Regional Distribution of Na,K-ATPase Activity in Porcine Lens Epithelium

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Purpose. It has been established that Na,K-ATPase activity is higher in lens epithelium than fibers. However, others have suggested the Na,K-ATPase enzyme may be inactive or absent in the central 10% of the epithelium. Studies were conducted to measure and compare Na,K-ATPase specific activity and to examine Na,K-ATPase protein expression in the anterior and equatorial regions of porcine lens epithelium.

Methods. Na,K-ATPase activity was determined by measuring the ouabain-sensitive rate of adenosine triphosphate (ATP) hydrolysis. Western blot analysis was used to detect Na,K-ATPase catalytic subunit (α) and glycoprotein subunit (β) protein as well as β-actin which was used as a loading control.

Results. Na,K-ATPase specific activity was more than two times higher in the equatorial epithelium than the anterior 50% of the epithelium. However, the abundance of Na,K-ATPase α1 isoform protein was similar in the two regions. Neither the α2 nor α3 Na,K-ATPase isoform could be detected in the anterior or equatorial epithelium, but Na,K-ATPase β1 protein was detected in both regions. In contrast to the observed regional difference in Na,K-ATPase activity, the activity of a different P-type ATPase, plasma membrane Ca-ATPase (PMCA), was not significantly different in the anterior and central epithelium. Western blot analysis indicated the presence of two PMCA isoforms, PMCA2, and PMCA4.

Conclusions. Na,K-ATPase activity is significantly higher at the equatorial region of the epithelium compared with the anterior, even though the level of Na,K-ATPase protein is similar in the two regions. It is possible that nonuniform distribution of functional Na,K-ATPase activity contributes to the driving force for circulating solute movement through the lens fiber mass. (*Invest Ophthalmol Vis Sci.* 2003;44:4395–4399) DOI: 10.1167/iovs.03-0287

The lens must be transparent to focus light on the retina. Maintenance of ionic balance is a key requirement for lens transparency. The sodium pump, Na,K-ATPase, contributes to ionic regulation, not only by maintaining the necessary high potassium and low sodium concentration within lens cells but also by establishing the sodium gradient necessary for sodium-coupled transport mechanisms that shift amino acids and other solutes.[1] Another P-type ATPase, the plasma membrane calcium ATPase (PMCA), serves an important but different role in maintaining the intracellular calcium concentration at a low level. Abnormally high sodium and calcium concentrations have been found in opaque human cataractous lenses compared with transparent control lenses.[2,3] Furthermore, ouabain, a specific Na,K-ATPase inhibitor, has been shown to induce lens opacification.[4] Raising lens calcium concentration by incubation of lenses in high calcium medium has also been shown to result in opacification of cultured lenses.[5] These results illustrate the importance of maintaining low sodium and calcium concentrations within the lens and necessity for proper Na,K-ATPase and PMCA function.

Investigators have demonstrated that Na,K-ATPase specific activity is not equally distributed throughout the lens, with fibers having lower specific activity than the epithelial monolayer.[6,7] There are results indicating nonuniform sodium pump function, even within the lens epithelium. In the frog lens epithelial cells, patch-clamp measurement of the Na,K-ATPase–derived ouabain-sensitive electrical current suggested Na,K-ATPase activity could be higher in the equatorial epithelium than the anterior epithelium.[8] More recently, using the considerably larger rabbit lens, Candia and Zamudio[9] showed that ouabain-inhibitable current is entirely undetectable in the most central region of the lens epithelium. These investigators suggested that in the central 10% of the anterior surface, the Na,K-ATPase enzyme may be either inactive or absent. The present study was undertaken to measure and compare Na,K-ATPase enzyme activity in anterior and equatorial porcine lens epithelium and also to examine Na,K-ATPase protein expression in the two regions.

For the purpose of comparison, the activity of another P-type ATPase, plasma membrane Ca-ATPase (PMCA), was also examined in the anterior and equatorial lens epithelium. Bian et al.[10] have shown that mRNA of all isoforms (PMCA types 1–4) is present in bovine lens epithelium, with PMCA3 being the most abundant. In contrast, Nabekura et al.[11] found only PMCA1b mRNA in the rat lens epithelium. Nabekura et al. also analyzed material from the nuclear lens fibers but were unable to detect any PMCA mRNA. To our knowledge, there is no previous report on the regional expression of PMCA proteins within the lens epithelium.

Materials and Methods

Sample Preparation

Materials were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Lenses were dissected posteriorly from porcine eyes kindly donated by Swift Meat Packing Co. (Louisville, KY). The eyes were used within 4 hours of death. The average lens weight was 430 mg (range, 370–470 mg) and the average equatorial diameter was 8 mm. The use of animal tissues was approved by the University of Louisville Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Samples of lens capsule–epithelium were obtained separately from the anterior and the periphery (equatorial region) of the lens surface. The anterior epithelium (50%–60% of the anterior surface) was isolated by a curvilinear tear with the aid of surgical scissors. The remaining

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equatorial epithelium was then separated from the fibers. Anterior and equatorial epithelium samples were homogenized separately in ice-cold buffer containing (in mM) 150 sucrose, 5 HEPES, 4 EGTA, 0.8 dithiothreitol, and protease inhibitors (in μM) 2 antipain, 2 leupeptin, 1 pepstatin A, 1 phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL aprotinin. Protein concentration was determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL), and homogenates were used for Na,K-ATPase activity assay or Western blot analysis.

Measurement of Na,K-ATPase Activity

Na,K-ATPase activity was determined as described by Okafor et al.[12] Briefly, lens epithelium homogenate was suspended at a final concentration of 0.25 μg protein/μL in buffer containing a final concentration of (in mM) 100 NaCl, 10 KCl, 3 $MgCl_2$, 1 EGTA at pH 7.4 as well as alamethicin (10 μg/mL) added to permeabilize membrane and vesicles to ions and ATP.[13] To half of the samples, ouabain was added at a final concentration of 1 mM. After a preincubation period of 5 minutes at 37°C, ATP was added to a final concentration of 1 mM. All tissue samples were subjected to identical ionic conditions and ATP concentration. ATP hydrolysis reaction was stopped after 45 minutes by the addition of ice-cold trichloroacetic acid. ATP hydrolysis was quantified by determining the amount of inorganic phosphate in each sample by using a colorimetric method based on the measurement of absorbance at 750 nm after the addition of 1% ammonium molybdate and 4% ferrous sulfate in 1.32 N hydrochloric acid. Na,K-ATPase activity was defined as the difference in ATP hydrolysis (inorganic phosphate release) measured in the presence and absence of ouabain.

Measurement of Plasma Membrane Ca-ATPase Activity

PMCA activity was determined as described by Dean et al.[14,15] In this technique, ATP hydrolysis is coupled to reduced nicotinamide adenine dinucleotide (NADH) oxidation by pyruvate kinase, using phosphoenolpyruvate and lactate dehydrogenase. Samples of lens epithelium homogenate were centrifuged at 100,000*g* for 7 minutes to obtain crude membrane preparations. Fifty micrograms of crude membranes and 2 μL of supernatant (cytosolic component) was added to 200 μL of buffer containing (in mM) 20 HEPES, 130 NaCl, 10 $MgCl_2$, 0.01 $CaCl_2$, 1 ATP, and the coupled assay components, as well as 1 mM ouabain, 1.5 μM thapsigargin (a specific inhibitor of sarcoplasmic and endoplasmic reticulum Ca-ATPase) and 7.5 mM sodium azide (a mitochondrial inhibitor). The background rate of ATP hydrolysis was first measured under baseline conditions, and then 50 μg of membrane material was added to the reaction mixture and the rate measured again. Half the samples contained EGTA (5 mM) to chelate calcium. The ATP hydrolysis rate was determined by measuring the change of absorbance at 340 nm with a plate reader (Powerwave X; Bio-Tek instruments, Winooski, VT). PMCA activity was calculated as the difference between the ATP hydrolysis rate, with and without EGTA. The data are calculated as nanomoles phosphate released per milligram protein per minute.

Western Blot Analysis

Proteins in lens epithelium homogenates were solubilized in Laemmli[16] buffer and separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane. Blocking of the nitrocellulose membrane for 1 hour with 5% nonfat dry milk in TTBS (30 mM Tris, 150 mM NaCl, 0.5% [vol/vol] Tween-20 [pH 7.4]) was followed by a 1-hour incubation with the primary antibody. The antibodies used in this study were as follows: monoclonal anti-Na,K-ATPase α1 isoform (Sigma-Aldrich); polyclonal rabbit anti-Na,K-ATPase α2 and α3 isoforms (obtained from Thomas A. Pressley, Texas Tech University Health Science Center, Lubbock, TX); monoclonal anti-β-actin (Sigma-Aldrich); monoclonal anti-Na,K-ATPase β1 isoform, anti-Na,K-ATPase β2 isoform rabbit antiserum. and polyclonal rabbit anti-Na,K-ATPase β3 isoform, a monoclonal anti-PMCA panspecific clone (5F10; Upstate Biotechnology, Lake Placid, NY); and polyclonal rabbit anti-PMCA1 to -3 isoforms and monoclonal anti-PMCA4 isoform clone JA9 (Affinity Bioreagents, Golden, CO). After five washes in TTBS for 5 minutes each, the nitrocellulose membrane was incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour. After three washes in TTBS for 5 minutes each, enhanced chemiluminescence substrate (Pierce) was applied for 1 minute to the nitrocellulose membrane, and the proteins were visualized by exposure to x-ray film.

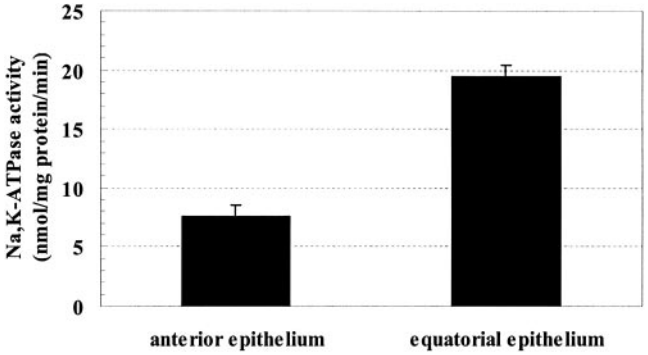

FIGURE 1. Na,K-ATPase specific activity measured in the anterior and equatorial epithelium obtained from fresh porcine lenses. Data are the mean ± SE of results from 11 samples. The difference in Na,K-ATPase activity between the two regions was significant ($P < 0.05$).

Data Analysis

Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Ouabain-sensitive Na,K-ATPase activity was measured separately in freshly isolated samples obtained from two different regions of the lens epithelium: the anterior and the equator. Na,K-ATPase activity was significantly higher in the equatorial region ($P < 0.05$) than in the anterior epithelium (Fig. 1). To determine whether differences in Na,K-ATPase protein expression potentially could cause the observed differences in Na,K-ATPase activity, samples from both regions were probed by Western blot for the Na,K-ATPase catalytic α1 subunit, the ubiquitously expressed Na,K-ATPase isoform.[17] A dense ~100-kDa band was observed in both the anterior and equatorial epithelium (Fig. 2a). Although the activities from the two regions were different (Fig. 1), the abundance of the catalytic α1 subunit was similar (Fig. 2a). To confirm equal loading of proteins, the blots were stripped and reprobed for β-actin, a housekeeping protein. As shown in Figure 2b, the abundance of β-actin in the two regions of the lens epithelium was similar.

To determine whether there are regional differences in Na,K-ATPase β subunit expression that could influence Na,K-ATPase activity, Western blot analysis was performed for the Na,K-ATPase β1 glycoprotein subunit, the most widely expressed β isoform. A dense band at ~45 kDa was observed in both the anterior and equatorial epithelia (Fig. 3). A similar ~45-kDa band was observed in brain, together with an ~50-kDa band, which probably signifies a pool of β1 protein with different glycosylation. Attempts to probe for the Na,K-ATPase β2 and β3 isoforms were not successful. The β2 antibody produced numerous potentially spurious bands, whereas the β3 antibody failed to cross-react (results not shown).

Western blot studies were conducted to determine whether expression of Na,K-ATPase isoforms α2 and α3 could explain the observed difference in Na,K-ATPase activity between the anterior and equatorial epithelium. Isoform-specific polyclonal

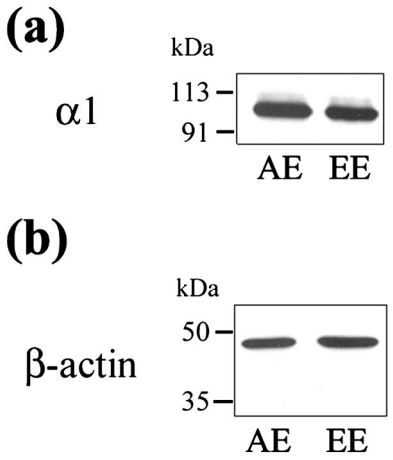

FIGURE 2. Western blot of (**a**) Na,K-ATPase α1 polypeptide and (**b**) β-actin determined in anterior (AE) and equatorial (EE) porcine lens epithelium. *Left*: Molecular weight markers (in kilodaltons). A similar amount of protein (50 μg) was applied to each lane. Density of Na,K-ATPase α1 polypeptide and β-actin bands was similar between the two regions.

antibodies, anti-HERED for the Na,K-ATPase α2 isoform and anti-TED for the Na,K-ATPase α3 isoform, which has been shown to cross-react with several mammalian species,[18] were used for this purpose. As shown in Figure 4, Western blot using the anti-HERED and anti-TED antibodies revealed dense immunoreactive bands at ~100 kDa in membrane material isolated from porcine optic nerve and brain but not from kidney medulla, which is known to express only α1.[17] The findings confirm the Na,K-ATPase α2 and α3 isoform specificity and cross-reactivity of the antibodies with porcine material. Neither the anterior nor equatorial regions of lens epithelium revealed detectable Na,K-ATPase α2 or α3 bands.

For the purpose of comparison with Na,K-ATPase activity distribution, PMCA activity was measured in samples isolated from the anterior and equatorial lens epithelium. Unlike Na,K-ATPase activity, no significant difference was observed between PMCA activity in the central and equatorial regions (Fig. 5). To examine PMCA protein expression, we subjected epithelium samples from the two regions to Western blot with isoform-specific antibodies for PMCA types 1 to 4 and a panspecific antibody that recognizes all four isoforms. The PMCA1 and -3 isoforms were not detectable (results not shown). Samples from the anterior epithelium displayed an immunoreactive

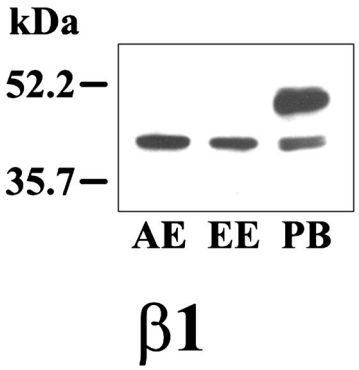

FIGURE 3. Western blot of Na,K-ATPase β1 polypeptide determined in anterior (AE) and equatorial (EE) porcine lens epithelium. *Left*: Molecular weight markers (in kilodaltons). Homogenate isolated from porcine brain (PB) was used as a positive control. A similar amount of protein (100 μg) was applied to each lane.

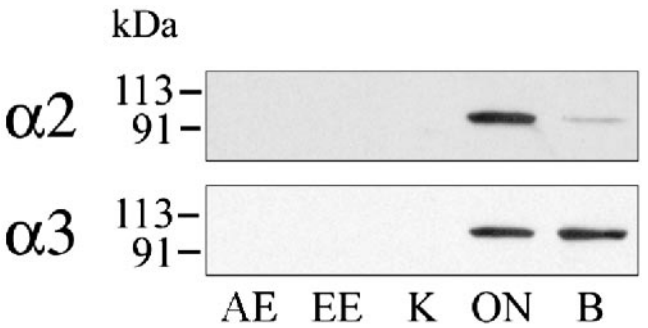

FIGURE 4. Western blot of Na,K-ATPase α2 and α3 polypeptide determined in anterior (AE) and equatorial (EE) porcine lens epithelium. *Left*: Molecular weight markers (in kilodaltons). Homogenates isolated from porcine optic nerve (ON) and brain (B) were used as positive controls. Purified porcine kidney medulla membrane (K) was used as a negative control. No immunoreactive bands were detected in lens samples or the kidney sample.

band at ~130 kDa when probed with the PMCA2 isoform-specific antibody (Fig. 6). The same band was also observed in samples from the equatorial epithelium but with weaker intensity. In addition, two other PMCA2-immunoreactive bands were observed at 70 to 80 kDa and another band at ~130 kDa. Distinct bands were detected in samples from both regions of the lens epithelium, by using the PMCA4 isoform-specific antibody (Fig. 6). The major band observed in material from the equatorial region had a slightly higher molecular weight than that in the anterior region. On most occasions, the anterior epithelium also displayed a weak band aligning with the upper band from the equatorial region. The panspecific PMCA antibody detected the bands recognized by the PMCA4 antibody but not the PMCA2 antibody (Fig. 6). This was confirmed by stripping a blot initially probed by the PMCA panspecific antibody and reprobing with either the PMCA2- or -4–specific antibody.

DISCUSSION

Abnormally high sodium content has been demonstrated in cataractous human lenses and experimental disturbance of sodium concentration has been shown to induce lens opacification.[2–4] It has been recognized for a long time that Na,K-ATPase is responsible for sustaining low sodium within the lens and that lens epithelium has much higher specific Na,K-ATPase activity than the fibers.[19] Enrichment of Na,K-ATPase

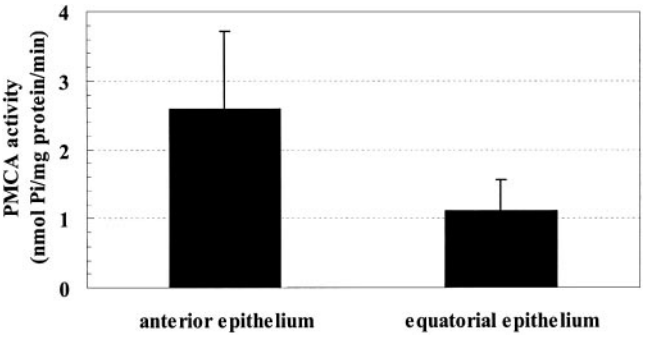

FIGURE 5. PMCA-specific activity measured in the anterior and equatorial epithelium obtained from fresh porcine lenses. Data are the mean ± SE of 18 measurements obtained from five different pools of membrane material. The difference in PMCA activity between the two regions was not significant.

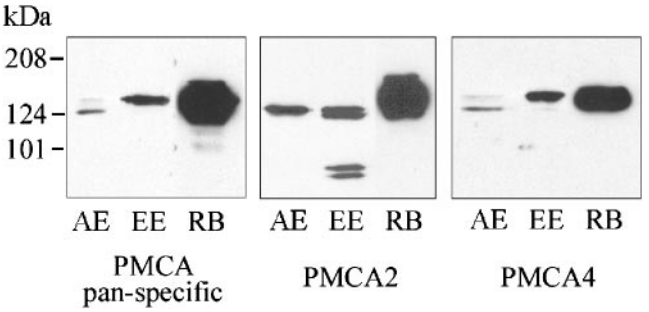

FIGURE 6. Western blot of PMCA peptides determined in anterior (AE) and equatorial (EE) porcine lens epithelium. Samples were probed with a PMCA panspecific antibody and PMCA2- and -4–specific antibodies. *Left*: Molecular weight markers (in kilodaltons). A rat brain microsome preparation (RB) was used as a positive control.

activity in the epithelium suggests that it plays a significant role in conducting active sodium-potassium transport for the entire lens. However, the discovery of circulating currents around the lens signify nonuniform ionic current across the lens epithelium[20–22] suggesting there may not be uniform expression of Na,K-ATPase and ion channels across the lens epithelium. More recently, Gao et al.[8] have shown in frog that the magnitude of sodium pump current is larger in the lens equator than in the anterior epithelium. In the central 10% of the rabbit lens epithelium, sodium pump current is undetectable.[9] In the current study, Na,K-ATPase activity in the equatorial epithelium was more than two times the activity in the anterior 50% to 60% of the epithelium. In contrast, PMCA activity was not significantly different in the anterior and equatorial epithelium.

In the frog lens, Gao et al.[8] have demonstrated that differences in sodium pump current are the apparent result of differences in Na,K-ATPase catalytic subunit isoform distribution across the epithelium. Na,K-ATPase α2 isoform protein was detected at the anterior and α1 isoform protein at the equator. In the present study, we detected only the α1 isoform of the Na,K-ATPase catalytic subunit in the porcine lens epithelium. A noteworthy finding was that the abundance of the Na,K-ATPase α1 isoform was similar in the anterior and equatorial regions. Both regions also displayed robust expression of Na,K-ATPase β1 subunit. The glycoprotein β subunit is thought to be necessary for folding of the α, β heterodimer and transport of the complex to the plasma membrane.[23] Taken together, the results suggest that lower Na,K-ATPase activity in the anterior epithelium of the porcine lens is not the result of reduced Na,K-ATPase α and β protein isoform expression.

Na,K-ATPase catalytic subunit expression by the lens epithelium appears to vary widely between species. Rat lens epithelium expresses three isoforms: Na,K-ATPase α1, α2, and α3.[24] Bovine lens epithelium also expresses all three Na,K-ATPase isoforms at the equatorial region, but it does not have the α2 isoform in the anterior region.[25] In contrast, the frog lens epithelium lacks the Na,K-ATPase α3 isoform and expresses mainly the Na,K-ATPase α2 isoform in the anterior region and Na,K-ATPase α1 isoform in the equatorial region.[8] The present study confirms earlier reports that α1 is the only Na,K-ATPase isoform normally detectable in the porcine lens epithelium.[26,27] However, it should be pointed out that Na,K-ATPase α2 protein can be expressed in the epithelium of porcine lenses subjected to culture under conditions that cause cytoplasmic sodium elevation.[26,27]

The anterior and equatorial epithelium express a similar abundance of Na,K-ATPase catalytic subunit yet have a different Na,K-ATPase–specific activity. The mechanisms underlying the observed difference in Na,K-ATPase activity in the two regions have yet to be identified. Various mechanisms such as oxidation[28,29] or phosphorylation[30,31] have been shown to modify Na,K-ATPase activity. A recent study by Cui et al.,[32] using the human lens epithelial cell line B3, suggested Na,K-ATPase activity may be influenced by the turnover of Na,K-ATPase protein. There could be a difference in protein turnover rate between the two regions. Epithelial cell function at the anterior and the equator is different in numerous respects including the ability to proliferate, differentiate, and migrate.[33,34] There are also regional differences in receptor expression and function.[35,36]

A number of mechanisms including PMCA, sarcoplasmic and endoplasmic reticulum Ca ATPases, mitochondria, sodium calcium exchangers, and calcium channels contribute to cytoplasmic calcium regulation.[37] In this study, we focused on PMCA, which, together with the sodium calcium exchangers, is responsible for transporting calcium outward across the plasma membrane.[38] Unlike Na,K-ATPase activity, PMCA activity was not significantly different between the anterior and equatorial regions of the epithelium. The results indicate the presence of PMCA2 and -4 isoforms in the porcine lens epithelium. Dense ~130-kDa PMCA2 bands were observed in both the anterior and equatorial epithelium. PMCA2-immunoreactive bands at 70 to 80 kDa, which could represent proteolytic fragments, were observed only in the equator. The bands detected using the panspecific monoclonal antibody 5F10, which cross-reacts with all isoforms,[39] colocalized with the PMCA4 band but not the PMCA2 band. PMCA4, which is found in most adult cells,[38] was more abundant at the equator than the center of the epithelium. Moreover, the PMCA4 band in the anterior region had a lower molecular weight by approximately 15 kDa than did the major band in the equatorial region. This could be due to the cleavage of the isoform by calpain, as reported by James et al.[40]

In summary, the results of this study clearly demonstrate a nonuniform distribution of Na,K-ATPase activity within the lens epithelium, with equator having significantly higher activity than the anterior lens epithelium. Na,K-ATPase activity which is high at the lens equator may contribute to the driving force for circulating ionic currents that flow outward at the equator and inward at the anterior and posterior poles.[41] Although the mechanism underlying the difference in Na,K-ATPase activity is not clear, it does not appear to be due to differences in Na,K-ATPase protein levels or differences in the pattern of Na,K-ATPase isoform expression. In contrast, the PMCA activity was not significantly different between the two regions.

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