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Characterization of aquaporin 4 protein expression and localization in tissues of the dogfish (Squalus acanthias)

Christopher P. Cutler1,2 *, Sheena Harmon1,2, Jonathon Walsh1,2 and Kia Burch1,2

INTRODUCTION
Aquaporin 4 (AQP4) is a member of the water-selective sub-group of aquaporin water channel cell-membrane proteins found in all organisms so far investigated. This sub-group in mammals also includes other aquaporins such as AQP0, AQP1, AQP2, AQP5, and AQP6 (Ishibashi et al., 2009; Zelania, 2010). Other subgroups such as aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) have additional transport properties (e.g., transport of urea and glycerol, etc.). In mammals, AQP4 is widely expressed in a variety of tissues including the brain (Zelania, 2010), retina (Hirrlinger et al., 2011), salivary gland (Delporte and Steinfield, 2006), trachea (Borok and Verkman, 2002), heart and muscle (Butler et al., 2006; Wakayama, 2010), gastrointestinal tract (Ma and Verkman, 1999; Xu et al., 2009), and kidney (Nejsum, 2005), but is not expressed in the lung itself or liver tissues (Ishibashi et al., 2009). While aquaporins such as AQP4 have been studied in a wide variety of (mostly) higher vertebrates, no complete studies have yet been published on the role of aquaporins in elasmobranch fish such as the dogfish shark (Cutler et al., 2005; Cutler, 2006, 2007). A companion paper (Cutler et al., 2012) has characterized aqp4 mRNA expression in dogfish tissues, this article gives the first information on the Aqp4 protein and its localization in tissues. In mammals such as the rat, AQP4 appears as two bands on Western blots, a non-glycosylated form of 30–32 kDa (sometimes spliceforms with different N-terminal ends exist) and a putative 50 kDa glycosylated form (Terris et al., 1995; Nicchia et al., 2008). Immunohistochemical staining of mammalian epithelial cells generally yields a basolateral localization for AQP4 proteins, however, AQP4 staining both within the cell and in the apical membrane is thought to occur in some cells (Terris et al., 1995; Nejsum, 2005; Mobasheri et al., 2011). As indicated in the companion paper (Cutler et al., 2012), dogfish aqp4 mRNA expression in tissues is largely ubiquitous. The questions this article sets out to address are, whether Aqp4 protein expression is similarly ubiquitous and if so, where
within the tissues that are important for the control of water balance in sharks (gills, kidney, rectal gland, etc.), is the Aqp4 protein located?

**MATERIALS AND METHODS**

**FISH**

All animal experiments were performed in accordance with IACUC regulations and had IACUC approval from both MDIBL and Georgia Southern University. Animals for experiments were housed in a stock tank with running seawater at ambient temperature and were sacrificed by decapitation followed by immediate pithing of the spinal cord. Various tissues were then removed from the animal by dissection for further processing in Western blotting and immunohistochemistry experiments.

**POLYCLONAL ANTIBODY PRODUCTION**

Custom-made polyclonal antibodies were produced commercially against peptides whose amino acid sequence was derived from the dogfish aqp4 nucleotide sequence. The first of these AQP4/1 (produced by ProSci, San Diego, CA, USA) was located at the C-terminal end of the protein (at positions 329–346 of the amino acid sequence) and had the sequence NH2–CGGNEEKKDATKELSSV–COOH. As part of that sequence, the two glycine amino acids were added as spacers at the N-terminal end. The second antibody AQP4/2 (produced by GenScript, Piscataway, NJ, USA), was produced much more recently and was located a little further in from the C-terminal end than the first peptide (at positions 290–308 of the amino acid sequence), and had the sequence NH2–CKSTQPSGDKYAEGEDNRSQ–COOH. Peptides for both antibodies had an N-terminal cysteine amino acid added for coupling to the protein carrier. The Aqp4 peptides were coupled to keyhole limpet hemocyanin (KLH) prior to injections of the antigens into different pairs of rabbits. The resulting anti-sera were affinity purified using the same peptide (that was used for immunization), attached to a purification column.

**TISSUE CELL-MEMBRANE PREPARATION**

Dogfish tissues for Western blotting experiments were kept briefly on ice and then homogenized in Tris (25 mM), sucrose (0.25 M) buffer, also containing 78 mg/ml diithiothreitol (DTT), and either protease inhibitor cocktail I (Research Product International, Mount Prospect, IL, USA) or Halt protease inhibitor cocktail (Pierce), was used according to manufacturers instructions. Hard tissues such as muscle, kidney, liver, and rectal gland, etc., were homogenized using a polytron homogenizer (Kinematica, Luzern Switzerland). Soft tissues such as brain or scraped epithelia were homogenized using a syringe and 16 gage needle. Epithelia were scraped from gill arches using a single sided razor blade and from intestine and esophagus/cardiac stomach using a glass microscope slide.

Homogenized samples were then sieved through several layers of cotton gauze. The filtrate was then centrifuged in a SS-34 (Sorvall, Asheville, NC, USA) rotor at approximately 50,000 g max for 1 h at 4°C. The resulting crude membrane pellet was then resuspended in the same buffer as previously used and measured for protein content using a Bradford’s protein assay (Boston Bioproducts, Ashland, MA, USA). Crude membrane homogenates were stored frozen at −20 or −80°C prior to use in Western blotting experiments.

**WESTERN BLOTTING**

Crude protein homogenates (300 µg protein/lane) were separated based on their size, on 10% Laemmli SDS-polyacrylamide gels (Laemmli, 1970) using a Protein II gel apparatus (Biorad). The gel was then transferred to a methanol-activated high protein capacity sequi-blot PVDF filter (Biorad) using a trans-blot cell electroblotter (Biorad, Taunton, MA, USA), at 30 V overnight. The resulting filters were then cut into strips for each experiment. Filter strips were incubated in TNT buffer [10 mM tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20], containing 5% Blotto (fat-free dry milk powder) for 30 min room temperature. They were then washed four times in TNT buffer and primary antibody added (in TNT buffer) at 1 in 400 dilution (or 1 in 4000 dilution for peptide-blocking experiments) used because there can be a problem blocking high antibody concentrations sometimes due to the limits of peptide antigen solubility. Lower antibody concentrations allows the same result to be obtained with less peptide for 1 h at room temperature. The filters were then washed four times in TNT buffer and incubated in 1 in 4000 dilution of alkaline phosphatase enzyme cross linked – highly cross-absorbed – donkey anti-rabbit IgG secondary antibody for 1 h at room temperature. Filters were washed again twice in TNT buffer and twice in 10 mM tris (pH 8.0), 150 mM NaCl, and finally incubated in NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3’-indoly phosphate p-toluidine salt) alkaline phosphatase enzyme substrate containing 1 mM levamisole endogenous alkaline phosphatase inhibitor. The presence of the bound secondary antibody/alkaline phosphatase enzyme yielding a purple/blue colored product.

**IMMUNOHISTOCHEMISTRY**

Dissected tissues were fixed in filtered standard phosphate buffered saline (PBS; Oxoid, Lenexa, KS, USA) containing 4% paraformaldehyde, for 1 h at room temperature. Tissues were then cut into segments to fit in standard histological cassettes. The cassettes were rinsed twice in PBS and then dehydrated through a series of alcohols (50, 70, 85, 95, and 100% ethanol), 1 h in each. Subsequently, cassettes were placed twice in histochrome clearing medium (Amresco, Solon, OH, USA) and then into molten paraffin wax three times (Paraplast) held between 56 and 58°C in an oven, 1 h each wax solution. The tissue pieces were then placed in stainless steel molds, which were filled with molten wax and were finally mounted with the back of the cassette placed on top. Once the wax had cooled and set, the molds were removed, revealing the wax-embedded tissue blocks for section cutting. Five micron thick sections were cut using a microtome (Leica, Buffalo Grove, IL, USA), these were placed on the surface of a warm waterbath (37°C) and floated onto glass microscope slides (Superfrost plus). The slides were heated at 37°C for 1 h to adhere tissue sections to the positively charged surface of the slide. Slides for experiments were then taken back through two incubations (5 min each) in histo-choice clearing agent to remove the wax and through a descending series of alcohol concentrations (5 min each; 100, 95, 85, 70, 50 ethanol) to re-hydrate the tissue. Finally slides were incubated in PBS.
Slides were then placed horizontally in a slide box that had moist tissue in the bottom for humidification. The tissue on the slides was rinsed using a hydrophobic barrier pen (to retain subsequent solutions on the tissue) and a solution of PBS with sodium chloride (17.5 g/l) and 0.02% Tween 20 detergent was added for 10 min to permeabilize the tissue. The slides were washed twice with PBS and then incubated for 5 min in PBS containing (2.68 g/l) ammonium chloride, to block any free aldehyde groups of the fixative. The slides were washed again twice in PBS and then incubated in Image-iT FX blocking solution (Invitrogen, Grand Island, NE, USA) for 30 min. The slides were washed again twice in PBS and then incubated in a second blocking solution of PBS containing (10 g/l) bovine serum albumin (BSA; Promega, Madison, WI, USA) and (1 g/l) gelatin (Boston Bioproducts) for 10 min. The slides were washed again twice in PBS and then incubated in 1 in 100 dilution of primary antibody in PBS for 1 h, room temperature. The slides were then washed four times in PBS and then incubated in 1 in 1000 dilution of secondary antibody (Alexa 488-, Dylight 549-, or Alexa 555-labeled, highly cross-absorbed-anti-rabbit) in PBS for 1 h, room temperature. From this point onward slide boxes were kept closed in a draw between manipulations, to reduce light exposure. The slides were then washed four times in PBS and then were mounted in Prolong Gold mounting medium containing the nuclear counterstain, DAPI (Invitrogen). The slides were then covered with a coverslip ready for microscope viewing.

Four-color immunohistochemical co-localization studies, were carried out as above with the following modifications. A rabbit anti-sculpin V-type ATPase antibody (a gift from Dr. J. B. Claiborne) was used initially and detected using a highly cross-absorbed Alexa 488 (green) fluorescently labeled anti-rabbit secondary antibody. The secondary antibody was then blocked using normal rabbit serum (sections incubated for 1 h at room temperature). The rabbit anti-dogfish AQP4/1 antibody was directly labeled with a (red) Dylight 633 fluorescent dye (using a Pierce microscale antibody labeling kit) and then used on sections. Subsequently, a mouse anti-Na, K-ATPase α5 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used and detected using a highly cross-absorbed (orange) Alexa 555 anti-mouse secondary antibody. A DAPI nuclear counterstain (blue) was also utilized as before.

RESULTS

Western blotting of crude membrane protein extracts from a variety of different dogfish tissues and using the AQP4/1 antibody showed that Aqp4 is expressed ubiquitously in all the tissues studied (Figure 1A). In most tissues, there were two protein bands on the blot with estimated molecular weights of 35.5 and 49.5 kDa respectively, and this tissue distribution was similar to that of aqp4 mRNA (see companion paper; Cutler et al. (2012)). Additionally visible in the sample from rectal gland, there were two other faint bands of 37.5 and 38.5 kDa (see also Figure 3). Also in liver there was another strongly staining band at 57 kDa. The 35.5 kDa band common to most tissues is similar in size to the estimated molecular weight of the protein (37.2 kDa) based on the amino acid sequence derived from the gene sequence. The larger 49.5 kDa band common to most tissues is similar in size to the estimated molecular weight of the protein (37.2 kDa) based on the amino acid sequence derived from the gene sequence. The larger 49.5 kDa band was considerably more abundant in the rectal gland and appeared to be absent in the brain. The second AQP4/2 antibody was also used in Western blotting of tissue crude membrane extracts (Figure 1B). This blot showed a lot more bands than when using AQP4/1 antibody, although the 49.5 kDa was present.
in each tissue similar to the AQP4/1 antibody blot, except that the
49.5 kDa protein band was also present in the brain. There was
also some indication of the 35.5 kDa band on the AQP4/2 blot,
but the intensity of this was variable between tissues. There were
additionally some higher molecular weight bands of 99 kDa and
around 140 kDa suggesting the presence of dimers or Aqp4 aggre-
gates. There was no indication of 37.5 and 38.5 kDa bands in the
rectal gland sample as seen with the AQP4/1 antibody.

To test whether the antibody staining was specific, various con-
trol blots were performed (Figure 2). While the staining appeared
as in the tissue blot (Figure 1) when using the AQP4/1 or AQP4/2
antibody alone, when the antibody was pre-blocked using its pep-
tide antigen, staining was almost entirely abolished in the case
of either antibody. Additionally, there was no similar staining
when using either serum taken from the rabbit prior to immu-
низацию с the Aqp4 antigens (pre-immune serum) or from the
secondary antibody used on its own (no primary Aqp4 antibody).

As the exact nature of the 49.5 and 57 kDa bands on the
AQP4/1 Western blot was worth further investigation, it was
decided that, heat denaturation of the samples might cause some
kind of aggregation (of the 35.5 kDa protein) to occur. Rect-
al gland crude membrane homogenate protein samples were
therefore produced and blotted, that were either heated (as normal)
and not heated (ambient temperature incubation, Figure 3). The
lack of heat denaturation in comparison to normal had no
effect on the 35.5 or 49.5 kDa bands, but the minor 37.5 and
38.5 kDa bands were absent in the un-heated protein sample lane
and another diffuse band of around 32 kDa appeared instead.

To further test whether any of the protein bands identified with
the AQP4/1 antibody were glycosylated forms, crude membrane
protein homogenates were treated with the enzyme PNGase F
(New England Biolabs), which removes core N-glycosylated moi-
eties from glycoproteins reducing their apparent molecular weight
(Figure 4). However in either rectal gland or liver samples, PNGase
F had no effect on the mobility of proteins identified by the
AQP4/1 antibody, in comparison to similarly incubated control
samples (no enzyme). This suggests that none of the protein bands
represent glycosylated forms of Aqp4.

When the AQP4/1 and AQP4/2 antibodies were used for
immunohistochemical staining of tissues, somewhat similar
images were obtained with both antibodies but there were some
differences, with the AQP4/2 antibody appearing to be more spe-
cific. In kidney (Figure 5), both antibodies labeled a sub-set of
renal tubules both in the packed areas of the lateral bundle zone
(Figures 5A–C) and in the sinus zone (Figure 5D). However, the
AQP4/1 antibody (as is typical for other tissues also) stained the
whole of the cell cytoplasm including in the region of the DAPI
stained cell nucleus. Staining in renal tubule cells also was some-
what more intense toward the apical pole of the cell but was
otherwise uniform throughout the cell. With the AQP4/2 anti-
body, fluorescence localized to the cytoplasm excluding the nuclear
area stained by DAPI. Also there appeared to be no staining to the
apical side of the nucleus in many cells. Serial sections stained
with either AQP4/1 (Figure 5E) or AQP4/2 (Figure 5F) showed
that the two antibodies stain the same segments of the similar
renal tubule, although the type and intensity of staining was some-
times different between the two antibodies. With either antibody
there appeared to be a sub-set of renal tubules (approximately
1–10% of the total stained) that showed basolateral membrane
staining but with little or no cytoplasmic staining (Figure 6). In
Figure 6B, a tubule has been cut through in longitudinal section
but due to the depth of the section (5 μm), the bottom of the
tubule is also visible. Interestingly, the membranes of an apparent
stellate-shaped tubule cell have also been stained by the AQP4/1
antibody.

Similar to the situation in renal tubule cells, in the rectal gland
both antibodies fluorescently labeled all the secretory tubules of
the gland. As in renal tubules AQP4/1 antibody stains the whole
cell including the DAPI stained nuclear region with more intense
labeling near the apical pole in many tubule cells (Figures 7A,B).
The AQP4/2 antibody also stains the cytoplasm of tubule cells but
with more intense staining toward the basal and apical poles of
the cell and with lower intensity in the vicinity of the nucleus

![Figure 2](image2.png)

**FIGURE 2** | Control Western blots using tissue homogenates (300 μg) from rectal gland (RG) and/or liver (LIV). Normal Western blots used either AQP4/1 (A) or AQP4/2 (B) primary antibodies (Aqp4 Antibody). Control Western blots used either the Aqp4 antibody pre-blocked with the peptide antigen used to raise it (Aqp4 Antibody + peptide antigen), no primary antibody, i.e., only TNT buffer (secondary antibody alone) or serum taken from the rabbit before immunization with dogfish Aqp4 antigen (pre-immune serum). Control blots used the same primary or secondary antibody concentrations as in the normal blots. All blots were incubated 1 min in NBT/BCIP substrate. Sizes were determined using kaleidoscope pre-stained molecular weight marker proteins (M).
Major staining was also seen in tubule-like structures in dogfish cardiac stomach (Figure 8). Again both antibodies stained these tubules strongly although there was also some less intense staining in cells underlying the epithelium (e.g., see Figures 8A,C). In these putative cardiac stomach secretory tubules, again the AQP1/4 antibody stained the whole of the cell with more intense staining toward the apical pole. Staining with the AQP4/2 antibody was somewhat more patchy and diffuse in the cytoplasm (than with the AQP1/4 antibody) but was of lower intensity in the nuclear region.

Lastly, strong staining was seen in large "chloride cell"-like cells of both the filament epithelium and the lamellae of the gill (Figure 9). The AQP4/1 antibody gave uniform cytoplasmic staining in many of these cells, and staining localized more to the plasma membrane and in the nuclear region in a minority of the other stained cells (Figures 9C,D). The AQP4/2 antibody also stained large cells in the primary filament epithelium and the secondary lamellae of the gill (Figures 9E,F) but here the staining in many of these cells was localized entirely in the region of the plasma membrane, while in others there was also some cytoplasmic staining.

Previously, Piermarini and Evans (2001), and Wilson et al. (2002), showed that there are different large mitochondria-rich (MR) or "chloride cell"-like cells present in elasmobranch gill, that stained either for the ion transport enzyme Na\(^+\),K\(^+\)-ATPase, or V-type ATPase. Thus a four-color localization study was performed to determine whether the cells staining with the Aqp4 antibodies co-localize with either transport enzyme (Figures 10 and 11). Initial studies using AQP4/1, Na\(^+\),K\(^+\)-ATPase, and V-type ATPase antibodies on serial dogfish gill sections suggested some co-localization of the three antibodies (data not shown). However, four-color staining with all the antibodies on the same section clarified the situation. Although it is not clear from the wide-field image of the gill (Figure 10), essentially all of the cells staining with the AQP1/4 antibody (red) co-localize with either Na\(^+\), K\(^+\)-ATPase (orange), or V-type ATPase (green) fluorescence. Only one cell was seen that appeared to express Aqp4 alone (and this one may have occurred as a consequence of the sectioning technique used). An example higher magnification image (Figure 11), clearly shows the co-localization of AQP4/1 fluorescence with the Na\(^+\), K\(^+\)-ATPase (orange), or V-type ATPase (green) fluorescence. Only one cell was seen that appeared to express Aqp4 alone (and this one may have occurred as a consequence of the sectioning technique used). An example higher magnification image (Figure 11), clearly shows the co-localization of AQP4/1 fluorescence with the Na\(^+\), K\(^+\)-ATPase (orange), or V-type ATPase (green) fluorescence. Only one cell was seen that appeared to express Aqp4 alone (and this one may have occurred as a consequence of the sectioning technique used). 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Na\(^+\), K\(^+\)-ATPase expressing cells were on the secondary lamellae with only a few cells in the primary filament epithelium.

**DISCUSSION**

This study utilized two polyclonal antibodies that were raised against peptide antigens whose sequences were located in different non-overlapping regions of the dogfish Aqp4 derived amino acid sequence. Work with custom polyclonal antibodies is not always straightforward as they are often more specific in immunohistochemistry than Western blotting or vice versa, or sometimes only work in one of those techniques. In this case, a second independent antibody was raised (AQP4/2) because unlike with the Western blot results with the AQP4/1 antibody, the immunohistochemical sub-cellular localizations, being largely cytoplasmic but also showing a nuclear location, were highly unusual for AQP4, which is normally a plasma membrane protein. To test the veracity of these results, AQP4/2 antibody was made and while it produces a larger array of bands in Western blotting (see Figure 1), as far as can be determined in this study, it produces staining in the same cells as the AQP4/1 antibody but shows a much more restricted sub-cellular localization, in particular showing either no or greatly reduced nuclear staining. It is possible to propose hypotheses that would explain the differences in results between the two antibodies (for example, it may be that only versions of Aqp4 with the C-terminal end removed (i.e., the region used to raise AQP4/1) can associate to form higher molecular weight dimers, trimers, and
tetramers, hence explaining the high molecular weight banding seen with AQP4/2 (see Figure 1). However, the most parsimonious explanation is that the AQP4/1 binding is less specific in immunohistochemistry than AQP4/2, but that the opposite is true in Western blotting.

As with the tissue distribution of dogfish *aqp4* mRNA expression (see Cutler et al., 2012), dogfish Aqp4 protein expression was ubiquitous and consequently dogfish Aqp4 is more widely expressed than is the case in mammals (Ishibashi et al., 2009). In particular, unlike in mammals, Aqp4 is expressed in dogfish gill and liver.

The AQP4/1 antibody produced two bands (49.5 and 35.5 kDa) in Western blots, which are essentially similar to the two bands obtained with mammalian AQP4 (Terris et al., 1995). The abundance of the 35.5 kDa protein band was similar between tissues but there was somewhat more Aqp4 protein in rectal gland, liver and brain, and with lower levels in (pyloric) stomach and kidney. In the case of mammalian AQP4, the larger band was suggested to be a glycosylated form of AQP4. While the Aqp4 amino acid sequence from dogfish as well as other species, possess putative consensus N-glycosylation sites, often glycoproteins on gels/blots yield broad diffuse bands and the 49.5 kDa band here and the 50 kDa AQP4 band in mammals are both discrete bands. It would also be expected that if the 49.5 kDa proteins were glycosylated, these bands would have their molecular weights reduced by the enzyme PNGase F, and this was not the case in this study (see Figure 4). There is some evidence from unpublished work from this laboratory concerning eel aquaporins expressed in *Xenopus* oocytes (i.e., the presence of dimers, trimers and tetramers), that standard Laemmli SDS-reducing gels do not abolish all interactions between proteins and also that some proteins appear to run apparently smaller than their molecular weight, suggesting incomplete unfolding (Lignot et al., 2002). Consequently it would seem likely that the 49.5 kDa band either represents Aqp4 with an accessory protein still attached to it, or an Aqp4 dimer that has run much smaller than its expected size (74.4 kDa). Lastly the 49.5 kDa band could represent a dimer that has undergone partial protease digestion. Similarly the 57 kDa protein seen in liver homogenates is likewise likely to occur due to one of the three aforementioned options. Finally it is not clear why the 37.5 and 38.5 kDa bands were absent from the un-heated rectal gland crude membrane homogenates (see Figure 3), but its likely the diffuse 32 kDa band present instead, represents some form of folded Aqp4 protein that when heat denatured runs at a larger more accurate molecular weight. It is also not clear why the 37.5 and 38.5 kDa bands were absent when using the AQP4/2 antibody on blots of rectal gland crude membrane homogenates, although the region of Aqp4 used as an antigen to raise the AQP4/2 antibody, contains predicted serine and tyrosine kinase phosphorylation sites (NetPhos 2.0; Blom et al., 1999) and if 37.5 and 38.5 kDa represent phosphorylated Aqp4 proteins, then the AQP4/2 antibody might not be able to bind them.

**KIDNEY**

Clearly from the immunohistochemistry performed with either antibody, dogfish Aqp4 is expressed in a sub-set or particular parts of renal tubules. Marine elasmobranch renal tubules are complex with two loops [with various neck (I–II), proximal (I–IV), intermediate (I–VI), distal (I–II), and collecting duct (I–II) segments] compared to the single loop of Henle found in mammals (Lacy and Reale, 1995). There are also lateral bundle zones with tightly packed tubules and sinus zones with blood sinuses seen as open areas. Based on the work of Lacy and Reale (1995), very tentative localizations for dogfish renal Aqp4 staining can be made. The

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**FIGURE 6** Immunohistochemistry of 5 μm cross-sections through the dogfish kidney showing basolateral (BL) plasma membrane staining in around 1–10% of stained tubule segments, viewed with a Zeiss Axiovert microscope. (A,B) Stained with the AQP4/1 primary and a Dylight 549 anti-rabbit secondary antibody (orange). (C) Stained with the AQP4/2 primary and an Alexa 555 anti-rabbit secondary antibody (orange). Nuclear counterstain, DAPI (blue). N=Aqp4 staining throughout the cells of the tubule. Arrows indicate the extent of the cell processes of a stellate cell (S).
**FIGURE 7** | Cross-sections through dogfish rectal gland stained with AQP4/1 (A,B) or AQP4/2 (C,D). (A) Lower magnification image acquired with a Zeiss 510 Meta confocal microscope showing tubule staining and the gland’s central duct (RD). (B) Higher magnification image using a Zeiss Axiovert microscope, showing AQP4/1 – Alexa 488 secondary antibody (green) staining throughout the cytoplasm of tubule cells with higher intensity in the apical pole (arrows). (C,D) Images showing AQP4/2 – Alexa 555 secondary antibody (orange) staining using a Zeiss Axiovert microscope, and showing stronger staining toward the basal pole of cells. Nuclear counterstain, DAPI (blue).

**FIGURE 8** | Cross-sections through dogfish cardiac stomach stained with AQP4/1 (A,B) and acquired with a Zeiss 510 Meta confocal microscope or with AQP4/2 (C,D) and acquired with a Zeiss Axiovert microscope. (A) Lower magnification image showing tubule staining and the cardiac stomach lumen (L). (B) Higher magnification image, showing AQP4/1 – Alexa 488 secondary antibody (green) staining throughout the cytoplasm of tubule cells with higher intensity in the apical pole. (C,D) Images showing AQP4/2 – Alexa 555 secondary antibody staining (orange), and showing patchy cytoplasmic staining but with less staining in the vicinity of the nucleus than in the case of the AQP4/1 antibody. Nuclear counterstain, DAPI (blue).
majority of tubules segments staining are reminiscent of interme-
diate tubule segments, such as In-IV and In-V (or possibly In-II
though In-IV) of the second renal tubule loop. This is because the
Aqp4 antibodies stain open tubules without any apparent brush
border (found in proximal segments) and these stained tubules
are largely found in the lateral bundle zone. The In-IV segment is
known as the “diluting segment” due to the occurrence of sodium
chloride re-absorption in this region of the nephron (Friedman
and Hebert, 1990). The presence of Aqp4 in this region would
be curious as its thought to have low water permeability (Fried-
man and Hebert, 1990). If Aqp4 was trafficked into the basolateral
membrane as occurs with some AQPs in mammals, provided their
was no apical water conduit this would be consistent with low
tubule water permeability. Additionally this would suggest Aqp4
may be involved in cell volume regulation in this segment. Because
some tubules showing Aqp4 staining are also found in the sinus
zone this suggests that these regions are likely to be other interme-
diate segments (i.e., the first part of In-VI or less likely In-I). The
presence of Aqp4 in the In-VI would make sense, as this segment
is thought to have high water permeability and may be involved
in osmotic equilibration due to water egress from the renal tubule
(Friedman and Hebert, 1990). However Aqp4 would have to reside

FIGURE 9 | Longitudinal sections cut through the dogfish gill,
stained with the AQP4/1 antibody and an Alexa 488 secondary
antibody (green), and viewed with a Zeiss 510 Meta confocal
microscope (A–D). Further sections were stained with the
AQP4/2 antibody and an Alexa 555 secondary antibody (orange),
and viewed with a Zeiss Axiovert microscope (E,F). Nuclear
counterstain, DAPI (blue). Where F, filament, L, lamellae, and EE,
external environment.
in the plasma membrane for this to be the case and its currently not clear that that is the case. Again regulated trafficking of Aqp4 to the plasma membrane might also be an explanation for that as mention above. Additionally, the very occasional tubule showing Aqp4 staining, did appear to have some brush border material present, this suggests Aqp4 may also stain a small part of the proximal tubule. Lastly, the tubule segments showing basolateral staining probably represent a different part of the intermediate segment (In-III?). However the localization of Aqp4 to particular tubule segments currently remains difficult. Different renal tubule segments have been identified using various lectins (Althoff et al., 2006) but the results of this study suggest it would not be trivial to reliably localize Aqp4 (or other proteins) to particular tubule segments using their methods, and that is therefore beyond the scope of the current article.

RECTAL GLAND

The fact that Aqp4 was expressed in the dogfish rectal gland is also of interest as the gland has been a major model tissue historically for studies involving fluid secretion (Burger and Hess, 1960; Bonting, 1966; Hayslett et al., 1974; Epstein et al., 1983; Greger et al., 1988; Riordan et al., 1994; Forest, 1996; Silva et al., 1996; Evans, 2010). Despite the many studies concerning ion transport, the only study to look at water permeability of the gland suggested it probably did not express aquaporins due to low apparent membrane water permeability (Zeidel et al., 2005). However, a series of illuminating studies by Solomon et al. (1984a,b, 1985) showed that ion secretion by the rectal gland was not stimulated when animals were perfused with hypertonic shark ringer solution (plasma salinity was raised without changing body fluid volume), but was stimulated when body fluid volume was increased using isotonic shark ringer. This strongly suggests that the principle function of the rectal gland is actually to remove excess water but due to the fact that water transport is passive, ions have to be transported to allow the water to follow by osmosis. Additionally, almost every example of secretory tissues/cells investigated has shown that the cells involved (in fluid secretion) invariably express some kind of aquaporin isofrom. So it might be expected that elasmobranch rectal gland secretory tubule cells would express aquaporins. However, in the case of the staining with either of the AQP4/1 or AQP4/2 antibodies, with the respective apical or basal staining, in neither case does staining appear present in the plasma membrane itself to any great extent, and this may explain why the study of Zeidel et al. (2005), found no significant water permeability associated with rectal gland plasma membranes. However, the question would be, why have Aqp4 then? The dogfish used in this study were normal unfed animals whose rectal glands are unlikely to have been particularly active. The answer to the question therefore may be that a significant amount of Aqp4 may not reside in the plasma membranes of tubule cells until the gland is stimulated to secrete, whereupon Aqp4 may be inserted into the plasma membrane. Regulated insertion of aquaporins has been shown to occur in mammals and is a particularly important mechanism for renal AQP2 (Nejsum, 2005). The possibility of regulated trafficking of dogfish rectal gland Aqp4 may be tested by further experiments in the future.

CARDIAC STOMACH

During a screen of different dogfish tissues to see where Aqp4 was expressed, particularly strong staining was found in the cardiac stomach, which is an extension of the esophagus (anterior to the pyloric stomach) that has a totally distinct morphology in comparison to the esophagus itself (smooth brown epithelium rather than a surface covered with white cartilaginous conical structures). The Aqp4 staining appears to be localized particularly to secretary tubule structures that are likely to represent acid secreting gastric glands. The cardiac stomach of dogfish has been shown to have an acidic lumen with a pH in the range of 2–4 (Wood et al., 2007). Elasmobranchs have also been shown to express the H⁺, K⁺-ATPase enzyme in proximal stomach, which is associated with stomach acid secretions in mammals (Smolka et al., 1994; Choe et al., 2004; Shin et al. (2009)). As often occurs with ion secretions, they usually represent fluid secretions and consequently water is also secreted. This may move via the paracellular pathway, but a transcellular route via aquaporin water channels is more easily controlled. In dogfish cardiac stomach gastric glands, the immunohistochemical results in this study suggest fluid secretion may well involve Aqp4, but again there is no particular staining clearly associated with the gland cell plasma membranes although the AQP4/1 antibody shows staining toward the apical pole of cells. As mentioned previously, the dogfish in this study were unfed and it may be that Aqp4 is only inserted into the plasma membranes
after secretion is stimulated by feeding. This is therefore another avenue for further study.

**GILL**

Another location showing strong Aqp4 staining was the epithelial cells of the gill. Studies in the even more ancient Agnathan hagfish show staining for Aqp4 only in the gill but not other tissues, suggesting this may be the original cell localization for Aqp4 in vertebrates (Nishimoto et al. (2007)). In these cyclostomes, however, Aqp4 was found only in the pavement cells of lamellae. In this study, Aqp4 staining was found in both the filament epithelium and the in lamellae, in large “chloride cell”-like or “MR”-like cells. In particular with the AQP4/2 antibody (but also to some extent the AQP4/1 antibody) there were two different staining patterns
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