Comparative Biochemistry and Physiology, Part A 154 (2009) 535–540

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Immunolocalization of Na^+/K^+–ATPase and $Na^+/K^+/2Cl^-$ cotransporter in the tubular epithelia of sea snake salt glands

L.S. Babonis [a,*], K.A. Hyndman [a,b], H.B. Lillywhite [a], D.H. Evans [a]

[a] Department of Biology, University of Florida, Gainesville, FL, USA 32611
[b] Vascular Biology Center, Medical College of Georgia, Augusta, GA, USA 30912

ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 27 August 2009
Accepted 27 August 2009
Available online 12 September 2009

Keywords:
Histology
Immunohistochemistry
Na^+/K^+–ATPase
$Na^+/K^+/2Cl^-$ cotransporter
Osmoregulation
Sea snake
Salt gland
Western blot

ABSTRACT

The sublingual salt gland is the primary site of salt excretion in sea snakes; however, little is known about the mechanisms mediating ion excretion. Na^+/K^+–ATPase (NKA) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) are two proteins known to regulate membrane potential and drive salt secretion in most vertebrate secretory cells. We hypothesized that NKA and NKCC would localize to the basolateral membranes of the principal cells comprising the tubular epithelia of sea snake salt glands. Although there is evidence of NKA activity in salt glands from several species of sea snake, the localization of NKA and NKCC and other potential ion transporters remains unstudied. Using histology and immunohistochemistry, we localized NKA and NKCC in salt glands from three species of laticaudine sea snake: *Laticauda semifasciata, L. laticaudata,* and *L. colubrina*. Antibody specificity was confirmed using Western blots. The compound tubular glands of all three species were found to be composed of serous secretory epithelia, and NKA and NKCC were abundant in the basolateral membranes. These results are consistent with the morphology of secretory epithelia found in the rectal salt glands of marine elasmobranchs, the nasal glands of marine birds and the gills of teleost fishes, suggesting a similar function in regulating ion secretion.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Marine invasions have occurred, independently, multiple times among vertebrates. As most marine vertebrates maintain blood plasma at approximately 300 mOsm (about 1/3 the concentration of seawater), they experience salt accumulation and dehydration in marine environments (Evans, 2009). The evolution of specialized ionoregulatory tissues has, therefore, likely been responsible for ameliorating this ionic challenge, permitting the successful habitation of marine environments. One such specialized tissue, the salt gland, has evolved multiple times throughout the evolution of marine vertebrates. Among reptiles alone, five different cephalic salt glands have been described: the lachrymal gland in sea turtles and terrapins, the nasal gland in the marine iguana, the posterior sublingual and premaxillary glands in marine snakes, and the lingual gland in crocodilians (Dantzler and Bradshaw, 2009). While reptilian salt glands exhibit the greatest diversity and number of independent evolutionary origins among vertebrates, little is known about the mechanism of ion secretion in this group.

The anatomy and physiology of the rectal salt gland of marine elasmobranchs and the nasal salt glands of marine birds have been studied in great detail (for recent reviews, see: Hildebrandt, 2001; Evans et al., 2004). Glands from both groups have a compound tubular morphology composed of a series of branched secretory tubules that are bound by vascularized connective tissue arranged radially around the perimeter of a central duct (Sullivan, 1907; Marples, 1932). Secretory tubules bound into groups by connective tissue constitute the individual "lobules" of the gland; multiple such lobules are joined by the connection of their central ducts to a main duct, which serves to transfer the salt secretion from the mass of lobules to either the ileum of the intestine (elasmobranchs) or the nasal passage (birds) from where it is expelled. The salt secretory function of these glands is thought to be mediated by the specialized cells comprising the individual secretory tubules (reviewed by: Shuttleworth and Hildebrandt, 1999). Two cell types are typically found in the tubules: "principal" cells are often the most abundant as they populate the length of the secretory epithelium, while "peripheral" cells comprise the blind ends of the tubules. Principal cells have dense mitochondria and either deeply invaginated basal membranes (birds) or extensive lateral evaginations (elasmobranchs) which provide the surface area necessary to house the suite of membrane-bound, ion transport proteins that typify vertebrate secretory cells (Kirschner, 1980; Lowy et al., 1989; Ernst et al., 1994; Riordan et al., 1994). In contrast, the peripheral cells are non-secretory and have little, if any, specialization of the plasma membrane. Among birds, the peripheral cells are thought to be generative in nature and have therefore been implicated

* Corresponding author. Department of Biology, 220 Bartram Hall, Gainesville, FL 32611, USA. Tel.: +1 352 392 1107; fax: +1 352 392 3704.
E-mail address: babonis@ufl.edu (L.S. Babonis).

1095-6433/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2009.08.022

in the "adaptive differentiation" of salt glands undergoing salt stress (Ellis et al., 1963). Although adult salt gland morphology has also been shown to vary with salinity in some species of elasmobranchs (Oguri, 1964; Gerzeli et al., 1976), a role for the peripheral cells in regulating this process remains to be demonstrated. No such investigation of the function of the peripheral cells has been undertaken in any reptilian taxon.

Excretion of NaCl from the salt glands of elasmobranchs and birds, as well as the gills of marine teleosts, is effected primarily by three ion transport proteins: Na^+/K^+–ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter isoform 1 (NKCC1), and cystic fibrosis transmembrane conductance regulator (CFTR) (Evans, 2009). Upon phosphorylation, NKA asymmetrically exchanges 3 Na^+ ions for 2 K^+ ions resulting in the extracellular accumulation of Na^+ ions and a potential difference across the basolateral membrane. Together, these phenomena create an electrochemical gradient which drives the uptake of Na^+, K^+, and Cl^- from the extracellular fluid at the basolateral surface of the cell via NKCC1. Ultimately, this process potentiates the apical loss of Cl^- through CFTR and the paracellular secretion of Na^+ through the leaky tight junctions between epithelial cells. While this model of ion transport across secretory epithelia appears to be conserved across taxa, the localization of the above ion transport proteins has yet to be identified in the secretory epithelia of marine reptile salt glands.

The objective of this study was to examine the secretory epithelia of marine snake salt glands to determine if the localization of NKA and NKCC in a marine reptile is consistent with the localization of these proteins in the vertebrate secretory cell model described above. Among marine snakes, two separate salt-secreting cephalic glands have been described: the posterior sublingual gland in sea snakes (Hydrophiidae and Laticaudidae; Dunson et al., 1971) and file snakes (Acrochordidae; Dunson and Dunson, 1973) and the premaxillary gland in old world watersnakes (Colubridae: Homalopsinae; Dunson and Dunson, 1979). Both are compound tubular glands, like those of elasmobranchs and birds, and both are comprised almost entirely of principal cells exhibiting the lateral evaginations typical of elasmobranch principal cells (Dunson and Dunson, 1973; Dunson and Dunson, 1979). Preliminary studies by Dunson and Dunson (1974, 1975) suggested that NKA activity is high in the salt glands of several sea snake taxa, including one freshwater species, and remains high even as environmental salinity is decreased. Further studies of salt gland function in estuarine turtles utilized NKA- and NKCC-specific blocking agents to demonstrate the involvement of these two ion transporters in activating NaCl excretion (Shuttleworth and Thompson, 1987). While no further investigation into ion transport mechanisms has been conducted in marine reptiles, studies of salt glands from desert iguanas also demonstrate basolateral localizations of NKA and NKCC (Ellis and Goertemiller, 1974; Hazard, 1999), consistent with their role in activating ion secretion.

In this study we build on the work of our predecessors by immunolocalizing NKA and NKCC in the secretory epithelia of salt glands from three species of laticaudine sea snake: *Laticauda semifasciata, L. laticaudata,* and *L. colubrina*. These three species are of special interest because they are commonly found in coastal areas and frequently experience fluctuations in environmental salinity. Furthermore, observations of their daily activity patterns suggest slight differences in habitat use whereby *L. semifasciata* tends to be more aquatic than either *L. laticaudata* or *L. colubrina* (Lillywhite et al., 2008). Thus, in addition to examining the localization of NKA and NKCC in the secretory epithelia, we aimed to determine if the ecological differences among these species were reflected in the anatomy of their salt glands. We found that, as in other vertebrates, NKA and NKCC localize to the basolateral membranes of the principal cells of the secretory tubules in all three species. Neither the gross anatomy, nor the localization of the examined ion transporters were found to differ among species.

2. Materials and methods

2.1. Animal collection

In June of 2006, three species of laticaudine sea snake (*L. semifasciata, L. laticaudata,* and *L. colubrina*) were collected from the shallow coastal inlets around the perimeter of Orchid Island, Taiwan. Animals ($n = 6$ per species) were captured by hand and maintained in mesh bags during transportation to the laboratory at National Taiwan Normal University in Taipei. In the laboratory, animals remained in the mesh bags and were allowed to dehydrate in air for 14 days (for the experiment published in: Lillywhite et al., 2008). All animals were fasted through the entire dehydration period. Throughout the experimentation period, all animals were treated in accordance with the standard of ethics put forth by the University of Florida's Institutional Animal Care and Use Committee.

2.2. Tissue collection

Following the 14-day dehydration period, each animal was euthanized and the posterior sublingual salt gland was excised and cut in half lengthwise. Half of each gland was snap frozen in liquid nitrogen, transported back to the University of Florida, and stored at −80 °C for Western blot analysis. The other half of each gland was fixed in Bouin's solution (71% saturated picric acid, 24% formaldehyde (37%), 5% glacial acetic acid) for 24 h at room temperature (RT, 27 °C). Following fixation, tissues were washed in three rinses of 10 mM phosphate buffered saline (PBS) and stored in 75% ethanol for transport back to the University of Florida. In preparation for histology and immunohistochemistry, fixed salt glands were dehydrated through a series of ethanol washes of increasing concentration (75 to 100%). Following dehydration, tissues were cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA, USA), embedded in paraffin wax (Tissue Prep 2, Fisher Scientific), and sectioned at 7 µm perpendicular to the long axis of the gland. Sections were mounted on charged glass microscope slides (Superfrost Plus, Fisher Scientific) and dried for 24 h at 30 °C.

2.3. Histology

For analysis of salt gland tissue morphology, we used the Lillie (1940) modification of the Masson Trichrome technique (Humason, 1972). To further examine the secretory nature of the various cell types we used a modified Periodic Acid Schiff (PAS) technique (Humason, 1972). In brief, tissue sections were de-paraffinized in Citrisolv and rehydrated through a series of ethanol baths of decreasing concentration (100 to 35%). Rehydrated sections were then rinsed in 10 mM PBS for 5 min followed by a 1 min rinse in de-ionized (DI) water. Sections were then placed into 0.5% periodic acid (in DI water) for 5 min at RT, rinsed for 1 min in DI water, and placed into Schiff's reagent (Sigma Aldrich, St. Louis, MO, USA) for 1 min at RT. Hematoxylin was used to counter-stain before sections were dehydrated through a series of ethanol baths of increasing concentration, cleared with Citrisolv, and mounted with coverslips using Permount (Fisher Scientific). Alcian blue was used to detect acidic mucopolysaccharides following a modification of the protocol outlined in Humason (1972). Briefly, rehydrated sections were incubated in 3% acetic acid (in DI water) for 3 min at RT and then placed directly into 1% Alcian blue 8GX (in 3% acetic acid, pH 2.5) for an additional 30 min at RT. Sections were then rinsed in running tap water for 5 min, rinsed in DI water for 1 min, dehydrated, cleared, and mounted.

2.4. Immunohistochemistry

To localize specific ion transporters in tubular epithelia, we followed the immunohistochemical techniques of Piermarini et al.

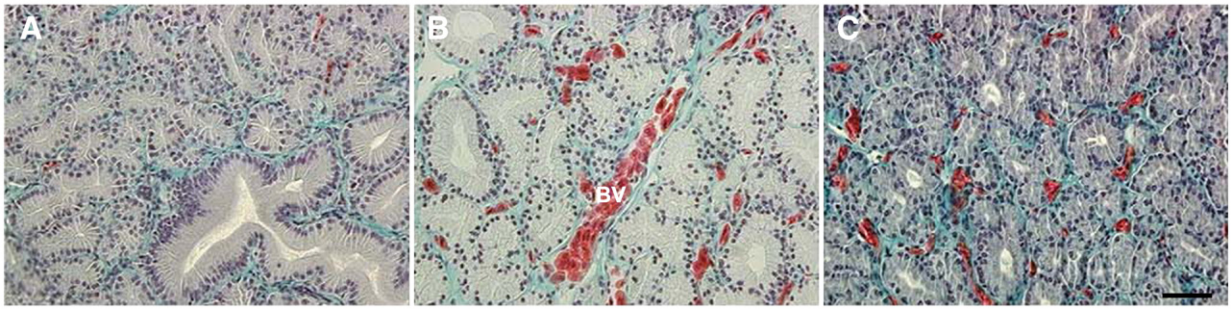

Fig. 1. Cross sections of sublingual salt glands from (A) *L. semifasciata*, (B) *L. laticaudata*, and (C) *L. colubrina* (Masson Trichrome). Blood vessels (BV) are easily distinguishable by the presence of red blood cells. Collagen fibers (green) surround each secretory tubule. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(2002). Briefly, rehydrated tissue sections were washed in 10 mM PBS, encircled with a hydrophobic barrier using a PAP pen (Electron Microscopy Sciences, Hatfield, PA, USA), and incubated in 3% H_2O_2 (in DI water) for 30 min at RT. Tissues were again washed in 10 mM PBS and incubated in a Biogenex Protein Block (BPB; normal goat serum with 1% bovine serum albumin, 0.09% NaN_3, and 0.1% Tween 20; San Ramon, CA, USA) for an additional 20 min at RT. Tissues were again rinsed in 10 mM PBS followed by incubation in anti-NKA (1/100; diluted in BPB) or anti-NKCC (1/2000) overnight at 4°C. The primary antibody was then removed with 10 mM PBS and tissues were prepared for visualization using the horseradish peroxidase Super Sensitive™ Link-Label IHC Detection System (Biogenex). To begin, tissues were incubated in "Link" (peroxidase-conjugated streptavidin) for 20 min in a humidified chamber at RT. Following a 10 mM PBS wash, tissues were incubated in "Label" (biotinylated anti-immunoglobulins) for an additional 20 min. Visualization was achieved through a final 5-min incubation in 3, 3′-diaminobenzidine tetrahydrochloride (DAB; Biogenex) at RT. Following visualization with DAB, tissue sections were dehydrated and mounted. Negative controls were produced following a modification of the aforementioned procedures whereby tissues were incubated in BPB rather than primary antibody.

2.5. Primary antibodies

Monoclonal anti-NKA (α5) developed by Dr. Douglas Fambrough and monoclonal anti-NKCC (T4) developed by Drs. Christian Lytle and Bliss Forbush III were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. While α5 recognizes an epitope specific to the α_1 subunit of NKA (Takeyasu et al., 1988), T4 was made against a conserved epitope in the carboxyl tail of NKCC (Lytle et al., 1995) and is therefore unable to distinguish between NKCC isoforms 1 (NKCC1) and 2 (NKCC2).

2.6. Western blot analysis

Frozen salt glands were homogenized in ice-cold lysis buffer (10 mL of buffer per 1 g of tissue; Cell Signaling Technology, Danvers, MA, USA) and centrifuged at 16,000 × *g* for 10 min at 4 °C. The supernatant was then removed and stored on ice. To quantify protein in each sample, we used the BCA detergent compatible protein assay kit (Pierce, Rockford, IL,USA). Following addition of 2% β-mercaptoethanol and 0.01% bromophenol blue, each sample was heated at 65 °C for 10 min. We then loaded 15 μg of total protein from each sample into a 7.5% Tris–HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and electrophoresed each gel for 2 h at 100 V. Separated proteins were then transferred to an Immuno-blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) following the manufacturer's protocol. Following transfer, membranes were rehydrated in 100% methanol for 5 min and rinsed twice in deionized water. To block non-specific proteins, membranes were incubated, while shaking, in Blotto: 5% non-fat dry milk in Tris-buffered saline (TBS 25 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl; pH 7.4), for 1 h at RT followed by an incubation with anti-NKA (α5, 1/100) or anti-NKCC (T4, 1/4000) overnight at RT. Membranes were then washed three times (15 min each) with TTBS (0.1% Tween-20 in TBS; pH 7.4) and incubated in alkaline-phosphatase-conjugated goat anti-mouse IgG (1/3000 diluted in Blotto) at RT for 1 h and washed in TTBS. To visualize antibody binding, membranes were incubated in a chemiluminescent signal (Immun-star chemiluminescent kit, Bio-Rad) following the manufacturer's protocol. Total protein present on the membranes was visualized by incubating membranes in 0.02% Coomassie blue stain (diluted in 50%

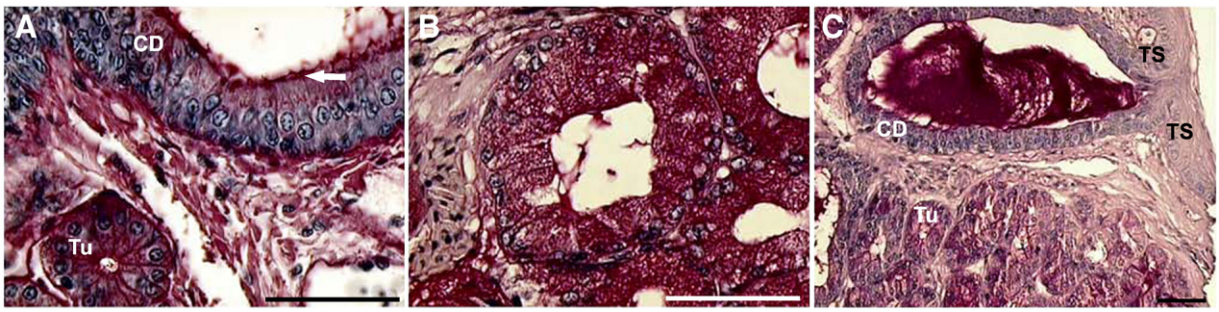

Fig. 2. Periodic Acid Schiff (PAS) reaction reveals the presence of polysaccharide (magenta color) in the tubules of all three species. (A) Secretory tubules (Tu) can be distinguished from central ducts (CD) by the size of the lumena and the morphology of the epithelium. Tubules have relatively small lumena and simple cuboidal to columnar epithelia; ducts have large lumena and pseudostratified columnar epithelia. Additionally, while central duct epithelium is PAS-positive apically (arrow), (B) the PAS-positive material appears to be more evenly distributed through the cytoplasm of the secretory tubules. (C) Ultimately, central ducts join distally with the tongue sheath epithelium (TS) to facilitate passage of secreted products (magenta mass) into the tongue sheath. (A *L. semifasciata*, B *L. colubrina*, and C *L. laticaudata*) Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Cross sections of salt glands from all three species (A *L. semifasciata,* B *L. laticaudata,* and C *L. colubrina*) Both tubules and ducts are negative for Alcian blue stain at pH 2.5, which indicates that these cells are not secreting acidic mucopolysaccharides. Scale bar = 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

methanol, 10% acetic acid, and 40% water) for 1 min. Exposed films and Coomassie blue stained membranes were digitally imaged using a flatbed scanner and brightened using Adobe Photoshop CS3 (San Jose, CA USA).

3. Results

3.1. Anatomical description of salt glands

The sublingual salt gland in each species is typified by branched secretory tubules encased in a matrix of collagen fibers (Fig. 1A–C). Abundant blood vessels populate the connective tissue surrounding each tubule. The central ducts can be distinguished from the secretory tubules by their pseudostratified columnar epithelia and large lumena (Fig. 2A–C). In contrast, secretory tubules are simple and cuboidal to columnar, have relatively smaller lumena and thereby smaller apical than basal surfaces. Distally, ducts join and become continuous with the stratified squamous epithelium of the tongue sheath (Fig. 2B); this provides the opportunity for the secreted salt solution to be expelled by tongue-flicking (Dunson and Taub, 1967; Dunson and Dunson, 1979). Both central ducts and secretory tubules are PAS-positive apically, suggesting the presence of polysaccharides (magenta coloration, Fig. 2A–C). However, neither ducts nor tubules stained positively for mucopolysaccharides (all were Alcian blue-negative; Fig. 3A–C).

3.2. Immunolocalization of ion transport proteins in salt gland epithelia

Immunolocalization of the α-subunit of NKA was detected in the basolateral membrane of the cuboidal cells of the secretory epithelia in all three species (brown coloration, Fig. 4A–C). A similar basolateral

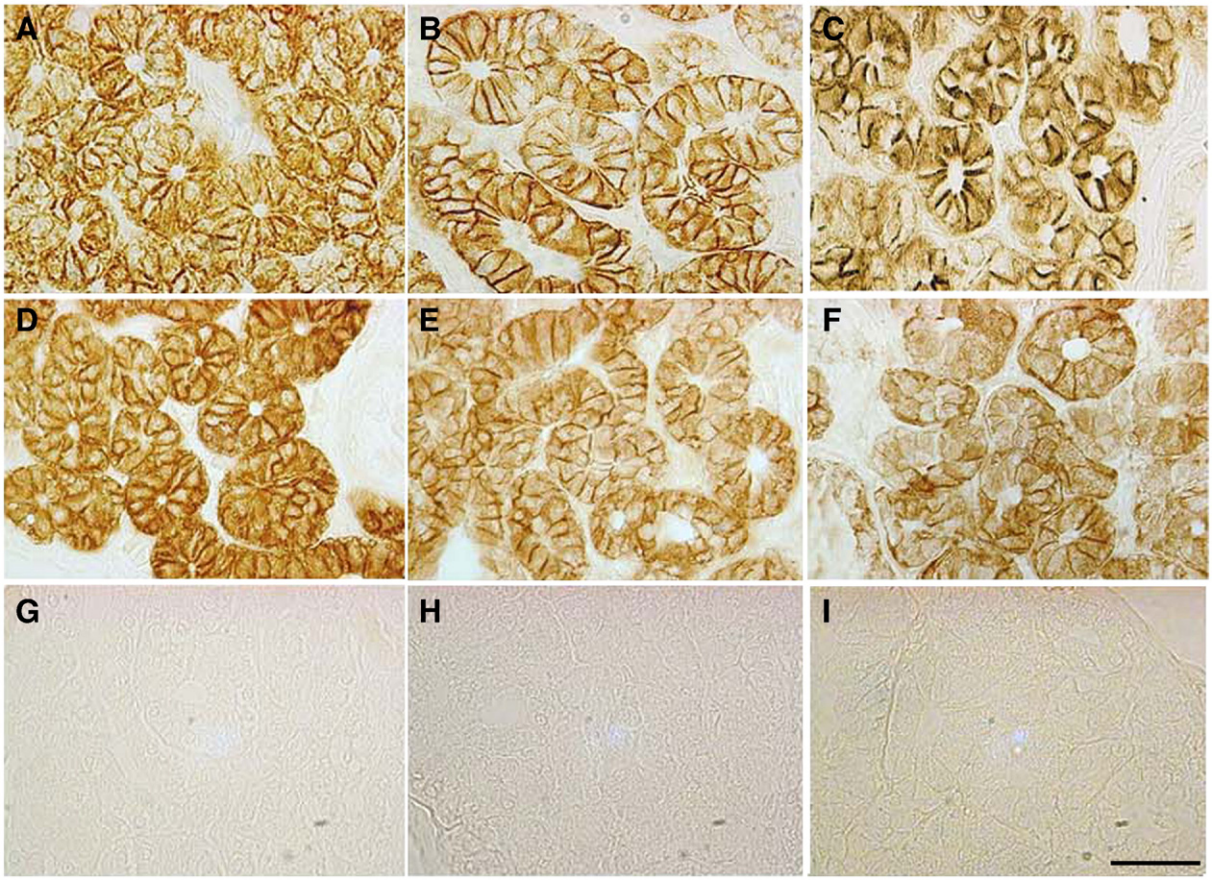

Fig. 4. IHC showing the localization of (A–C) NKA and (D–F) NKCC to the basolateral membranes of the cells comprising the tubular epithelia in all three species (A/D/G *L. semifasciata*; B/E/H *L. laticaudata*; and C/F/I *L. colubrina*). Negative controls for NKA and NKCC (D–F) show no staining. Scale bar = 50 µm.

localization was detected for NKCC (Fig. 4D–F). While there appears to be weak cytoplasmic staining in NKA- and NKCC-positive cells, this likely reflects the localization of NKA and NKCC to the interdigitating lateral membranes of the secretory cells. The localization of these two proteins does not appear to differ among the species examined. Control sections lack staining for either NKA or NKCC (Fig. 4G–I).

3.3. Primary antibody specificity

The anti-NKA antibody (α5) detected a protein with a molecular weight of approximately 110 kDa in each species (Fig. 5A), which is consistent with the molecular weight of NKA in other vertebrates (Blanco and Mercer, 1998). Additionally, anti-NKCC (antibody T4) detected a single band of approximately 195 kDa (Fig. 5B), also within the range published for the molecular weight of this protein in other vertebrates (Lytle et al., 1995). Coomassie blue stains total protein (Fig. 5C) in the same lanes shown for primary antibody (Fig. 5A–B). Detection of only a single product of the appropriate size (110 kDa and 195 kDa for α5 and T4, respectively) in the presence of the full complement of proteins extracted from the salt glands supports specificity of these antibodies for their target proteins.

4. Discussion

Our results confirm that the morphology of salt glands from three species of laticaudine sea snake is similar to that of all other vertebrate salt glands studied to date (Hildebrandt, 2001; Evans et al., 2004; Dantzler and Bradshaw, 2009). While no peripheral cells were identified in this study, the principal cells, which comprise the tubular epithelium of the salt gland from all three species, were found to be predominantly serous in nature. In all three species the nuclei from the principal cells are round and positioned in the basal portion of the cell. The presence of PAS-positive polysaccharide granules throughout the cytoplasm of the secretory cells (Fig. 2C) and the absence of mucopolysaccharides (Fig. 3A–C) further confirms the serous nature of this gland. As most cephalic secretory glands (primarily salivary glands) are typified by more equivalent proportions of serous and mucous cells (Burns and Pickwell, 1972; Baccari et al., 2002), the primarily serous nature of vertebrate salt glands might, in fact, reflect a developmental pathway leading to the evolution of this gland type from an unspecialized seromucous precursor (Dunson, 1969; Peaker and Linzell, 1975; Barnitt and Goertemiller, 1985).

Additionally, we demonstrate that the localization of NKA and NKCC in marine snake salt glands is consistent with the localization of these proteins in all other vertebrate salt secreting tissues studied to date (Evans, 2009). In all three species of sea snake NKA and NKCC were localized to the basolateral membranes of the cuboidal/columnar cells comprising the epithelia of the salt gland tubules (Fig. 4A–F). The localization of these two proteins is consistent with their roles in the active uptake of $Na^+/K^+/Cl^-$ across the basolateral membrane of the secretory cells (Shuttleworth and Thompson, 1987) and suggests conservation of a similar mechanism for ion transport at the level of the vertebrate secretory cell. While the identity of the putative apical Cl^- transporter remains to be determined, the presence of CFTR in taxa ranging from elasmobranchs to birds suggests that this ion transporter is also likely conserved among the reptiles. In fact, we have cloned a partial sequence for CFTR from the salt gland of *L. semifasciata* (unpublished); however, our attempts to localize CFTR protein in the tubular epithelium have failed (despite our use of several antibodies both commercially available and donated from other laboratories). Thus, further studies of the role of CFTR and other potential apical chloride channels are necessary before hypotheses about conservation of the full ion secretory mechanism can be evaluated.

Finally, despite apparent differences in both habitat use and dehydration rate (Lillywhite et al., 2008), no differences in either gross morphology or the localization of either NKA or NKCC were seen among the species examined. While our Western blots suggest that variation may exist in the abundance of both NKA and NKCC among species, further investigations aimed at quantifying this pattern are necessary before conclusions about the relationship between habitat use or dehydration rate and ion transporter abundance can be made.

The consistency with which salt gland form and function have been conserved throughout the evolution of marine vertebrates suggests that the genetic mechanism leading to the development of this tissue type may also be conserved. Indeed studies of the regulation of salt gland development may reveal a mechanism by which these glands have been co-opted from unspecialized gland precursors (Dunson, 1969; Peaker and Linzell, 1975; Barnitt and Goertemiller, 1985). In this context, studies aimed at understanding the development and distribution of principal cells in the secretory epithelia as well as the development of the compound tubular structure of the gland would be of special interest.

Acknowledgements

We thank Dr. Ming-Chung Tu and his students for the use of their laboratory at National Taiwan Normal University and their assistance with animal collections. We also thank Coleman M. Sheehy III for his assistance with animal and tissue collections, the administrative officials at Orchid Island for permission to remove animals for this work, and Michael W. McCoy for his assistance with manuscript edits. This work was supported by National Geographic funding to HBL (8058-06), NSF funding to DHE (IOB-056273), and Sigma Xi funding to LSB.

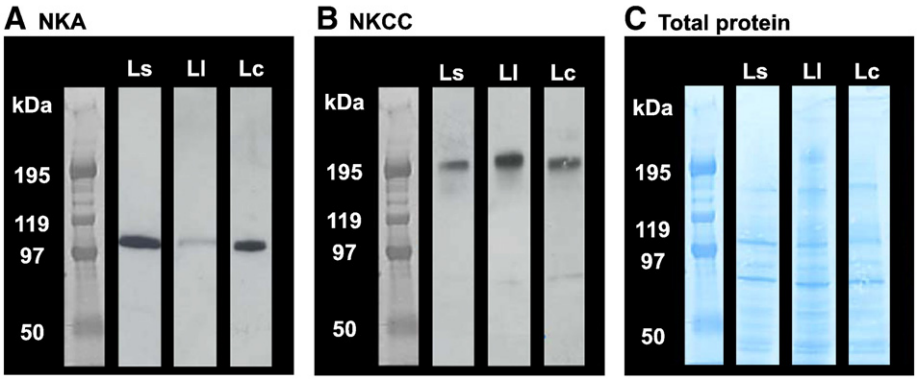

Fig. 5. Western blots support the specificity of the antibodies used in IHC for all three species (*Ls* = *L. semifasciata*, *Ll* = *L. laticaudata*, *Lc* = *L. colubrina*.) (A) Antibody α5 (NKA) detects a protein of approximately 110 kDa in all three species of sea snake and (B) antibody T4 (NKCC) detects a protein of approximately 195 kDa. (C) Total protein is visualized on the same blot using Coomassie blue stain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

References

Baccari, G.C., Ferrara, D., Di Matteo, L., Minucci, S., 2002. Morphology of the salivary glands of three *Squamata* species: *Podarcis sicula sicula*, *Tarentola mauritanica*, and *Coluber viridiflavus*. Acta Zool. (Stockh.) 83, 117–124.

Barnitt, A.E., Goertemiller, C.C., 1985. Nasal salt-secreting glands of normal and hyperkalemically stressed *Sauromalus obesus* — histology and cytology. Copeia 403–409.

Blanco, G., Mercer, R.W., 1998. Isozymes of the Na–K–ATPase: heterogeneity in structure, diversity in function. Am. J. Physiol. 275, F633–F650.

Burns, B., Pickwell, G.V., 1972. Cephalic glands in sea snakes (*Pelamis*, *Hydrophis* and *Laticauda*). Copeia 547–559.

Dantzler, W.H., Bradshaw, S.D., 2009. Osmotic and ionic regulation in reptiles. In: Evans, D.H. (Ed.), Osmotic and ionic regulation: Cells and animals. CRC Press, Boca Raton, FL, pp. 443–503.

Dunson, W.A., 1969. Reptilian salt glands. In: Botelho, S.Y., Brooks, F.P., Shelley, W. B. (Eds.), The exocrine glands. University of Pennsylvania Press, Philadelphia, pp. 83–103.

Dunson, W.A., Dunson, M.K., 1973. Convergent evolution of sublingual salt glands in the marine file snake and true sea snakes. J. Comp. Physiol. 86, 193–208.

Dunson, W.A., Dunson, M.K., 1974. Interspecific differences in fluid concentration and secretion rate of sea snake salt glands. Am. J. Physiol. 227, 430–438.

Dunson, M.K., Dunson, W.A., 1975. Relation between plasma Na concentration and salt gland Na–K ATPase content in the diamondback terrapin and the yellow-bellied sea snake. J. Comp. Physiol. 101, 89–97.

Dunson, W.A., Dunson, M.K., 1979. Possible new salt gland in a marine Homalopsid snake (*Cerberus rhynchops*). Copeia 661–673.

Dunson, W.A., Taub, A.M., 1967. Extrarenal salt excretion in sea snakes (*Laticauda*). Am. J. Physiol. 213, 975–982.

Dunson, W.A., Packer, R.K., Dunson, M.K., 1971. Sea snakes: an unusual salt gland under the tongue. Science 173, 437–441.

Ellis, R.A., Goertemiller Jr., C.C., 1974. Cytological effects of salt-stress and localization of transport adenosine triphosphatase in the lateral nasal glands of the desert iguana, *Dipsosaurus dorsalis*. Anat. Rec. 180, 285–297.

Ellis, R.A., Delellis, R.A., Goertemiller, C.C., Kablotsky, Y.H., 1963. Effect of a salt water regimen on development of salt glands of domestic ducklings. Dev. Biol. 8, 286–308.

Ernst, S.A., Crawford, K.M., Post, M.A., Cohn, J.A., 1994. Salt stress increases abundance and glycosylation of CFTR localized at apical surfaces of salt gland secretory cells. Am. J. Physiol. 267, C990–C1001.

Evans, D.H., 2009. Osmotic and Ionic Regulation: Cells and Animals. CRC Press, Boca Raton, FL, p. 590.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2004. Homeostasis: osmoregulation, pH regulation, and nitrogen excretion. In: Carrier, J.C., Musick, J.A., Heithaus, M.R. (Eds.), Biology of Sharks and Their Relatives. CRC Press, Boca Raton, FL, pp. 247–268.

Gerzeli, G., De Stefano, G.F., Bolognani, L., Koenig, K.W., Gervaso, M.V., Omodeo-Sale, M.F., 1976. The rectal gland in relation to the osmoregulatory mechanisms of marine and freshwater elasmobranchs. In: Thorson, T.B. (Ed.), Investigations of the Ichthyofauna of Nicaraguan Lakes. School of Life Sciences, University of Nebraska, Lincoln, Nebraska, pp. 619–627.

Hazard, L.C., 1999. Ion secretion by the nasal salt gland of an herbivorous desert lizard, *Dipsosaurus dorsalis*. Ph.D. Dissertation. University of California, Riverside, CA, p. 148.

Hildebrandt, J.P., 2001. Coping with excess salt: adaptive functions of extrarenal osmoregulatory organs in vertebrates. Zoology 104, 209–220.

Humason, G.L., 1972. Animal Tissue Techniques. W.H. Freeman and Company, San Francisco.

Kirschner, L.B., 1980. Comparison of vertebrate salt-excreting organs. Am. J. Physiol. 238, R219–R223.

Lillie, R.D., 1940. Further experiments with the Masson trichrome modification of the Mallory connective tissue stain. Stain Technol. 15, 17–22.

Lillywhite, H.B., Babonis, L.S., Sheehy, C.M., Tu, M.C., 2008. Sea snakes (*Laticauda* spp.) require fresh drinking water: Implication for the distribution and persistence of populations. Physiol. Biochem. Zool. 81, 785–796.

Lowy, R.J., Dawson, D.C., Ernst, S.A., 1989. Mechanism of ion transport by avian salt gland primary cell cultures. Am. J. Physiol. 256, R1184–R1191.

Lytle, C., Xu, J.C., Biemesderfer, D., Forbush III, B., 1995. Distribution and diversity of Na–K–Cl cotransport proteins: a study with monoclonal antibodies. Am. J. Physiol. 269, C1496–C1505.

Marples, B.J., 1932. The structure and development of the nasal glands of birds. Proc. Zool. Soc. Lond. Part 4, 829–844.

Oguri, M., 1964. Rectal glands of marine and fresh-water sharks — comparative histology. Science 144, 1151–1152.

Peaker, M., Linzell, J.L., 1975. Salt Glands in Birds and Reptiles. Cambridge University Press, London, New York. p. 307.

Piermarini, P.M., Verlander, J.W., Royaux, I.E., Evans, D.H., 2002. Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R983–992.

Riordan, J.R., Forbush III, B., Hanrahan, J.W., 1994. The molecular basis of chloride transport in shark rectal gland. J. Exp. Biol. 196, 405–418.

Shuttleworth, T.J., Hildebrandt, J.P., 1999. Vertebrate salt glands: short- and long-term regulation of function. J. Exp. Zool. 283, 689–701.

Shuttleworth, T.J., Thompson, J.L., 1987. Secretory activity in salt glands of birds and turtles: stimulation via cyclic AMP. Am. J. Physiol. 252, R428–R432.

Sullivan, M.X., 1907. The physiology of the digestive tract of elasmobranchs. Contributions from the biological laboratory of the Bureau of Fisheries at Woods Hole, MA, vol. 27, pp. 3–27.

Takeyasu, K., Tamkun, M.M., Renaud, K.J., Fambrough, D.M., 1988. Ouabain-sensitive Na–K–ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. J. Biol. Chem. 263, 4347–4354.