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Cloning and expression of the epithelial sodium channel and its role in osmoregulation of aquatic and estivating African lungfish *Protopterus annectens*

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ABSTRACT

The epithelial sodium channel (ENaC) is a sodium (Na^+)-selective aldosterone-stimulated ion channel involved in Na^+ transport homeostasis of tetrapods. We examined full-length cDNA sequences and tissue distributions of ENaCα, ENaCβ, and ENaCγ subunits in the African lungfish *Protopterus annectens*. *Protopterus* ENaC (*p*ENaC) comprises 3 subunits: *p*ENaCα, *p*ENaCβ, and *p*ENaCγ. *p*ENaCα, *p*ENaCβ, and *p*ENaCγ subunits are closely related to α, β, and γ subunits of the Australian lungfish *Neoceratodus forsteri* ENaC (*n*ENaC), respectively. Three ENaC subunit mRNAs were highly expressed in the gills and moderately expressed in the kidney and rectum of *P. annectens*. During estivation for 2–4 weeks and 2–3 months, plasma Na^+ concentration was relatively stable, but plasma urea concentration significantly increased in comparison with the control fish kept in a freshwater environment. Plasma aldosterone concentration and mRNA expression of the ENaCα subunit gradually and significantly decreased in the gills and kidney after 2 months of estivation. Thus, aldosterone-dependent Na^+ absorption via ENaC probably exists in the epithelial cells of osmoregulatory organs of lungfish kept in fresh water, whereas plasma Na^+ concentration may be maintained by a mechanism independent of aldosterone-ENaC axis during estivation in lungfish.

1. Introduction

The transition from fish to tetrapod and a terrestrial life in vertebrates represents one of the greatest events in vertebrate evolution. The transition has involved considerable changes in osmoregulation such that the body fluid metabolism may appear particularly variable. Recent molecular studies indicate that lungfishes are the closest living relatives of tetrapods (Brinkmann et al., 2004). Thus, they may provide information on how early vertebrates evolved from aquatic to terrestrial environments under changing external conditions.

Estivation is a state of dormancy that is characterized by air breathing, slowdown of the general metabolism, and suppression of the kidney and digestive functions (Fishman et al., 1986). The African lungfish *Protopterus* sp. estivate in mud cocoons when their water habitat dries up, and they store urea in their body fluids until water is available again (Graham, 1997). *Protopterus* sp. normally estivates for periods of 7–8 months in the wild and several years under laboratory conditions (Greenwood, 1986). During both estivation and air exposure, estivating lungfish synthesize urea via the urea cycle and accumulate urea (Chew et al., 2004; Loong et al., 2012). Although urea production and metabolism have been previously investigated, only a few reports exist in literature concerning water and electrolyte balance that are controlled in lungfish during estivation or in water (Wright, 2007). Wilkie et al. (2007) observed that after 20 weeks of estivation, lungfish had no disturbances in blood osmotic and sodium (Na^+) and chloride (Cl^-) balances although plasma urea concentration increased 7–8 times compared with that of freshwater acclimated fish. In the hormonal control of osmoregulation, neurohypophysial hormones (arginine vasopressin, AVP; arginine vasotocin, AVT) and adrenocortical hormones have pivotal roles in regulating water and salt transfer across epithelial membranes of tetrapods (Bentley, 2002). In lungfish, in freshwater or during estivation, Na^+ and Cl^- uptakes and water retention are important, and a number of ion channels and transporters may be involved in the osmoregulatory organs such as the gills, lung, alimentary tracts, and kidneys.

Na^+ is the major osmotically active constituent in the extracellular fluid. Aldosterone is the most potent Na^+-retaining factor in the mammalian kidney and colon (Garty and Palmer, 1997). Aldosterone is present in the lungfish as well as tetrapods (Blair-West et al., 1977), whereas it is absent in teleosts, elasmobranchs, and agnathans (Bentley, 2002). Since lungfish has been shown to possess aldosterone, which is capable of increasing Na^+ retention, we sought to determine whether they also possess the amiloride-sensitive epithelial Na^+ channel (ENaC) as a precursor of osmoregulation for terrestriality. ENaC is

Abbreviations: epithelial sodium channel, ENaC; sodium ion, Na^+.

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essential for regulating Na^+ transport, mediating electrogenic Na^+ transport; it is rate limiting for Na^+ absorption in the epithelia of the kidney, colon, skin, airways, and taste cells in tetrapods (Studer et al., 2011). ENaC comprises at least 3 homologous subunits: ENaCα, ENaCβ, and ENaCγ. The ENaCα subunit appears to be the core conducting element, whereas the β and γ subunits are associated with trafficking and insertion of the channel in the cell membrane (Canessa et al., 1994; Kellenberger and Schild, 2002). The fourth subunit of ENaC, termed as ENaCδ, was also cloned in primates, but further studies are necessary to clarify the physiological role of ENaCδ (Ji et al., 2012). Although ENaCα, ENaCβ, and ENaCγ subunits have been cloned in tetrapods, no orthologs or paralogs have been identified in teleost fishes (e.g., zebrafish, fugu, stickleback, medaka). Genomic data reveal the presence of ENaC subunits in the coelacanth (*Latimeria chalumnae*) and the sea lamprey (*Petromyzon marinus*) (Ensemble Genome Research Database). Furthermore, we recently cloned full-length sequences of *Neoceratodus forsteri*, *n*ENaCα, *n*ENaCβ, and *n*ENaCγ subunits. *n*ENaCα immunoreactivity was observed on the apical membrane in the epithelial cells of the gills, kidney, and rectum. According to a physiological study using *Xenopus* oocyte expression system, it was shown that *n*ENaC potentially functions in Na^+ transport in aquatic environments (Uchiyama et al., 2012).

Thus, it is notable to investigate whether all living lungfishes possess ENaC similar to those of tetrapod species. The presence of ENaC subunits and their expression during estivation in *Protopterus* sp. under drought conditions would need to be studied to confirm that ENaC plays an important role in regulating Na^+ transport in the gills, kidney, and rectum under freshwater and estivation, relying on aldosterone along with an active ENaC system.

2. Materials and methods

2.1. Animals

Juvenile specimens of African lungfish, *P. annectens*, were purchased from a commercial supplier. The fish were fed twice weekly on a synthetic diet for carnivorous fish and were acclimated to laboratory conditions at 25–28 °C for at least 1 month. Twenty eight fish were divided into freshwater control and estivating groups. Body weights of the fish were measured prior to and after estivation periods. All experiments were performed according to the regulations of the ethics committee of the University of Toyama.

2.2. Estivation

Control fish (n = 5) were kept in dechlorinated tap water for 1 month. For the estivation treatment, 23 fish were placed individually on the surface of semi-liquid mud in plastic boxes. The fish began to burrow and formed an estivation nest. Thereafter, the mud began to dry; when the mud surface became crusty within 4 weeks, the lungfish were encased in a mud cocoon. The fish were maintained for 1 week to 3 months after the estivation treatment at room temperature (25–28 °C). The fish were killed after a designated period (1, 2, and 4 weeks and 2, and 3 months).

2.3. Blood sampling and analysis of plasma parameters

The fish were anesthetized with 0.1% ethyl 3-aminobezoate methanesulfonate (MS-222; Sigma, Saint Louis, MO, USA). Blood samples were collected by cardiac puncture either in heparinized 1-mL syringes or hematocrit (Ht) capillaries to measure electrolyte and hormone concentrations. All samples were immediately placed on ice and then centrifuged at 2000 ×*g* for 20 min at 4 °C. A part of the plasma sample was used to measure osmolality and electrolyte concentrations, and the remaining plasma was stored at −30 °C until aldosterone analysis. After sacrificing the animals, tissue samples were obtained and frozen immediately in liquid nitrogen and stored at −80 °C until use.

Plasma osmolality was measured using an osmometer (Vapro osmometer 5520; Wescor Biomedical Systems, UT, USA). Plasma Na^+, chloride (Cl^-), and glucose concentrations were measured using an ABL 625 analyzer (Radiometer, Copenhagen, Denmark). Plasma urea concentration was measured using the Wako Urea NB test (Wako Pure Chemical Industries, Osaka, Japan) in vitro enzymatic colorimetric method. Plasma aldosterone concentration was assayed using the Aldosterone EIA Kit (Cayman Chemical Company, MI, USA) with a microplate reader (Bio-Rad Laboratories, CA, USA).

2.4. Molecular cloning of P. annectens ENaC subunits

Total RNA was extracted from the gills of *P. annectens* using ISOGEN (Nippon Gene, Tokyo, Japan). Complementary DNA was synthesized from 1 μg of total RNA using a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan). Degenerate primers (Table 1) for each ENaC subunit were designed based on the alignment of the 3 subunits (α, β, and γ) of *N. forsteri*, amphibians, and mammals. 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 purified from the sliced gel and ligated into the pT7Blue T-Vector (Merk, Darmstadt, Germany). The ligated plasmid was transformed into the competent cell (XL1-Blue; Invitrogen,

Table 1
Primers used in this study.

Target gene	Application	Oligonucleotide sequences (5′–3′)
ENaCα	Degenerate PCR	Sense 1: AAYCGNATGAARACNGCNTTYTGG
		Sense 2: ACNTTYGGNCTNATGTAYTGG
		Antisense 1: TTRTARTARCARTGNCCCCA
		Antisense 2: CCRAACCANAGNGACCAYTG
	5′-RACE PCR	Antisense 1: CTGGAAAAACAAGTTTATCATAG
		Antisense 2: TGTTTGCAGGTGTATAATGG
		Antisense 3: TGCTTGTTAGAGGTATTGTTGC
	3′-RACE PCR	Sense 1: ACTGGTAATCGATCTCATTGC
		Sense 2: TCCTTCAGAGACTTATGTACATCC
		Sense 3: AATGAACATAGAGAAAGAGCACC
	RT-PCR	Sense: TCCTTCAGAGACTTATGTACATCC
		Antisense: TAATACTAGTCTGGCTCATTCGTC
	Real Time-PCR	Sense: TCCTTCAGAGACTTATGTACATCC
		Antisense: TAATACTAGTCTGGCTCATTCGTC
ENaCβ	Degenerate PCR	Sense 1: CTNCARAARGGNCCNGGNTA
		Sense 2: TTYGCNTGYCTNGTNTTYTGG
		Antisense 1: AGNACRTGRAABATCCARTCYTC
		Antisense 2: CCCATCCARAANCCRAAYTG
	5′-RACE PCR	Antisense 1: CTGAGAGAGACACGCTGACAC
		Antisense 2: AATTGTAACTGCTGGGAATGC
		Antisense 3: TGGAATACTTGAAAGGATTGG
	3′-RACE PCR	Sense 1: GATGGAGCCAACTACTGTGATAC
		Sense 2: CAACTCAGACTGGGTGTATTG
		Sense 3: AAGACTCCACAGATGCAAATG
	RT-PCR	Sense: CAACTCAGACTGGGTGTATTG
		Antisense: GAAGTTTAATCTGAGGACACCATC
ENaC γ	Degenerate PCR	Sense 1: ATGCARTGGTAYTGYYTSA
		Sense 2: ACNAAYACNCAYGGNTGYMG
		Antisense 1: GTCCAYTCYTTRAARCTRCA
		Antisense 2: GARCARCTCATCCANAGNCC
	5′-RACE PCR	Antisense 1: CTACTCACTTCAAATCTGCCTC
		Antisense 2: AATGGTCAATAGCTGTGGTTG
		Antisense 3: GGTGCTGAGTACTGTTCCTATG
	3′-RACE PCR	Sense 1: TCATCCAGAAGAGAAATAGTTG
		Sense 2: AATCCATACAGCTTCTCCAG
		Sense 3: TTTACAGGAATATCGTCAAGTG
	RT-PCR	Sense: GGTGCTGAGTACTGTTCCTATG
		Antisense: TATCTCAAAGTCCTTGGAATTGC
GAPDH	RT-PCR	Sense: AGTTTTCTGAGTGGCTGTATAAG
		Antisense: ATCCTGCTAACATCAAGTGGG
	Real Time-PCR	Sense: AGTTTTCTGAGTGGCTGTATAAG
		Antisense: ATCCTGCTAACATCAAGTGGG