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The epithelial sodium channel in the Australian lungfish, *Neoceratodus forsteri* (Osteichthyes: Dipnoi)

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Epithelial sodium channel (ENaC) is a Na^+-selective, aldosterone-stimulated ion channel involved in sodium transport homeostasis. ENaC is rate-limiting for Na^+ absorption in the epithelia of osmoregulatory organs of tetrapods. Although the ENaC/degenerin gene family is proposed to be present in metazoans, no orthologues or paralogues for ENaC have been found in the genome databases of teleosts. We studied full-length cDNA cloning and tissue distributions of ENaCα, β and γ subunits in the Australian lungfish, *Neoceratodus forsteri*, which is the closest living relative of tetrapods. *Neoceratodus* ENaC (*n*ENaC) comprised three subunits: *n*ENaCα, β and γ proteins. The *n*ENaCα, β and γ subunits are closely related to amphibian ENaCα, β and γ subunits, respectively. Three ENaC subunit mRNAs were highly expressed in the gills, kidney and rectum. Amiloride-sensitive sodium current was recorded from *Xenopus* oocytes injected with the *n*ENaCαβγ subunit complementary RNAs under a two-electrode voltage clamp. *n*ENaCα immunoreactivity was observed in the apical cell membrane of the gills, kidney and rectum. Thus, *n*ENaC may play a role in regulating sodium transport of the lungfish, which has a renin–angiotensin–aldosterone system. This is interesting because there may have been an ENaC sodium absorption system controlled by aldosterone before the conquest of land by vertebrates.

Keywords: evolution of ion channel; sodium transport; *Xenopus* oocyte; immunoreactivity; Dipnoi

1. INTRODUCTION

It is generally accepted now that the vertebrate transition from water to land was via a sarcopterygian fish ancestor. Sarcopterygians (lobe-finned fishes) separated from actinopterygians (ray-finned fishes) prior to the Devonian (great age of fishes), at least 416 Ma. Teleosts (the most numerous actinopterygian fish today) had not evolved at the time of this separation. Thus, they have been separated from tetrapods for more than 416 Myr. More recently, evidence has been accumulating to suggest that, of the living sarcopterygian fishes, lungfish (Dipnoi) are closer to the ancestral tetrapod than are the coelacanths [1]; and the Australian lungfish (*Neoceratodus forsteri*) is the most primitive lungfish [2]. So, it is to *Neoceratodus* that we must look for clues concerning the physiological pre-adaptations for terrestriality. One such pre-adaptation would be an efficient epithelial sodium channel (ENaC). The ENaC is a Na^+-selective, non-voltage-gated, non-inactivating ion channel of the ENaC/degenerin (ENaC/Deg) gene family [3–5]. The ENaC/Deg gene family has been proposed as being present in metazoans [6]; ENaC/Deg proteins participate in several physiological processes, ranging from ion homeostasis in epithelia to mechanosensation and synaptic transmission in neurons. In the ENaC/Deg family, ENaC is 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, keratinocytes and taste cells in tetrapods [7]. ENaC comprises at least three homologous subunits: ENaCα, β and γ [3,7–9]. Each subunit (size: 85–95 kDa) shares approximately 30–40% sequence identity with the other two. It has two presumed membrane-spanning domains, a large extracellular loop and intracellular N- and C-termini. ENaC subunit stoichiometry has been the subject of several studies since the initial cloning of three subunits. Recent crystallographic data obtained for related acid-sensing ion channels suggest that ENaC most likely functions as an ENaCα, β and γ heterotrimer in the plasma membrane [10–12]. The ENaCα-subunit appears to be the core-conducting element, whereas the β and γ subunits are associated

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with trafficking and insertion of the channel in the cell membrane. A fourth ENaC subunit, δ, has been cloned and is a member of the ENaC/Deg family. The ENaCδ is distinct from the ENaCα, β and γ subunits, known for their role in sodium homeostasis, as ENaCδ is expressed in the brain and activated by external protons [13]. The ENaC subunits have been cloned in some non-mammalian vertebrates: chicken, lizard, salamander, clawed frog, toad, coelacanth and lamprey [14–17 and Ensemble Genome Browser (http://www.ensembl.org/index.html)]. However, no orthologues or paralogues have been identified in teleost fishes (e.g. zebrafish, fugu, stickleback, medaka). Because of the findings of the same three subunits for the coelacanth, it is assumed that ENaC was lost in teleosts, probably at the time of the whole genome duplication when a number of genes were lost or rearranged rather than duplicated [18].

Our recent cloning of ENaCα subunit from a primitive urodele species, expressed in the external gills and pronephric ducts of larvae and in the ventral skin and mesonephros of adults, leads us to conclude that ENaC plays an important role in the osmoregulation of aquatic and terrestrial vertebrates. Thus, ENaC is important for the evolution of terrestriality during the evolution of early tetrapods, in addition to the acquisition of aldosterone as the most potent sodium-retaining factor in the kidney and colon [19]. Aldosterone is present in tetrapods and in *Neoceratodus* [20], whereas it is absent in teleosts, elasmobranchs and agnathans [21]. Because *Neoceratodus* has been shown to have aldosterone, which is capable of increasing sodium retention, we thought to find out whether it may also possess ENaCs as precursors of osmoregulation for terrestriality. We sought to identify the three subunits described in all tetrapod species examined to date.

2. MATERIAL AND METHODS

(a) *Animals*

Juvenile lungfish, *N. forsteri*, were obtained from the breeding colony at Macquarie University in Sydney, Australia (Macquarie University Animal Ethics Committee approval number: 2003/001). They were transported with CITES approval to the University of Toyama, Japan.

(b) *Molecular cloning of Australian lungfish* N. forsteri *epithelial sodium channel cDNA*

Total RNA was extracted from the *N. forsteri* gill, using ISOGEN (Nippon Gene, Tokyo, Japan). First-strand gill cDNA was synthesized with a first-strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). Full-length *n*ENaCα, ENaCβ and ENaCγ cDNAs were obtained by 5′- or 3′-rapid amplification of cDNA ends with adaptor primers (Takara Bio, Otsu, Japan) and ENaCα-, β- and γ-gene specific primers (see the electronic supplementary material, table S1).

(c) *Tissue distribution of* Neoceratodus *ENaCα, ENaCβ and ENaCγ mRNA*

Tissue expression of *n*ENaCα, β and γ mRNA was examined by reverse transcriptase PCR (RT-PCR). Total RNA was isolated from various tissues (brain, eye, gill, heart, liver, lung, muscle, small intestine and rectum), using ISOGEN (Nippon Gene). To prepare first-strand cDNA, total RNA (1 μg) was reverse-transcribed with a first-strand cDNA synthesis kit (Roche Diagnostics). Specific PCR primers were synthesized based on *n*ENaCα, β and γ. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard.

(d) *Expression and function of* Neoceratodus *epithelial sodium channel subunits in* Xenopus *oocytes*

Complementary RNAs (cRNAs) for *n*ENaCα, β and γ subunits were transcribed *in vitro* with an mMESSAGE mMACHINE T7 ultra kit (Ambion, USA). For functional expression studies, isolated *Xenopus* oocytes were treated with collagenase and then injected with the cRNAs. Oocytes were analysed electrophysiologically 24 h after cRNA injection, using a two-electrode voltage clamp technique, as described previously [22]. Briefly, oocyte membrane potential was held at 0 mV and stepped to test potentials from −100 to +60 mV in increments of 10 mV. Current was recorded in a solution containing 96 mM NaCl, 2 mM KCl, 3 mM $MgCl_2$ and 5 mM HEPES (pH 7.4). A macroscopic amiloride-sensitive nENaCαβγ current was obtained as the difference between the recorded currents from the same oocyte before and after adding 10 μM amiloride.

(e) *Immunohistochemistry and Western blot for* Neoceratodus *epithelial sodium channel in* Xenopus *oocytes*

For whole-mount immunostaining, oocytes were fixed overnight with 4 per cent paraformaldehyde at room temperature. Oocytes were incubated overnight in MAB (maleic buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) solution containing 1 : 2000 rabbit anti-*Bufo* ENaCα polyclonal antiserum [23] at 4°C. The anti-ENaCα polyclonal antiserum was raised in Japanese white rabbits with a synthetic peptide, NH_2–CNNTTIHGAIR–COOH, corresponding to amino acids 27–37 of the *Bufo* ENaCα-subunit. In the corresponding sites (aa 32–42) of the *n*ENaCα-subunit, only one amino acid, alanine (*Bufo* ENaCα 35), is different from threonine (*n*ENaCα 40). They were then incubated in blocking solution for 30 min, followed by incubation with a secondary antibody (1 : 500 Alexa-488 conjugated goat, anti-rabbit IgG; Invitrogen) for 2 h at room temperature. After rinses with PBT (1% bovine serum albumin, BSA/0.1% Triton X-100 in 1X phosphate-buffered saline, PBS), oocytes were observed with a dissecting microscope (Olympus SZX7) with a fluorescent module. For Western blot analysis of oocytes membrane preparations, whole membranes were isolated from oocytes. A total membrane sample was subjected to SDS–PAGE, using a 10 per cent resolving gel and a 5 per cent stacking gel. Proteins were transferred to nitrocellulose membranes, and Western blot analysis using an affinity-purified, anti-*Bufo* ENaC rabbit IgG fraction at 2 μg ml^{-1}.

(f) *Immunohistochemistry for* Neoceratodus *epithelial sodium channel in the kidney, gill and rectum*

Tissue samples that had been immersion-fixed with 4 per cent paraformaldehyde/PBS were dehydrated and embedded in paraffin. Deparaffinized sections (6 μm thick) were incubated overnight at 4°C with rabbit anti-*Bufo* ENaCα antiserum [16] at 1 : 500–4000 dilutions in 1 per cent BSA–PBS. Rabbit anti-salmon Na^+, K^+-ATPase α-subunit antiserum was also used [23]. After incubation, the sections were treated with Alexa Fluor 488-labelled, anti-rabbit IgG (dilution 1 : 200; Molecular Probes, USA) for 2 h at room temperature. Stained sections were observed and photographed under a

fluorescence microscope equipped with a digital imaging system (Axioplan/AxioCam; Zeiss, Germany).

(g) *Statistical methods*

Data are presented as the mean ± s.e. Statistical comparisons performed were by ANOVA followed by Bonferroni multiple comparisons post hoc test. A p-value of less than 0.05 was considered significant.

Further detailed methods and figures are provided in the electronic supplementary material, section 'materials and methods' and figure legends.

3. RESULTS

(a) *Molecular cloning of* Neoceratodus *epithelial sodium channel subunits*

Neoceratodus ENaC (*n*ENaC) comprises three subunits: *n*ENaCα, β and γ. A full-length cDNA sequence for *n*ENaCα was 2558 bp long with a 1965 bp open reading frame translating to a putative sequence of 655 amino acids (DDBJ accession no. AB675922; figure 1(α)). A full-length cDNA sequence for *n*ENaCβ was 2512 bp long with a 1953 bp open reading frame translating to a putative sequence of 651 amino acids (DDBJ accession no. AB675923; figure 1(β)). A full-length cDNA sequence for *n*ENaCγ was 2351 bp long with a 1959 bp open reading frame translating to a putative sequence of 653 amino acids (DDBJ accession no. AB675944; figure 1(γ)).

Hydropathy analysis using the Kyte–Doolittle algorithm for ENaCs predicted two putative transmembrane regions with N- and C-termini located in the cytoplasm; the large hydrophilic region was extracellular (see the electronic supplementary material, figure S1). ENaC subunits comprised two hydrophobic segments, the first (TM1) and second (TM2) transmembrane domains, which probably form transmembrane α helices. Predicted TM2 segment sequences show a high degree of homology among all members of the ENaC gene family identified in epithelial tissues of tetrapods. According to an ENaC pore model of mouse ENaC subunits by Kashlan & Kleyman [12], a Gly/Ser–X–Ser tract and selective sites for degenerin, amiloride blockade, voltage sensitivity and Na^+/K^+ selectivity are present in the TM2 domain of *n*ENaC subunits. A phylogenetic tree using the Ascidiadeg like (XP002121985) as an outgroup indicated that there were three main branches supported by a bootstrap value of 100 per cent: *n*ENaCα, *n*ENaCβ and *n*ENaCγ.

The *n*ENaCα protein was related to rodent ENaCα (52% identity) and salamander and *Bufo* (both 53% identity), coelacanth (49% identity) and lamprey (49% identify), based on the NCBI protein database research program (BLASTP). The *n*ENaCβ protein was related to ENaCβ of rodent (52% identity), chicken (54% identity), clawed frog (54% identity), coelacanth (50% identity) and lamprey (42%). The *n*ENaCγ protein was 52 per cent, 54 per cent, 54 per cent, 57 per cent and 39 per cent identical to the corresponding regions of rat ENaCγ, chicken, clawed frog, coelacanth and lamprey, respectively. Molecular phylogenetic relationships were analysed by the maximum-likelihood method, using the molecular evolutionary genetics analysis software (v. 4.0). The *n*ENaC subunits were most closely related to amphibian and coelacanth ENaC subunits (figure 2).

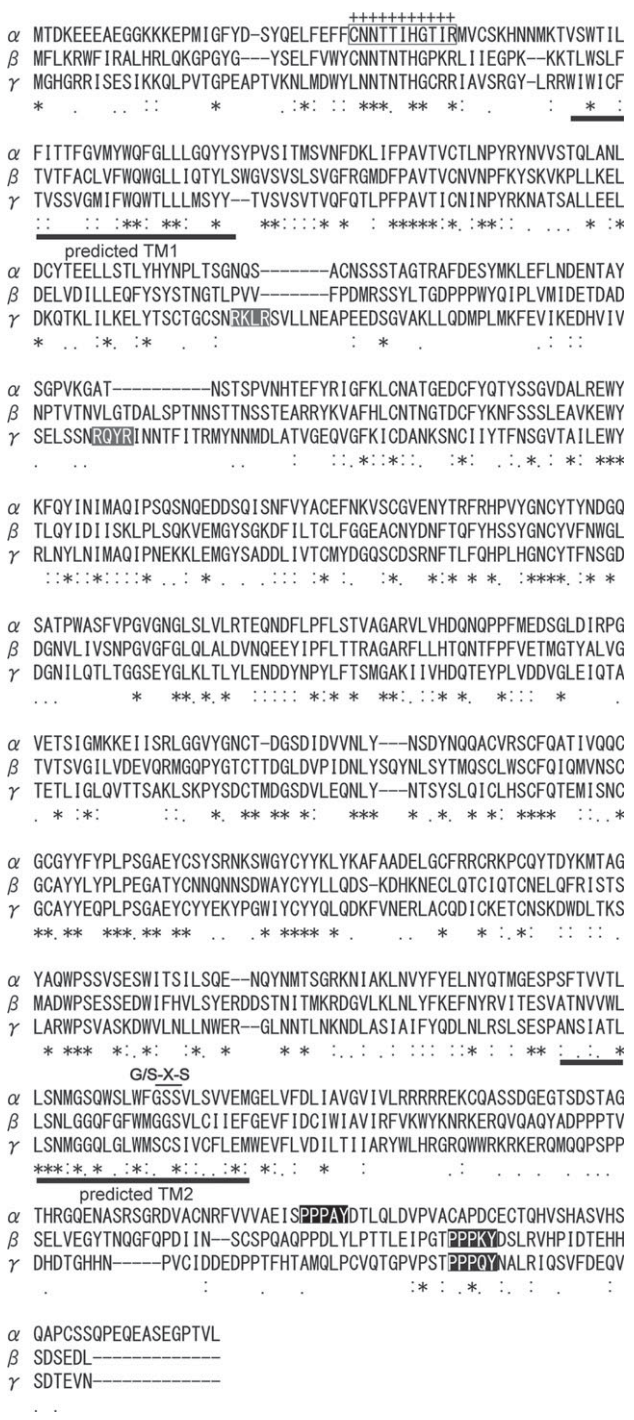

Figure 1. The deduced amino acid sequence of *n*ENaCα, β and γ proteins isolated from the gills of *Neoceratodus forsteri*. Plus symbols represent corresponding region of amino acids used as the anitigen to generate ENaC antibody. The grey shadows represent a consensus site for furin cleavage (RXXR). The dark shadows represent PY motif (PPxY) that is highly conserved in the C-terminus of ENaC subunits.

(b) Neoceratodus *epithelial sodium channel mRNA expressions and tissue distributions*

mRNA expressions and tissue distributions of *n*ENaCs were examined by RT-PCR, using total RNA isolated from various tissues. As shown in figure 3, *n*ENaCα mRNA expression was strong in the gill, kidney and rectum, and moderate or weak in the muscle, brain, heart, liver and intestine. No mRNA signal was detected in the eye and lung. *n*ENaCβ mRNA expression was

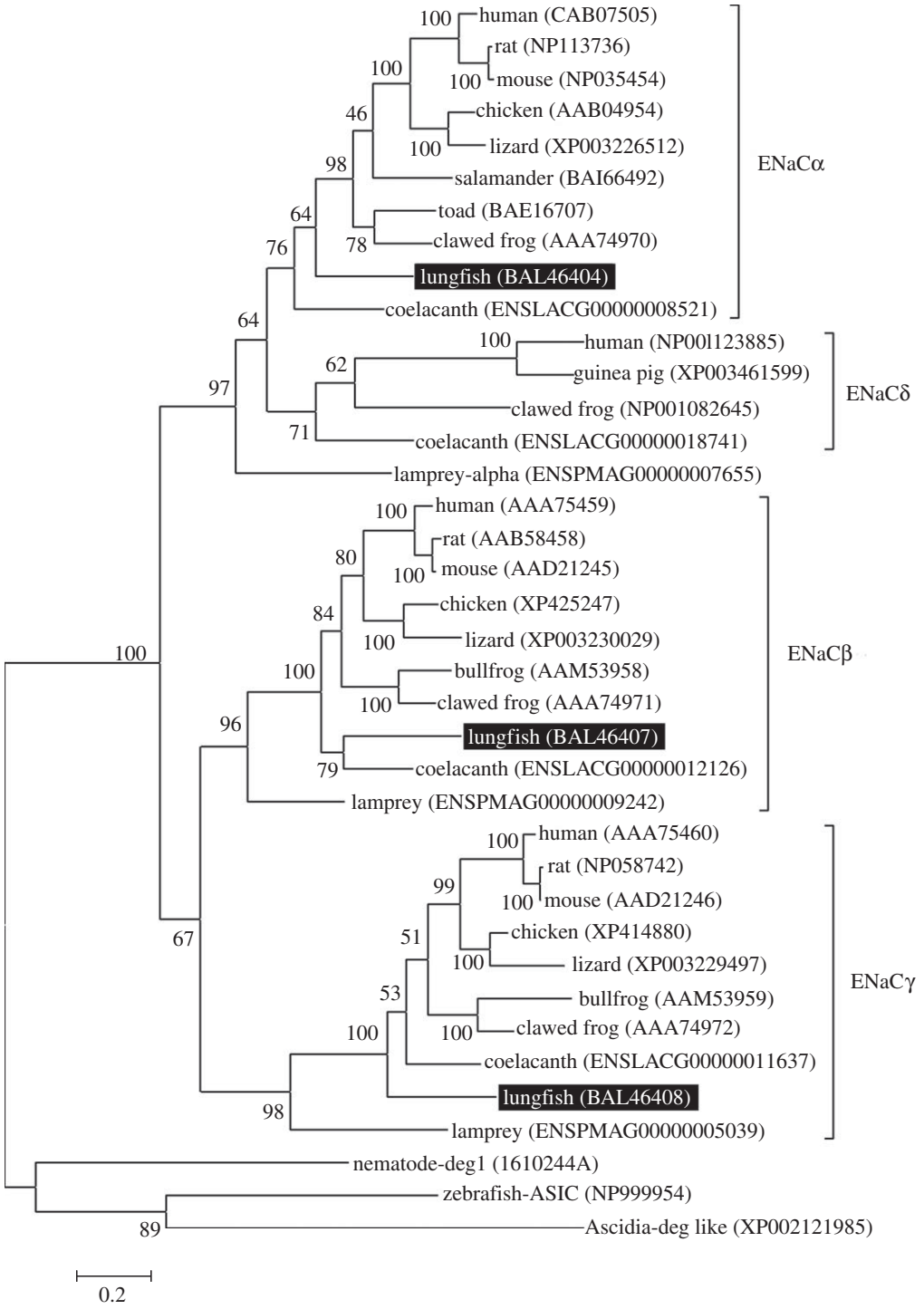

Figure 2. Phylogenetic tree showing the relative homologies of *n*ENaCα, β and γ subunit orthologues. Ascidia-deg like (XP002121985) was set as an outgroup. This analysis used the maximum-likelihood method with 100 bootstrap replicates. Numbers indicate the bootstrap values. Numbers in parentheses are the GenBank and Ensemble accession numbers of the sequences. ENaC, epithelial sodium channel; ASIC, acid-sensing ionic channel; deg, degenerin.

strong in the gill, kidney and rectum, and moderate in the brain, eye, liver and muscle. No mRNA signal was detected in the heart, lung and intestine. *n*ENaCγ mRNA expression was strong in the gill, liver, kidney and rectum, and moderate or weak in the heart, muscle and intestine. No mRNA signal was detected in the brain, eye and lung.

(c) *Electrophysiological recordings*

We recorded clear membrane currents under a two-electrode voltage clamp from *Xenopus* oocytes injected with *n*ENaCα, β and γ subunit cRNAs. Such a clear current was not obtained from non-injected or *n*ENaCα cRNA-injected oocytes under the same condition (figure 4*a*). The current–voltage relation for the *n*ENaCαβγ current showed a weak inward rectification (figure 4*b*). The resting membrane potential of oocytes expressing *n*ENaCαβγ was 23 ± 5 mV ($n = 6$). The less-depolarized value in comparison with expected reversal potential of Na^+ channel current (approx. +60 mV) was judged to be owing to a contamination of a slight leakage current. The reversal potentials of oocytes expressing *n*ENaCα only and non-injected

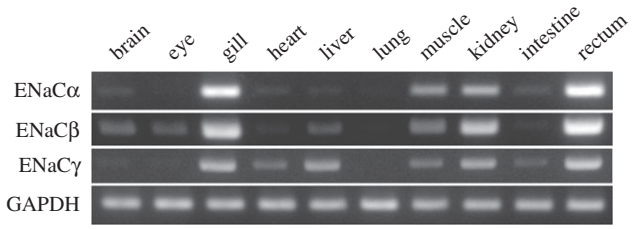

Figure 3. Tissue distributions of *n*ENaCα, β and γ subunit mRNAs by RT-PCR. Semi-quantitative RT-PCR used specific primers for the ENaC subunits and *n*GAPDH (*Neoceratodus* glyceraldehyde-3-phosphate dehydrogenase). mRNAs of ENaCα, β and γ subunits were strongly expressed in the gill, kidney and rectum, whereas no mRNA expression was observed in the lung or intestine.

oocytes were -13.8 ± 1.0 mV ($n = 5$) and -33.4 ± 5.2 mV ($n = 5$), respectively. *n*ENaC-mediated Na^+ currents excluding the leakage current were obtained as the difference between the currents recorded from the same oocyte before and 2 min after application of 10 μM amiloride in the bath solution. The subtracted current as well as its current–voltage relationship is shown in figure 4*c*. It showed an inward rectification, and the reversal potential was $+59.0 \pm 2.5$ mV ($n = 4$; figure 4*c*). These results indicate that amiloride-sensitive Na^+ current was expressed in *Xenopus* oocytes injected with *n*ENaCαβγ cRNA.

(d) *Immunochemical and immunohistochemical expressions of* Neoceratodus *ENaCα in the* Xenopus *oocytes*

Western blot analysis of total protein extracted from oocytes used an affinity-purified *Bufo* ENaCα polyclonal antibody. On the basis of its amino acid sequence, the predicted molecular weight of *n*ENaCα was 73.36 kDa, using the PeptideMass tool on the ExPASy server (http://www.expasy.org/tools/peptide-mass.html). The immunopositive bands on an SDS–PAGE gel were proteins of about 73 and 79 kDa (see the electronic supplementary material, figure S2). Immunofluorescence experiments using the same polyclonal antibody and low-power magnification revealed a polarized distribution of *n*ENaC in *Xenopus* oocytes (see the electronic supplementary material, figure S3), with a bright signal in the plasma membrane region at the vegetal pole. In non-injected or water-injected oocytes, no immunofluorescent signals were observed in the plasma membrane.

(e) *Immunohistochemical tissue localization of* Neoceratodus *ENaCα*

Immunohistochemistry using the polyclonal antibody was performed to determine the cellular expression and localization of *n*ENaCα in the kidney, rectum and gills. *Neoceratodus forsteri* gills consist of lobe-like filaments of varying sizes. *n*ENaCα immunoreactivity was observed in the cell membrane of oval-shaped cells in lobe-like filaments (figure 5*a*). *n*ENaCα was also located in the apical membranes of cells in the late distal tubule of the kidney. No *n*ENaCα-immunoreactive cells were found in the early distal nephron although Na^+, K^+-ATPase positive interdigitation in the basolateral membrane was prominent (figure 5*b*,*d*). In the rectum, the surface epithelial cells in the distal end of the spiral

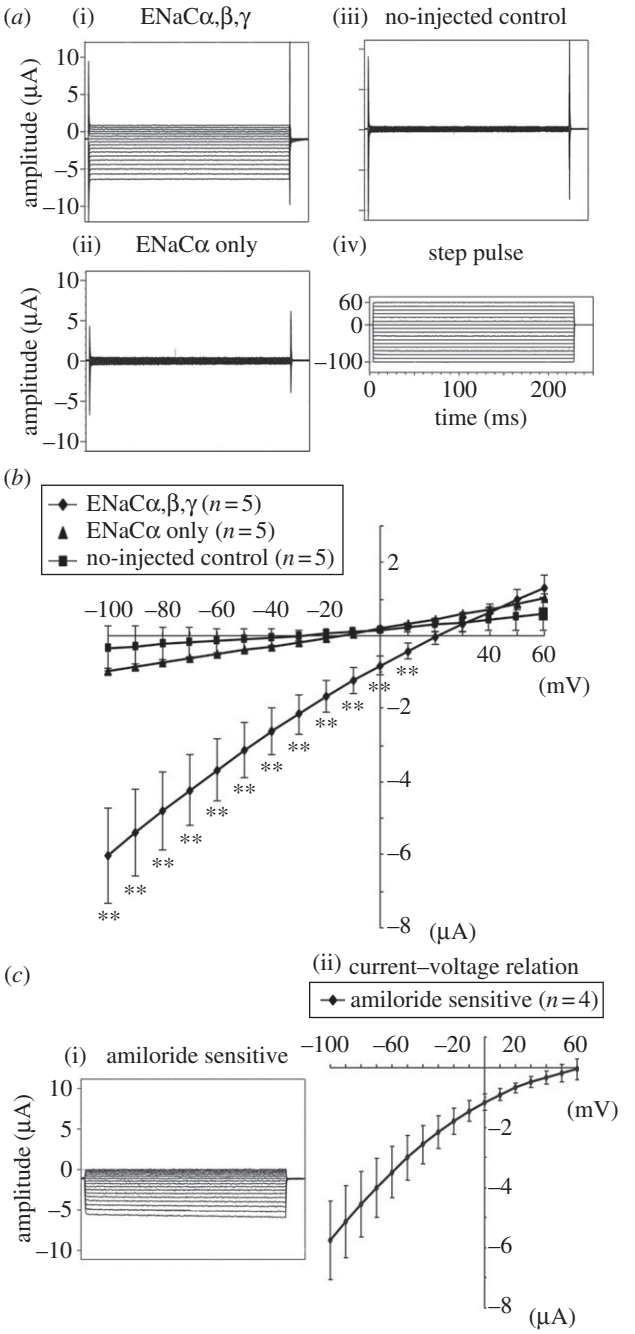

Figure 4. Current recordings under a two-electrode voltage clamp for *Xenopus* oocytes expressing *n*ENaCα and *n*ENaCαβγ. (*a*) Representative traces were obtained from an *n*ENaCαβγ-expressing oocyte (i: ENaCαβγ), and an oocyte-expressing *n*ENaCα (ii: *n*ENaCα only) and a non-injected oocyte (iii: non-injected control). (iv) Step pulse. Oocytes were voltage-clamped at 0 mV and step pulses were given from −100 to +60 mV in increments of 10 mV. (*b*) Current–voltage relationships for oocytes expressing *n*ENaCα only and *n*ENaCαβγ, and no-injected oocytes were shown. Results from five oocytes in each group were summarised. There were no significant differences in current values for ENaCα-expressing oocytes compared with the values for non-injected controls. There were significant inwardly rectifying currents in *n*ENaCαβγ-expressing oocytes compared with the non-injected control oocytes. (*c*) Subtracted current before and after application of 10 μM amiloride from the same oocyte-expressing *n*ENaCαβγ. Average of current–voltage relationship is shown in the right-hand side of the figure. Plotted data are shown as mean ± s.e. $^{**}p < 0.01$ different from control.

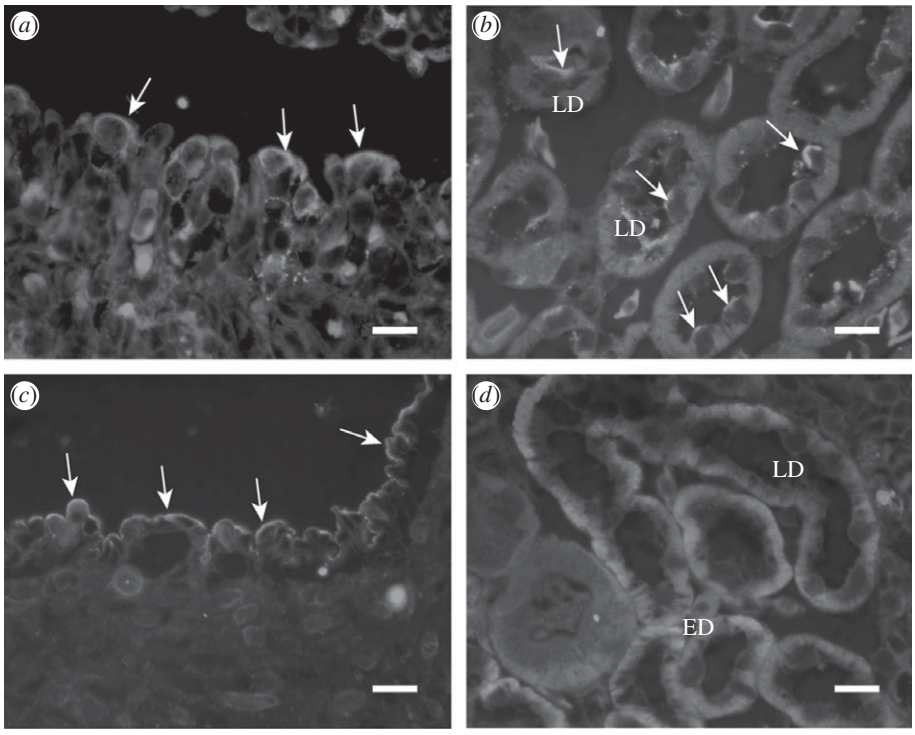

Figure 5. Immunohistochemical localization of *n*ENaCα and Na^+, K^+-ATPase in the gills, kidney and rectum, using a polyclonal *Bufo* ENaCα antibody or a polyclonal Na^+, K^+-ATPase antibody. (*a*) Immunoreactivity (green fluorescence) for *n*ENaCα was observed in the apical cell membrane (arrows) in the epithelial cells of gill lamella. (*b*) Apical cell membranes (arrows) of some cells in the late distal tubules of the nephron were also immunoreactive. (*c*) Immunoreactivity (green fluorescence) was observed in the apical membrane of rectum epithelium. (*d*) Na^+, K^+-ATPase immunoreactivity was observed in the basolateral cell membrane of the cells along the early distal (ED) to the late distal tubules (LD) in the kidney. Scale bars, 20 μm.

valve showed dense immunostaining (figure 5*c*). By contrast, immunostaining with the anti-ENaCα antibody was absent in the ventral skin of *N. forsteri*, where ENaCα-immunopositive cells are prominently present in amphibians.

4. DISCUSSION

This is, to our knowledge, the first report on the cDNA and genomic sequences of ENaC (α, β and γ) subunits and their expression pattern in the tissues of Australian lungfish *N. forsteri*. Taken together with the presence of a functional aldosterone system for ion uptake and salt secretion in *N. forsteri* [20], this further confirms the position of *N. forsteri* as the closest living ancestor to the tetrapods (land vertebrates).

(a) *Molecular identification and characterization of* Neoceratodus *epithelial sodium channel subunits*

The *n*ENaCα, β and γ subunit sequences were most closely related to amphibian ENaC subunits (54%, 56% and 54% identities, respectively). Homology among *n*ENaC subunits was low; each *n*ENaC subunit shared only about 30 per cent sequence identity with the other subunits, suggesting that gene duplication occurred early in this subfamily. The *n*ENaCα, β and γ subunits (*ca* 650 amino acids) had two presumed membrane-spanning domains (TM1 and TM2) connected by a large extracellular loop (>60% of the total protein) with short intracellular N- and C-termini projecting into the cytosol. Although little is known about the overall channel architecture and secondary structures of ENaC, the second membrane-spanning domain (TM2) is thought to contribute to the channel pore.

All three *n*ENaC subunits had conserved proline-rich regions conforming to the consensus PPXY motifs, where P is proline, Y is tyrosine and X is any amino acid, in their C-terminal end. The PPXY motif is important for interactions with the ubiquitin–protein ligases Nedd4 and Nedd4-2, which promote the ubiquitination, endocytosis and proteasomal degradation of ENaC [24]. ENaC activity is regulated by numerous intracellular and extracellular factors through alterations of either channel density, open probability or both. Channel activity is regulated by several serine proteases, including prostasin, furin and elastase. Hughey *et al.* [25] reported that furin cleaved specific sites within the extracellular loops of the ENaCα- and γ-subunits. Consensus sequences for furin-dependent proteolysis at two sites within ENaCα- and at single sites within γ-subunits are R–X–X–R, where R is arginine and X is any residue [25,26]. These consensus sequences of the ENaCα- and γ-subunits interosculate among the ENaC subunits of tetrapods. In this study, there was no furin cleavage consensus site within the *n*ENaCα-subunit. Conversely, there were two sites of consensus sequences for furin-dependent proteolysis within the *n*ENaCγ subunits (figure 1). Thus, a furin cleavage site domain is not conserved in *n*ENaCα-subunits. This may be related to our results that insignificant amiloride-sensitive sodium current was observed in *Xenopus* oocytes that expressed *n*ENaCα cRNA only.

(b) *Expression of* Neoceratodus *ENaCα, β and γ subunit mRNA and ENaCα protein*

In amphibians, ENaC mRNA and protein are expressed in the urinary bladder and ventral skin in addition to the kidney [16,19]. We recently studied the ontogeny of ENaC expression in the external gills and the kidney in a primitive urodele, *Hynobius nigrescens* [19]. *Hynobius* ENaC mRNA and protein were expressed in the external gills and pronephric ducts of aquatic larvae, and in the urinary bladder, ventral and dorsal skin and kidney in amphibious adults. It would seem that ENaCs change their tissue expression sites to play an important role in hydromineral regulation throughout life. For lungfishes, a few studies have outlined the structures of gills, kidneys and the alimentary canal, although the functions of these organs were not described in detail [27–29]. In this study, mRNA for ENaCα, β and γ subunits were highly expressed in common in the gills, kidney and rectum. ENaCα protein was also highly expressed immunohistochemically in the gills, kidney and rectum. Fish gills are major sites for multiple functions [30]. The expression pattern of ENaCα immunoreactive cells differed from *Hynobius* ENaCα expression in the external gills of salamander larvae. High *Hynobius* ENaCα immunoreactivity was observed in the apical cell membrane of a population of pavement cells and in mitochondria-rich (MR) cells in the primary filaments and secondary lamellae of the external gills [23]. Lungfish have MR cells in the gills and skin epithelia [27]. In this study, *n*ENaCα was localized on the apical membrane of several cells, possibly MR cells. In the kidneys of mammals and amphibians, ENaC are expressed on the apical membranes of the principal cells in the distal nephron [3,16]. The major stimulus for Na^+ reabsorption by principal cells is the hormone aldosterone, which stimulates both ENaC and Na^+, K^+-ATPase activities [5]. It was recently proposed that the renin–angiotensin–aldosterone system and angiotensin itself directly control ENaC activity and expression in the distal nephron of mammalian kidney [31]. Because angiotensin regulates aldosterone secretion in the Australian lungfish [20], as it does in mammals, a renin–angiotensin–aldosterone system may control ENaC expression and its function in the lungfish. In freshwater, ingested food may substantially contribute to ion uptake.

In this study, *n*ENaCα was expressed specifically on the apical membrane of epithelial cells of the rectum, and the distal end of the spiral valve but not in the midgut region [29]. The present observations may indicate that dietary sodium uptake contributes significantly to ion regulation in the lungfish. In mammals, ENaC has been found to regulate airway mucosal hydration and mucus clearance in the lung. It was not observed in *N. forsteri* lung, however, probably because this lungfish preferentially uses its gills for respiration and cannot survive long out of water, unlike the other two genera of living lungfish, which are renowned for their ability to aestivate out of water for long periods of drought [32].

According to recent studies, terrestrialization of vertebrates occurred in two steps: (i) the first tetrapods diverged from sarcopterygians during the Late Devonian period (*ca* 380 Ma); (ii) this was followed by adaptation to terrestrial life during the earliest Carboniferous period (*ca* 350 Ma). During the first step, the lungfishes represent an important step in the direct transition from water breathing to air-breathing vertebrates in that they possess both gills and lungs [32]. *Neoceratodus forsteri* has a single dorso-lateraly placed lung and preferentially uses its four pairs of holobranch gills for respiration and only uses its lungs when oxygen levels in water are very low. Thus, ENaC may play an important role in regulating sodium transport in gills, kidney and rectum under normal circumstances, relying on its rennin–angiotensin–aldosterone system along with an active ENaC system. The other two genera of lungfish are more recently derived and appear to have moved towards a different path of semi-terrestriality, involving aestivation when water-deprived. The presence of ENaC subunits and their expressions during aestivation for *Protopterus* spp. and *Lepidosiren* would need to be studied to confirm or not, that all living lungfishes possess ENaC similar to those of tetrapod species.

All experiments were performed according to the regulations of the Ethics Committee of the University of Toyama.

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