (Wako Pure Chemical, Tokyo, Japan) *in vitro* enzymatic colorimetric method.

cDNA cloning

Total RNA was extracted from the kidney of the FW specimens using the Isogen reagent (Nippon Gene, Tokyo, Japan). First-strand kidney cDNA was reverse-transcribed from DNase I-treated total RNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa Bio, Otsu, Shiga, Japan). Degenerate primers (Table 1) for AVT receptors were designed based on the alignment of the three subtypes (V1a, V2, V1b/V3) of the mammalian AVP receptors (Lolait et al., 1992; Morel et al., 1992; Sugimoto et al., 1994), amphibian AVT receptors (Kohno et al., 2003; Acharjee et al., 2004; Hasunuma et al., 2007) and teleost V1a-type receptors (Mahlmann et al., 1994; Warne, 2001). Polymerase chain reaction (PCR) was performed using BIOTAQ DNA polymerase (Bioline, London, UK) as follows: 94°C for 2 min, 35 cycles at 94°C for 1 min, 55°C for 30 s, 72°C for 40 s, and finally 72°C for 10 min. The PCR products were separated by electrophoresis, and the major band of the predicted size was purified from the sliced gel and ligated into the pT7Blue T-Vector (Novagen, San Diego, CA, USA). The ligated plasmid was transformed into the competent cell (XL1-Blue, Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated by a modified alkaline/SDS method (Rapid Plasmid Purification Systems, Marligen Bioscience, Ijamsville, MD, USA). The sequencing reaction was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster

Table 1. PCR primers for cDNA cloning and PCR

Target genes	Application	Oligonucleotide sequences (5′–3′)
AVT precursor	Real-time PCR	Sense: TGTATGTCATGTGGTCCACGG Antisense: ATCTCAAAGTCTCTGCGGTGC
VT1aR	Degenerate PCR	Sense: GARGARCTNGCNAARATHGA Antisense: TGNCCNGARAARAACATRTA
	5′-RACE PCR	Antisense1: AACCAGGTCAGCTAAACTAAGATG Antisense2: AGAGCAAGCAACACACTAATG
	3′-RACE PCR	Sense1: CAGTAAAAATGACATTCGTGATTG Sense2: GGATGATCAGTTCTCTTGGGA
	RT-PCR	Sense: GGATGATCAGTTCTCTTGGGA Antisense: GTCTGATCCTTGTTTTGTAGTGG
	Real-time PCR	Sense: GAAGGCAAACCTCTCTAACCAAGA Antisense: GCGATTCCTTCCAGGTTCCT
VT2R	Degenerate PCR	Sense: TAYATGATHGTNGCNATGAC Antisense: CCANGGRTTNGTRCARCTRTT
		Antisense1: CTTGAAAGTACTTGACAGCTCGG Antisense2: GATAACAATTTGTGGGAGACTGAG
		Sense1: GTTATTGTGGTTATCTATATCGCTTG Sense2: TTATCTATATCGCTTGCTGGGCT
	RT-PCR	Sense: GTTATTGTGGTTATCTATATCGCTTG Antisense: ATTTTAATACAGAGATTCTTTGGG
	Real-time PCR	Sense: GATCCCCACGCACCAAAG Antisense: CAGCTGTTCAGACTTGCTAATAGCA
GAPDH (internal standard)	RT-PCR	Sense: AGTTTTCTGAGTGGCTGTATAAG Antisense: ATCCTGCTAACATCAAGTGGG
	Real-time PCR	Sense: GGGAGCCAGGCAGTTGGTA Antisense: ATGAAAAGTACGACAACAGTCTGACA

VT1aR, V1a-type receptor; VT2R, V2-type receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

City, CA, USA). The nucleotide sequence was determined using an ABI PRISM 310 or 3100 DNA sequencer (Applied Biosystems). Full-length cDNAs were obtained by 5′- or 3′-rapid amplification of the cDNA ends (RACE) using adaptor and gene-specific primers (Table 1).

Molecular phylogenetic analysis

The deduced amino acid sequences of the lungfish AVT receptors were aligned with those of the other neurohypophysial hormone receptors using ClustalX version 1.83 (Thompson et al., 1997). The aligned amino acid sequences were analyzed by the neighbor-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods. The NJ and MP analyses were conducted using MEGA4 software (Tamura et al., 2007), and the ML analysis was conducted using the Bio Edit program version 7.08 (Ibis Biosciences, Carlsbad, CA, USA). To estimate the reliability of the trees, bootstrapping of the data (1000 replicates) was performed.

Functional analyses of the cloned receptors

Intracellular accumulation of Ca^{2+} and cAMP was analyzed to determine the intracellular signaling system of the cloned receptors. Functional analyses of cloned receptors were conducted using Chinese hamster ovary (CHO) cells transiently expressing lungfish V1a-type or V2-type receptor. The CHO cells were cultured in alpha-MEM (Gibco, Invitrogen) containing 10% fetal calf serum (FCS) at a density of 1×10^6 cells in a 10-cm dish for 24 h. Expression vectors (pcDNA3.1/V5-His TOPO; Invitrogen) containing the coding region of lungfish V1a-type or V2-type receptor (2.5 μg) were transfected with FuGENE6 (Roche Diagnostics) according to the manufacturer's protocol. Twenty-four hours after transfection, the transfected CHO cells were plated onto a normal 96-well black plate at a density of 3×10^4 cells per well. The measurement of intracellular Ca^{2+} mobilization was performed using FLIPRtetra (Molecular Devices, Menlo Park, CA, USA). Twenty hours after plating, the culture medium was aspirated, and 100 μl fluorescent dye solution containing 4.4 μmol l^{-1} Fluo-4AM (Invitrogen), 1% FCS and 0.045% pluronic acid (Sigma) in a working buffer [1×Hank's Balanced Salt Solution (Gibco):20 mmol l^{-1} Hepes buffer containing 250 μmol l^{-1} probenecid (Sigma)] was loaded onto each well. The plate was incubated for 1 h at 37°C in a CO_2 incubator or at room temperature. After washing three times with the working buffer, 100 μl of AVT and AVP (Peptide Institute, Osaka, Japan) in a working buffer containing 0.001% Triton

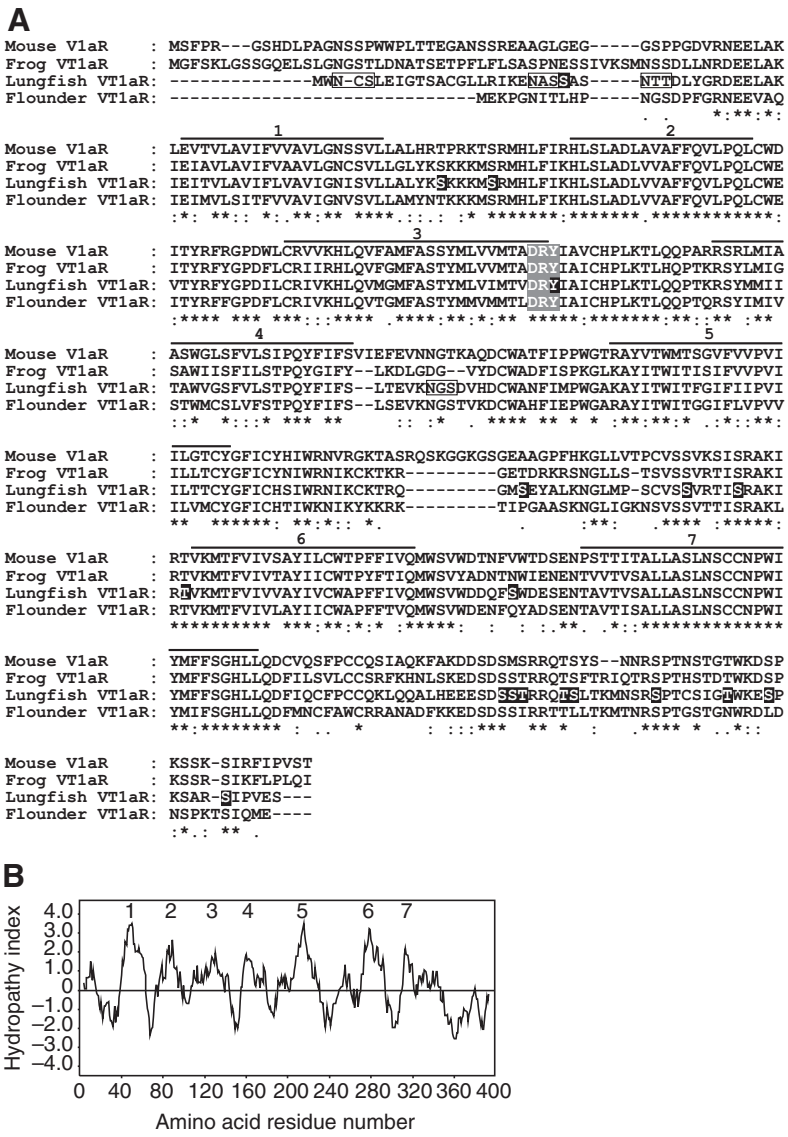

Fig. 1. Comparison of the deduced amino acid sequences of the V1a-type AVP/AVT receptors (V1aR/VT1aR). The deduced amino acid sequence of lungfish VT1aR was aligned with mouse (NP058543), frog (AAQ22364) and flounder (AAF00506) V1aR/VT1aR using the Clustal algorithm (A). The putative transmembrane regions are indicated by a line. The open boxes and black boxes indicate putative N-linked glycosylation sites and putative phosphorylation sites, respectively. The V1aR/VT1aR consensus sequence (Asp-Arg-Tyr) is indicated by the gray box. The asterisks indicate identical amino acid residues. The Kyte-Doolittle hydropathy profile of the deduced amino acid sequence of lungfish VT1aR predicts the presence of seven putative transmembrane regions (B).

X-100 were automatically added to the well by the FLIPR system. Intracellular Ca^{2+} changes were measured by excitation at 488 nm and emission at 500–560 nm.

For the cAMP assay, the cells were washed twice with Dulbecco's-PBS (TaKaRa Bio) prior to the experiment and were preincubated for 10 min at room temperature in a stimulation buffer (pH 7.4) containing 0.2 mmol l^{-1} 3-isobutyl-l-methylxanthine (IBMX), 0.1% BSA, 5 mmol l^{-1} Hepes and 1×Hank's BSS. The cells were incubated at room temperature for 1 h in the stimulation buffer containing AVT, AVP and ghrelin. After incubation, the cells were treated with 1% Tween 20 at room temperature for 1 h, and the supernatants were then transferred to 96-well optical plates. Intracellular cAMP accumulation was measured by using the Alphascreen cAMP Functional Assay Kit (Perkin Elmer, Shelton, CT, USA) according to the manufacturer's protocol.

RT-PCR

The tissue distribution of lungfish V1a- and V2-type receptor mRNAs was examined by RT-PCR. Total RNA was extracted from various tissues (brain, eye, internal gill, lung, heart, liver, gall bladder, pancreas, intestine, kidney, ovary, testis, muscle and ventral skin) of the FW and EST specimens using the Isogen reagent (Nippon Gene). After treatment with DNase I (Invitrogen) to remove the genomic DNA, cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). PCR was performed using specific primers (Table 1) for individual genes [V1a-type receptor, V2-type receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. GAPDH mRNA cloned from *P. annectens* (DDBJ accession no. AB426480) was used as an internal standard to estimate the relative levels of objective mRNA expression. The PCR conditions comprised 34 cycles (V1a-type receptor), 32 cycles (V2-type receptor) and 24 cycles (GAPDH) of 40 s at 94°C (denaturation), 30 s at 55°C (annealing) and 40 s at 72°C (extension) in 20-μl reaction mixtures.

Quantitative real-time RT-PCR

Total RNA was extracted from the hypothalamus and kidney of the FW and EST specimens using the Isogen reagent (Nippon Gene). After treatment with DNase I (Invitrogen), 5 μg of RNA was used as the template for reverse transcription using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The PCR mixture (20 μl) contained Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nmol l^{-1} (AVT, V1a-type and V2-type receptors) or 100 nmol l^{-1} (GAPDH) forward and reverse primers, and standard cDNA (10^{-3} to 10^{-7} ng per reaction) or 50 ng of the reverse-transcribed cDNA

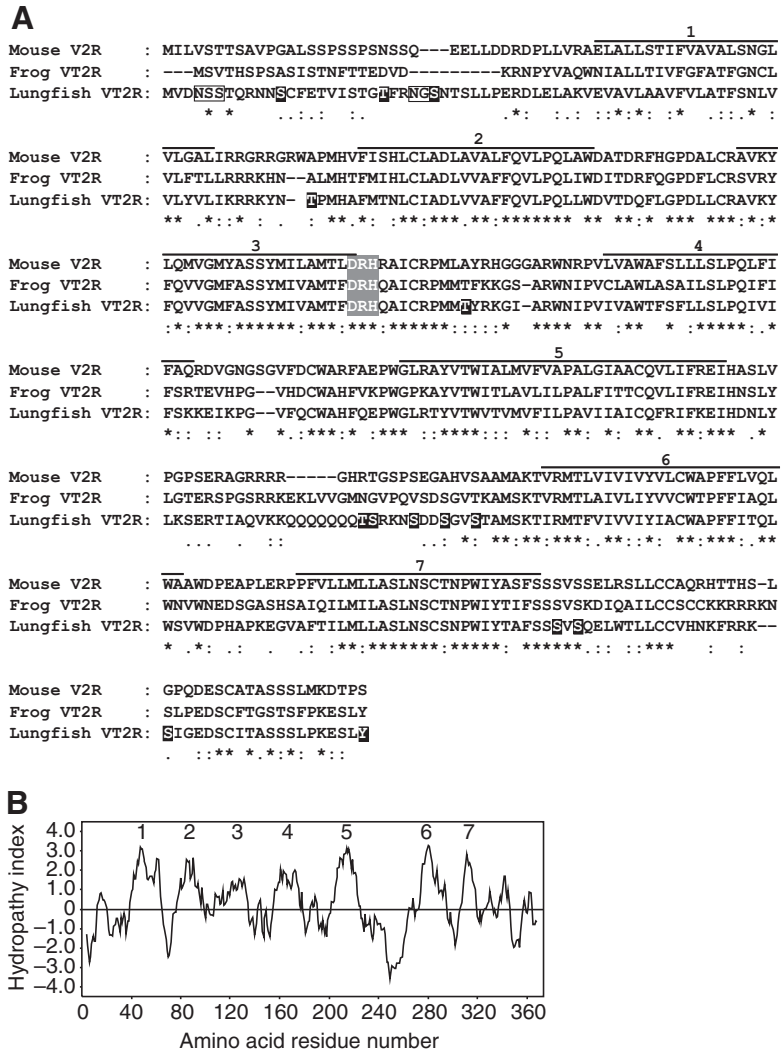

Fig. 2. Comparison of the deduced amino acid sequences of the V2-type AVP/AVT receptors (V2R/VT2R). The deduced amino acid sequence of lungfish VT2R was aligned with mouse (NP062277) and frog (BAC23056) V2R/VT2R using the Clustal algorithm (A). The putative transmembrane regions are indicated by a line. The open boxes and black boxes indicate putative N-linked glycosylation sites and putative phosphorylation sites, respectively. The V2R/VT2R consensus sequence (Asp-Arg-His) is indicated by the gray box. The asterisks indicate identical amino acid residues. The Kyte-Doolittle hydropathy profile of the deduced amino acid sequence of lungfish VT2R predicts the presence of seven putative transmembrane regions (B).

samples. After denaturation and activation at 95°C for 10 min, 40 cycles of amplification were carried out at 95°C for 15 s and at 60°C for 1 min. The sequences of the primers used in the assay are shown in Table 1. The reaction of each sample was carried out in triplicate.

Antibodies

A polyclonal antibody against lungfish V2-type receptor was raised by immunizing Japanese white rabbits with the synthetic peptide NH2-CVHNKFRRKSIGEDS-COOH, corresponding to amino acid residues 345–359 of lungfish V2-type receptor. The antiserum was collected and purified using an affinity column bearing the immobilized synthetic peptide with affinity gel beads (Affi-Gel 10; Bio-Rad, Hercules, CA, USA). Anti-vacuolar type H^+-ATPase α-subunit antiserum was raised by immunizing Japanese white rabbits with a synthetic peptide, NH_2-AEMPADSGYPAYLGAR-COOH, as described previously (Hayashi et al., 2000). This antiserum specifically recognizes intercalated cells of the late distal tubule and the collecting duct of the amphibian kidney (Uchiyama and Yoshizawa, 2002; Konno et al., 2006; Konno et al., 2007; Kumano et al., 2008).

Western blotting

Tissue samples from the kidney, heart and liver were homogenized in transmembrane protein extraction buffer I (ProteoExtract Transmembrane Protein Extraction Kit; Novagen) using a tissue homogenizer (Physcotron NS-310E, Nition, Chiba, Japan). The homogenates were centrifuged at 1000 ***g*** for 5 min at 4°C to collect the insoluble pellets containing the cell membrane. The pellets were solubilized in extraction buffer II (ProteoExtract Transmembrane Protein Extraction Kit; Novagen) and were then centrifuged at 16,000 ***g*** for 15 min at 4°C to collect the membrane fraction containing the transmembrane proteins. The total protein concentration of the membrane protein fractions was measured by the Bradford method (Bradford, 1976) using a Protein Assay Reagent (Bio-Rad). Samples containing 40 µg of transmembrane proteins were degenerated at 60°C for 15 min in Laemmli buffer, separated by SDS-PAGE using 10% polyacrylamide gel and then transferred from the gel to a nitrocellulose membrane (Hybond-C; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). To prevent non-specific binding, the blotted membranes were blocked with 5% skimmed milk for 2 h at room temperature and were then probed overnight at 4°C with the lungfish V2-type receptor antibody (dilution 1:2000 with 1% BSA-PBS). After washing with TBS-Tween 20, the membranes were incubated with HRP-conjugated anti-rabbit IgG (ECL plus Western Blotting Detection System; GE Healthcare Bio-Sciences) for 2 h at room temperature. After further washing of the membranes with TBS-Tween 20, immunodetection was performed by using an enhanced

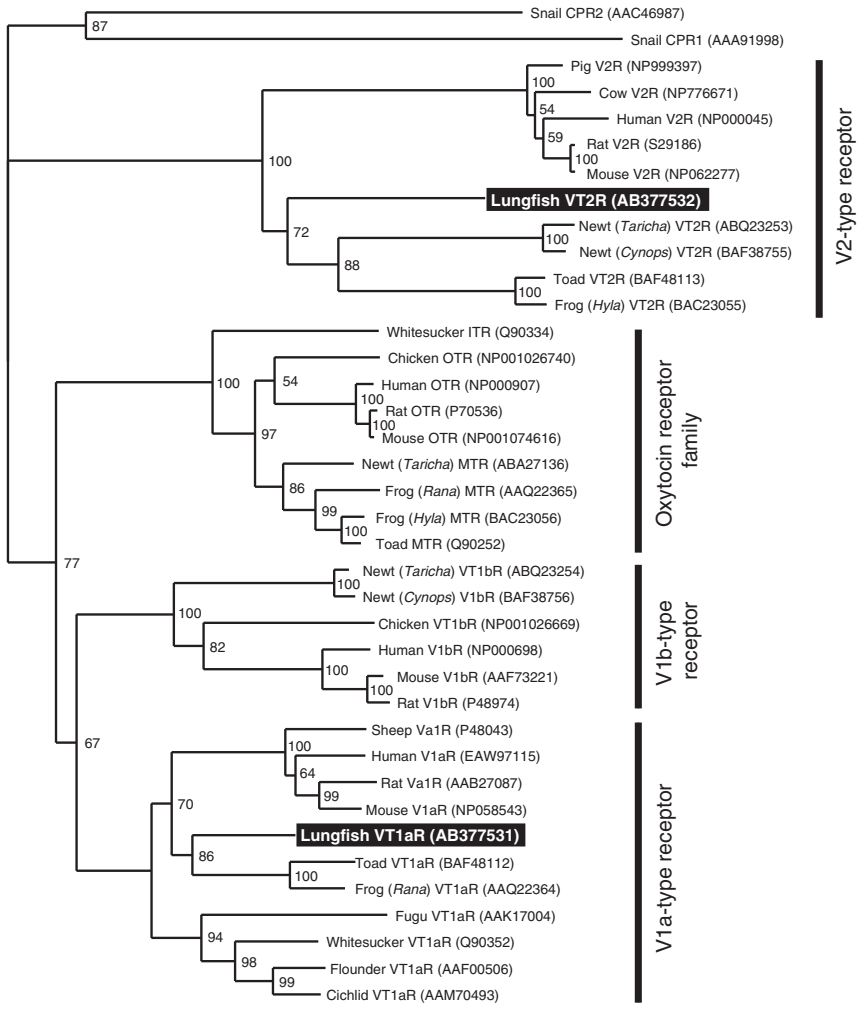

Fig. 3. A phylogenetic tree of the AVP/OT receptor family members inferred by the neighbor-joining method using ClustalX. The numbers at the branch points are derived from bootstrap analysis (1000 repetitions). The snail conopressin receptor (CPR) is regarded as the out-group. The scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. The positions of lungfish VT1aR and VT2R are indicated by the black boxes. OTR, oxytocin receptor; MTR, mesotocin receptor; ITR, isotocin receptor. Each sequence appears in the database of proteins (GenBank and DDBJ) with the following accession numbers: Snail CPR1, AAC46987; Human OTR, NP000907; Mouse OTR, NP001074616; Rat OTR, NP037003; Chicken OTR, NP001026740; Toad MTR, Q90252; Frog (*Hyla*) MTR, BAC23056; Frog (*Rana*), AAQ22365; Newt MTR, ABA27136; Sucker ITR, Q90334; Human V1aR, EAW97115; Sheep V1aR, P48043; Mouse V1aR, NP058543; Rat V1aR, CAA77748; Frog (*Rana*) VT1aR, AAQ22364; Toad VT1aR, BAF48112; Lungfish VT1aR, AB377531; Fugu VT1aR, AAK17004; Sucker VT1aR, Q90352; Flounder VT1aR, AAF00506; Cichlid VT1aR, AAM70493; Human V1bR, NP000698; Mouse V1bR, AAF73221; Rat V1bR, P48974; Chicken VT1bR, NP00102; Newt (*Cynops*) VT1bR, BAF38756; Newt (*Taricha*) VT1bR, ABQ23254; Human V2R, NP000045; Mouse V2R, NP062277; Rat V2R, S29186; Pig V2R, NP999397; Cattle V2R, NP776671; Frog (*Hyla*) VT2R, BAC23055; Toad VT2R, BAF48113; Newt (*Cynops*) VT2R, BAF38755; Newt (*Taricha*) VT2R, ABQ23253; Lungfish VT2R, AB377532.

chemiluminescence kit (ECL plus Western Blotting Detection System). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL; GE Healthcare Bio-Sciences). As a control, the primary antibody was replaced with the lungfish V2-type receptor antibody preincubated with 1 μg ml^{-1} of the immunogen peptide.

Immunohistochemistry

The kidney was fixed overnight at 4°C in Bouin's fixative without acetic acid (saturated aqueous picric acid, 75 ml; concentrated formalin, 25 ml). The tissues were dehydrated and embedded in paraffin. The kidney sections (6 μm) were immunohistochemically stained using the avidin-biotin-peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). After rehydration, the tissue sections were incubated with 2% normal swine serum in PBS (pH 7.4) for 2 h at room temperature, and then mirror-image sections were incubated with either the affinity-purified anti-lungfish V2-type receptor antibody (dilution 1:1000 with PBS) or the anti-vacuolar type H^+-ATPase α-subunit antibody (dilution 1:4000 with PBS) for 24 h at 4°C. After rinsing with PBS, the sections were incubated for 2 h at room temperature with biotinylated swine anti-rabbit IgG and then with the avidin-biotin-peroxidase complex. After rinsing with PBS, the immunoreactivity was visualized with 3′-diaminobenzidine solution (Sigma) containing 0.01% H_2O_2. Immunolabeling control for the lungfish V2-type receptor and vacuolar-type H^+-ATPase was performed by preabsorption of primary antibodies with excess peptides of each immunogen (1 μg ml^{-1}). All control experiments were negative for immunostaining.

Statistics

Values are expressed as means ± s.e.m. (standard error of the mean). To test the difference between the groups, two-tailed, paired and unpaired, Student's *t*-tests were used in the present study. Statistical significance was established at $P<0.05$ and $P<0.01$. Correlation was calculated by Spearman's correlation analysis. P values less than 0.01 were considered to be statistically significant.

RESULTS

Cloning of lungfish AVT receptors

We obtained of two types of AVT receptor cDNA from the kidney of *P. annectens*. These transcripts encoded different proteins composed of 398 (Fig. 1A; DDBJ accession no. AB377531) and 373 (Fig. 2A; DDBJ accession no. AB377532) amino acid residues. The deduced amino acid sequence of the former was shown to have the highest homology with amphibian V1a-type receptors (78–85%), and the latter with amphibian V2-type receptors (75–77%). Hydropathy analysis of both the deduced amino acid sequences indicated the presence of seven putative transmembrane domains that are characteristic of G-protein coupled receptors (Fig. 1B; Fig. 2B). The putative lungfish V1a- and V2-type receptors contained the consensus tripeptide of the V1a- (Asp-Arg-Tyr) and V2-type receptors (Asp-Arg-His), respectively, at the end of transmembrane region III. As shown in Fig. 1A and Fig. 2A, computer analyses for the phosphorylation and glycosylation sites using NetPhos2 and NetNGlyco1.0 software predicted that these receptor sequences include several putative phosphorylation and N-linked glycosylation sites.

Molecular trees were constructed using the amino acid sequences of the cloned receptors and other neurohypophysial hormone receptors. Since three different methods yielded the same tree, the tree inferred by the NJ method is shown in Fig. 3. Snail conopressin receptor (CPR) was used as the out-group. The receptors were classified into four groups, namely V1a-type, V1b-type, V2-type and oxytocin receptor. The two types of AVT receptors cloned from lungfish kidney were classified into the V1a- and V2-type receptor subtypes. In each subfamily, the lungfish AVT receptors form a monophyletic group with the amphibian receptors (Fig. 3). Based on these results, we concluded that AB377531 and AB377532 encode the lungfish V1a-type and V2-type receptors, respectively.

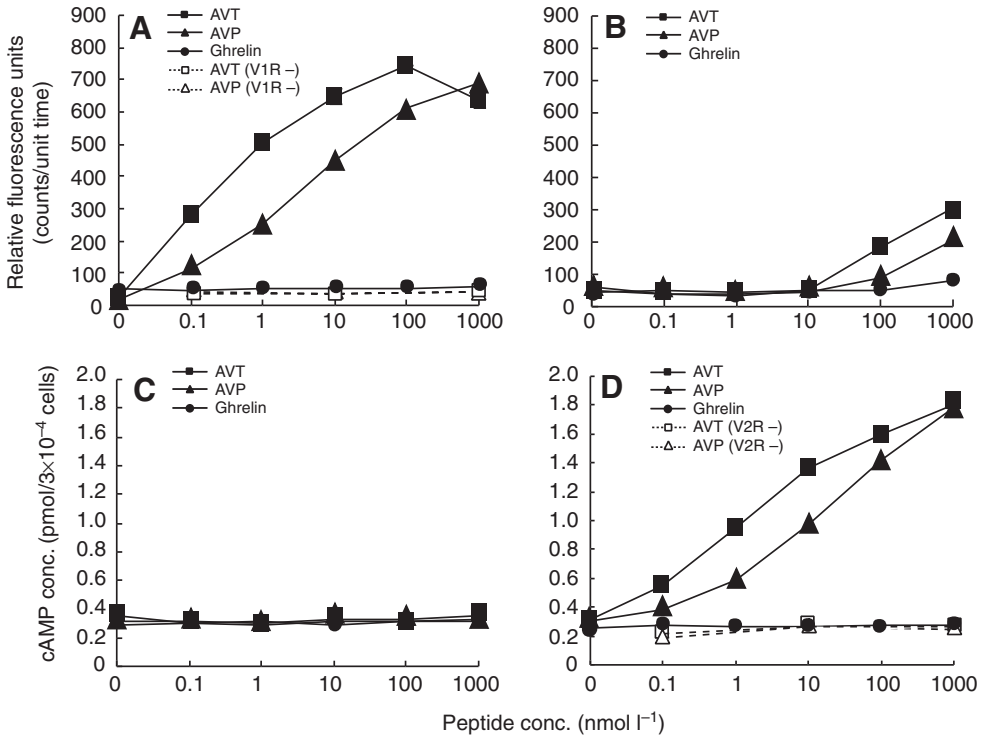

Fig. 4. Pharmacological characterization of lungfish V1a- (VT1aR) and V2-type (VT2R) receptors transfected to the CHO cells. The CHO cells transfected with lungfish VT1aR (A) or VT2R (B) were loaded with Fluo-4 Ca^{2+}-sensitive dye and were then stimulated with different doses (0.1–1000 nmol l^{-1}) of AVT, AVP and ghrelin (as a negative control). The fluorescence intensities for Ca^{2+} mobilization were determined using FLIPR. The cells transfected with lungfish VT1aR (C) or VT2R (D) were incubated with different doses (0.1–1000 nmol l^{-1}) of AVT, AVP and ghrelin. Intracellular cAMP accumulation was measured using an Alphascreen cAMP Functional Assay Kit. The broken lines represent the responses in the cells transfected with only the expression vector. The data are the means of triplicate wells from one experiment representative of two experiments.

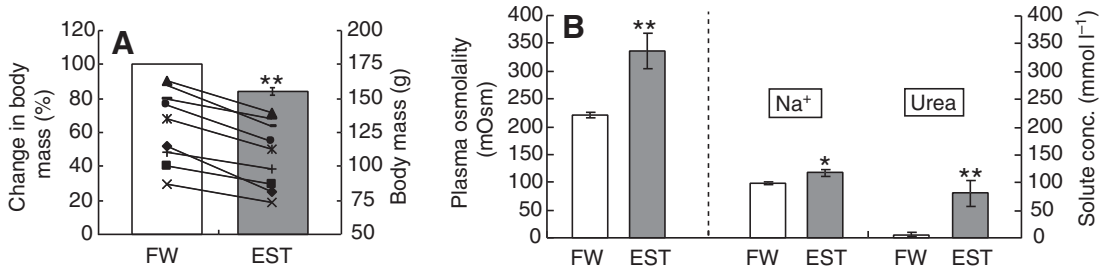

Fig. 5. Change in the body mass (A) and plasma parameters (B) in *P. annectens* under freshwater (FW) and estivation (EST) conditions for 90 days. (A) The percentage change in the body mass (columns) and individual body masses (lines) of the EST lungfish decreased significantly compared with those of the FW lungfish before EST treatment (N=9, $^{**}P$<0.01, two-tailed paired t-test). (B) The plasma osmolality and Na^+ and urea concentrations in the EST lungfish were increased compared with the corresponding values of the specimens maintained in the FW condition for 90 days (N=9, $^{*}P$<0.05, $^{**}P$<0.01, two-tailed Student's t-test). Values are means ± s.e.m.

Functional characterization of the lungfish AVT receptors

It has been shown that V1a- and V2-type AVP/AVT receptors couple with the PLC/PKC and AC/PKA signaling pathways, respectively (Liu and Wess, 1996). Therefore, we examined the effects of AVT administration on PLC/Ca^{2+} mobilization and AC/cAMP synthesis in CHO cells transfected with the cloned receptors. Addition of AVP or AVT to the cells transfected with lungfish V1a-type receptor resulted in an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner (Fig. 4A). On the other hand, in the CHO cells transfected with lungfish V2-type receptor, an extremely high dose of AVP or AVT resulted in a slight increase in $[Ca^{2+}]_i$; however, the responsiveness was 1000 times lower than that in the cells transfected with V1a-type receptor (Fig. 4B). Although addition of AVP or AVT to the cells transfected with V1a-type receptor did not increase intracellular cAMP levels (Fig. 4C), the cells transfected with lungfish V2-type receptor responded with the accumulation of cAMP in a concentration-dependent manner following AVP or AVT stimulation (Fig. 4D). Addition of ghrelin as a negative control did not affect the $[Ca^{2+}]_i$ and cAMP levels, similar to the results in the cells transfected with only the vector (Fig. 4A–D).

Expression of the AVT receptors in the freshwater and estivating lungfish

To examine the effects of long-term estivation (EST) on the expression of lungfish AVT receptors, the specimens were estivated for 90 days. In the EST lungfish, the mean body mass decreased from 129.4±9.1 to 108.4±8.2, and the percentage change in body mass before and after the treatment was –16.2% (Fig. 5A). The plasma osmolality and Na^+ concentration in the EST specimens was 1.5- and 1.2-fold greater, respectively, than the corresponding values of the specimens maintained in freshwater (FW; Fig. 5B). The plasma urea concentration in the EST specimens increased 13.1-fold compared with the FW specimens (Fig. 5B). Next, the tissue expression pattern of the cloned AVT receptor mRNAs was examined by RT-PCR in the FW and EST lungfish. In the FW lungfish, lungfish V1a-type receptor mRNA was ubiquitously expressed in all the tissues examined and was strongly expressed in the brain, eye, gill, lung and heart of the lungfish (Fig. 6A). Lungfish V2-type receptor mRNA was expressed in the brain, eye, gill, lung, heart, kidney, testis, muscle and skin of the FW specimens and was strongly expressed in the heart and kidney (Fig. 6A). No amplified product for lungfish V2-type receptor mRNA was observed in the liver, gall bladder, pancreas, intestine or ovary. In the EST lungfish, there was no obvious change in the expression pattern and levels of V2-type receptor mRNA between the FW and EST conditions (Fig. 6A,B). The expression pattern of V1a-type receptor mRNA also remained unchanged under the EST condition; however, the expression levels of V1a-type receptor mRNA appeared to have been increased under the EST condition in several tissues such as the liver, gall bladder and pancreas (Fig. 6A,B).

We quantitatively analyzed the mRNA expression levels of the hypothalamic AVT precursor and the renal AVT receptors in the FW and EST lungfish by a real-time RT-PCR method. The quantitative analysis showed a 5-fold increase in the amount of hypothalamic AVT mRNA under the EST condition as compared with the FW specimens (Fig. 7A). The expression level of AVT precursor mRNA in the hypothalamus significantly correlated with the plasma osmolality (Fig. 7B). However, there were no statistically significant differences between the FW and EST lungfish with regard to the expression levels of V1a- and V2-type receptor mRNA in

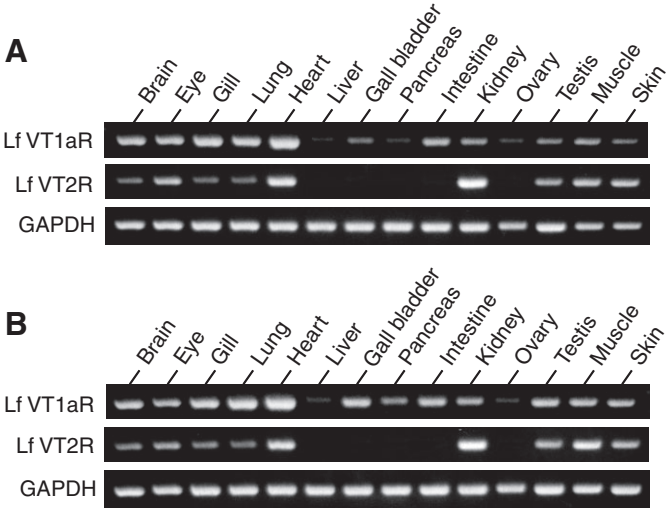

Fig. 6. Tissue distribution of lungfish V1a- (VT1aR) and V2-type (VT2R) receptor mRNAs in *P. annectens* under the FW and EST conditions. (A) Under the FW condition, lungfish VT1aR mRNA was ubiquitously expressed in all the tissues examined, and lungfish VT2R mRNA was strongly expressed in the heart and kidney. (B) Under EST, the expression pattern of VT1aR mRNA did not change; however, the expression levels of VT1aR mRNA appeared to have been increased by EST in several tissues such as the liver, gall bladder and pancreas. There was no obvious change in the expression pattern and levels of VT2R mRNA between the FW and EST conditions. GAPDH mRNA was used as an internal standard to estimate the relative levels of the objective mRNA expression.

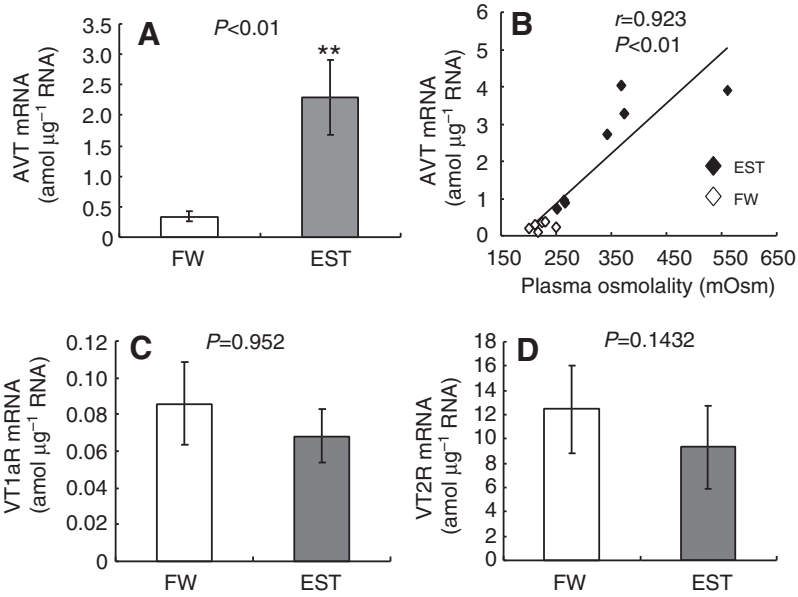

Fig. 7. Effect of estivation on the expression of AVT precursor mRNA in the hypothalamus and the two types of AVT receptor (VT1aR and VT2R) mRNA in the kidney. During the 90 days of EST, the expression level of AVT precursor mRNA in the hypothalamus increased significantly (A) and the expression level correlated significantly with the plasma osmolality (B). By contrast, there was no significant difference in the expression levels of either VT1aR or VT2R mRNA in the kidneys between the FW and EST lungfish (C,D). The values are means ± s.e.m. (N=7 or 8). The double asterisk indicates a statistically significant change (**P<0.01, Student's t-test) from the values under the FW condition.

the kidney (Fig. 7C,D). There was no significant difference in the expression levels of GAPDH mRNA, used as an internal control, between the two groups (data not shown). In addition, we examined whether there was a significant difference in the total RNA levels between the FW and EST groups and estimated the ratio of the amount of total RNA relative to the amount of genomic DNA extracted from the kidney (20 mg). As a result of the quantitative analysis, there was no significant difference between the groups (data not shown).

Immunochemical detection of lungfish V2-type AVT receptor

In the western blot analysis, a 61-kDa immunoreactive band was detected in the membrane fractions from the heart and kidney using the affinity-purified antibody to lungfish V2-type receptor.

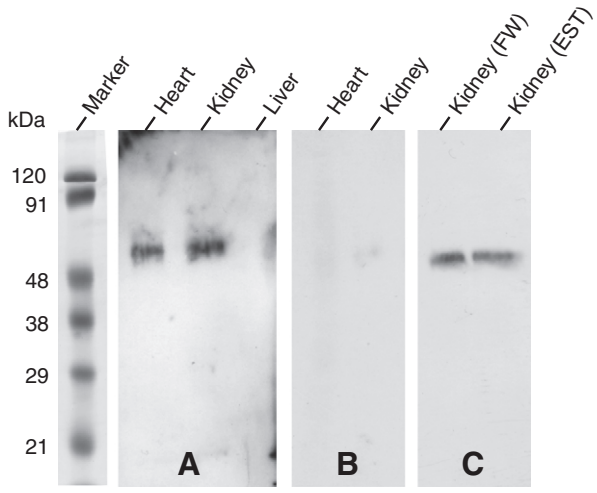

Fig. 8. Western blot analysis of lungfish V2-type receptor protein in the tissue extracts of *P. annectens*. An immunoreactive band was detected at 61 kDa in the extracts of the lungfish heart and kidney, but not the liver (A). When the antibody preabsorbed with the immunogen peptide (1 μg ml^{-1}) was used, the immunoreactive band was not observed (B). There was no difference in the protein expression levels in the kidney extracts between the FW and EST conditions (C).

However, the band was not detected in the fraction from the liver used as a negative control (Fig. 8A). The molecular mass of the immunoreactive band for lungfish V2-type receptor was similar to that for the glycosylated form (47–62 kDa) of mammalian V2 receptors (Fenton et al., 2007; Gutkowska et al., 2007). As shown in Fig. 8B, no immunoreactive band was observed after preabsorption with the immunogen (1 μg ml^{-1}). There was no significant difference between the FW and EST lungfish with regard to the amount of V2-type receptor protein levels (Fig. 8C), which coincides with the result of the V2-type receptor mRNA expression.

The renal nephron of the African lungfish *P. dolloi* is composed of a glomerulus, neck segment, proximal tubule, intermediate segment, early part of distal tubule, late part of distal tubule, and collecting tubule (Hentschel and Elger, 1987; Ojeda et al., 2006). In the present study, the nephron structure of *P. annectens* was observed to be similar to that of *P. dolli*. The kidney can be divided into three zones (ventral, middle and dorsal). The different renal tubule segments were identified based on the characteristics of the cell and arrangement of the segments reported in the previous studies (Hentschel and Elger, 1987; Ojeda et al., 2006). The early part of the distal tubule is mainly found in the ventral zone of the kidney, while the dorsal zone contains the proximal tubule and the collecting tubule. The glomerulus and the late part of the distal tubule are located in the middle zone of the kidney (Fig. 9A,B).

Immunoreactivity to lungfish V2-type receptor protein was observed in some tubules located in the middle zone of the kidney in the FW lungfish (Fig. 9C). The immunolabeling was mainly observed in the basolateral area of the immunoreactive cells (Fig. 9D). No immunoreactivity was observed in the early distal tubules, proximal tubules and blood vessels. Focusing on the distal nephron, the localization of lungfish V2-type receptor was compared with that of vacuolar-type H^+-ATPase using mirror-image sections (Fig. 9E–H). The H^+-ATPase antibody labeled the intercalated cells along a putative collecting tubule (Fig. 9F,H), whereas no immunoreactivity for lungfish V2-type receptor was observed in the collecting tubules (Fig. 9E,G). There was no obvious difference in the localization of immunoreactive V2-type receptor between the FW and EST lungfish (data not shown).

DISCUSSION

In the present study, we cloned two types of AVT receptors from the kidney of the African lungfish, *P. annectens*. The deduced amino acid sequences of both AVT receptors were shown to have the highest homology with amphibian V1a-type receptors (78–85%) and V2-type receptors (75–77%), respectively. The predicted receptors contained the consensus tripeptide of the V1a- (Asp-Arg-Tyr) and V2-type receptors (Asp-Arg-His), respectively, at the interface of TM III and the second intracellular loop. Molecular phylogenetic analysis also showed that the putative lungfish V2-type receptor belongs to the V2-type receptor group of tetrapods and was distinguishable from the V1-type receptor or other neurohypophysial hormone receptor groups. It has been known that the V1- and V2-type receptors in tetrapods are coupled to the PLC/PKC pathway and AC/PKA pathway, respectively (Wargent et al., 1999; Kohno et al., 2003). In the present study, administration of AVT to CHO cells transfected with lungfish V1a-type receptor or V2-type receptor resulted in intracellular Ca^{2+} mobilization and the accumulation of cAMP in a concentration-dependent manner, respectively. These results clearly indicate that both AVT receptors are as functional as those in tetrapods. This is the first direct evidence for the existence of V2-type receptor in fish, suggesting that V2-type receptor has emerged before the appearance of amphibians.

Balment and colleagues (Perrott et al., 1993; Warne, 2002) suggest that the AVT receptor associated with the AC/cAMP signaling pathway might be present in teleosts. In renal tubules isolated from rainbow trout, administration of AVT stimulated intracellular cAMP production in a dose-dependent manner (Perrott et al., 1993; Warne, 2002). However, there has been no molecular evidence for the existence of the V2-type AVT receptor in teleosts.

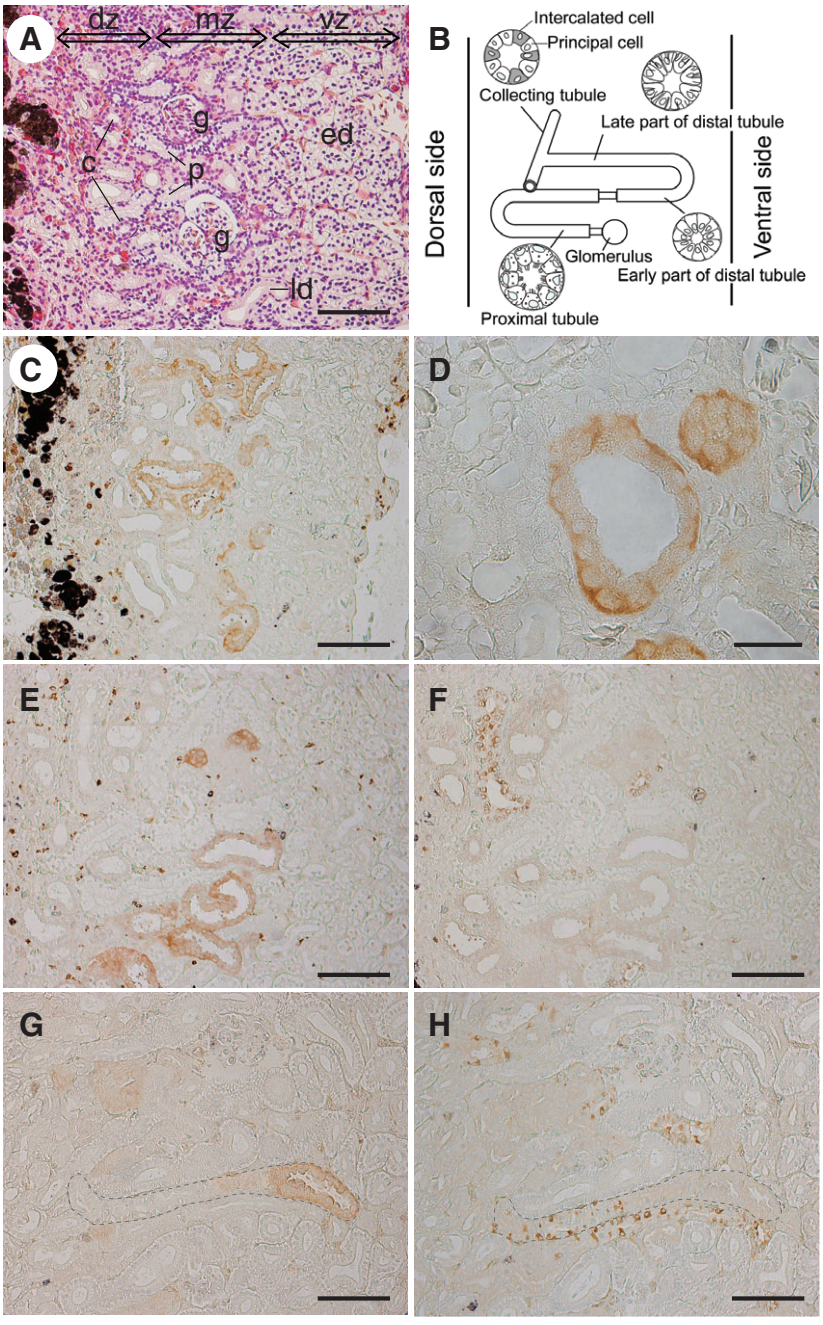

Fig. 9. Immunohistochemical localization of lungfish V2-type receptor (VT2R) in the kidney of *P. annectens*. (A) A longitudinal section of the lungfish kidney stained with hematoxylin and eosin. Some glomeruli (g) are located in the middle zone (mz) of the kidney. The ventral zone (vz) contains only the early part of the distal tubule (ed), whereas the dorsal zone (dz) contains other parts of the nephron segment (ld, late part of the distal tubule; c, collecting tubule; p, proximal tubule). (B) A schematic diagram to illustrate the positions of various segments and the constituent cells along the lungfish nephron is presented. (C) Cells immunoreactive for VT2R or H^+-ATPase stained brown with the diaminobenzidine (DAB). The collecting tubule consists of principal cells and intercalated cells. Immunostaining for lungfish VT2R (brown) was observed in the basolateral area of the cells of some tubules located in the middle zone. (D) Magnification of a VT2R-immunopositive renal tubule. In the mirror-image sections, no VT2R immunoreactivity (E,G) was detected in the collecting tubule where H^+-ATPase was expressed (F,H). Scale bars, 200 μm (A,C), 100 μm (E–H) and 50 μm (D).

In the databases for the V2-type AVT receptor sequences, we found several nucleotide sequences with homology to known V2-type receptor sequences in the databases of puffer fish (EMBL accession no. CAAE01014729 and CAAE01014991) and zebrafish (Genbank accession no. XP-001346005; EMBL accession no. CAN87971). However, the proteins deduced from these nucleotide sequences appear to be structurally incomplete, because these nucleotide sequences represent a deletion of the 3′ coding region or an elongated nucleotide sequence such as a fusion gene. The nucleotide sequences found in a Blast search may represent pseudogenes duplicated from an ancestral neurohypophysial hormone receptor gene because whole-genome duplication occurred during the evolutionary process to teleosts (Van de Peer, 2004). An exploration of the V2-type receptor in coelacanths and elasmobranchs, which diverged earlier than lungfish, will be necessary to elucidate the molecular evolution and physiological function of the AVP/AVT receptor family in vertebrates.

In the present study, we found that lungfish V2-type receptor mRNA was strongly expressed in the kidney. In mammals, the V2 receptor is predominantly expressed in the distal convoluted tubules and collecting ducts of the kidney and is involved in the regulation of permeability to water, Na^+ and urea in the renal tubule of the kidney (Bentley, 2002). Kohno et al. showed that in the Japanese tree frog, V2-type receptor mRNA was strongly expressed in the kidney, urinary bladder and pelvic skin, where absorption of water and ions occurs (Kohno et al., 2003). Therefore, the high expression of lungfish V2-type receptor in the kidney is consistent with the distribution of V2-type receptor in tetrapods, suggesting that the V2-type receptor is important for body fluid regulation in the kidney. The immunohistochemical study further revealed that the V2-type receptor protein is localized in the basolateral area of the cells of the late part of the distal tubules in the lungfish kidney. In tetrapods, water and Na^+ filtered in the glomerulus are reabsorbed in the distal tubule and the collecting tubule, which are the target sites of AVP/AVT (Bentley, 2002). In these segments, the V2-type receptor mediates the accumulation of aquaporin-2 water channel (AQP2) into the apical membrane through the activation of PKA (Lolait et al., 1992; Nielsen et al., 2002). In our preliminary experiment, lungfish AQP2 (DDBJ accession no. AB474277) was observed to be expressed on the apical cell membrane of the late part of the distal tubules in the kidney of *P. annectens* (N.K., S.H. and S. Uchiyama, unpublished results). The V2-type AVT receptor expressed in the kidney may be functionally coupled to the water channel and may be as involved in osmoregulation in the lungfish as it is in tetrapods.

In the lungfish, high V2-type receptor expression levels were also detected in the heart; however, the function of V2-type receptor in this organ remains to be elucidated. Moderate V2-type receptor expression was found in various tissues including the brain, gill, lung and skin. Consistent with our results, the expression of V2-type receptor mRNA has been found in various organs and tissues, even in frogs and newts (Kohno et al., 2003; Acharjee et al., 2004; Hasunuma et al., 2007). Therefore, lungfish V2-type receptor may be implicated in a broad range of physiological functions in primitive vertebrate groups.

It has been reported that *P. annectens*, *P. dolloi* and *P. aethiopicus* can experimentally estivate in mud or air for several months (Chew et al., 2004; Ip et al., 2005; Loong et al., 2008). In the present study, 90 days of EST using a plastic bag decreased the average body mass of the lungfish by approximately 16% compared with their mass before EST. The plasma osmolality and Na^+ and urea concentrations also increased significantly under the EST condition. It appears that water retention is facilitated during the EST period. However, we could not detect any obvious change in the expression levels of V1a-type and V2-type receptor mRNA in the kidney during the 90 days of EST. Similar results in the protein levels were found by western blot analysis with a specific antibody against lungfish V2-type receptor. There was no obvious difference in the density of the immunoreactive 61-kDa band in the kidney between the FW and EST lungfish. Conversely, 90 days of EST remarkably increased the expression levels of AVT precursor mRNA in the hypothalamus compared with that in the FW lungfish, and the mRNA levels significantly correlated with the plasma osmolality. Therefore, the present results suggest that the production of AVT may be accelerated in response to an elevation in the plasma osmolality and/or hypovolemia caused under EST. Warne et al. also reported that, although the hypothalamic AVT precursor mRNA and plasma AVT concentrations were significantly elevated after the hypertonic challenge of transfer of euryhaline flounder from FW to seawater, the expression levels of the V1a-type AVT receptor remained unchanged (Warne et al., 2005). It has been demonstrated that the expression of the V2 receptor in the rat kidney is downregulated during dehydration (Steiner and Phillips, 1988; Park et al., 1998; Machida et al., 2007), which opposes the conservation of water and sodium in dehydration; however, the physiological mechanisms that regulate the expression of the AVP receptors are not clearly understood. Identification of the promoter regions and transcriptional regulatory factors for the AVP/AVT receptors will be required to elucidate the regulation of expression of the receptors.

In conclusion, this was the first study to detect the functional V2-type AVT receptor in African lungfish. In the EST lungfish, a significant increase in the AVT precursor mRNA level was observed in the hypothalamus. The elevation of the AVT level induced by the EST condition may contribute to an antidiuretic action through both V1a- and V2-type receptors expressed in the lungfish kidney.

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AQP2	aquaporin 2
AVP	arginine vasopressin
AVT	arginine vasotocin
EST	estivation
FW	freshwater
NJ	neighbor joining
ML	maximum likelihood
MP	maximum parsimony
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

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