Isoforms of Na,K-ATPase in Rat Lens Epithelium and Fiber Cells

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In the normal transparent lens, the sodium concentration in the cytoplasm is 10% to 15% that of the sodium concentration in aqueous humor.1 Outwardly directed sodium transport by the active sodium–potassium pump (Na,K-ATPase) is responsible for maintaining this ion gradient between the interior of lens cells and the extracellular environment. Failure to preserve the low cytoplasmic sodium concentration results in considerable cell damage, and this can lead to lens opacification. Indeed, the cytoplasmic sodium content is elevated in almost all human cataractous lenses in which there is cortical opacification.2

The lens was one of the first tissues in which Na,K-ATPase activity was detected.3 However, the two cell types of the lens have very different Na,K-ATPase activity; the activity in the epithelial cells is considerably higher than in the fiber cells.3–5 In spite of this, the fiber cells appear to contain a significant amount of Na,K-ATPase polypeptide.6 The presence of Na,K-ATPase protein in lens fiber cells also is illustrated by the ability of lens fiber membrane to bind ouabain.7 We have suggested that Na,K-ATPase polypeptide in the rabbit lens fiber cell may be partially inactive.6
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Lens epithelial cells also appear to differ from fiber cells in that the epithelium, but not the fibers, have a demonstrated ability to synthesize new Na,K-ATPase protein when the lens ion permeability is increased.6 This suggests that the amount of Na,K-ATPase in lens epithelium may be dynamically regulated.

There are at least three different isoforms of the Na,K-ATPase catalytic (α) subunit.9 To examine in detail the differences between Na,K-ATPase in lens epithelium and fiber cells, we first tested by immunoblot whether the same isoforms of Na,K-ATPase catalytic (α) subunit can be detected in the two cell types. Second, we tested whether fiber and epithelial cells both have the machinery needed for ongoing Na,K-ATPase expression as judged by the detection of mRNA for Na,K-ATPase α subunit.

METHODS

Lens Dissection

Adult Wistar rats (each weighing approximately 200 g) were obtained from Harlan (Indianapolis, IN). Lenses from adult rats were obtained immediately after the animals were killed with an overdose of sodium pentobarbital. Lenses from neonatal rats (3 days of age) were obtained after the animals were killed by carbon dioxide inhalation. Procedures used in these studies were approved by the University of Louisville Institutional Animal Care and Use Committee, and they conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Lenses were removed from the eye by dissecting the posterior of the globe and gently cutting the suspensory ligaments, enabling the lens to be removed from the eye. The capsule–epithelium was removed by gently making an incision near the equator of the lens and peeling back the capsule using fine forceps. To slow proteolysis and mRNA breakdown, tissues were kept chilled during dissection. The lens capsule–epithelium and lens fiber tissue was frozen immediately in liquid nitrogen and stored at −70°C.

Lens Membrane Preparation

Membrane material was isolated from the lens capsule–epithelium or the lens fiber cell mass. Tissues were homogenized in ice-cold buffer A (150 mM sucrose, 0.9 mM HEPES, 0.03 mM EDTA containing the protease inhibitors antipain (16.5 μM), leupeptin (21 μM), pepstatin A (14 μM), PMSF (40 μM), and aprotinin (0.027 trypsin inhibitor U/ml)) for 1 hour and incubated for another hour with the primary Na,K-ATPase antibody (rabbit anti-rat Na,K-ATPase). Three polyclonal antibodies were used in this study. They were directed against the α1, α2, and α3 isoforms of rat Na,K-ATPase. These antibodies, purchased from Upstate Biotechnology (Lake Placid, NY), were prepared based on the method by Shyjan and Levenson12 in which portions of cDNA encoding each of the rat Na,K-ATPase isoforms were expressed in Escherichia coli.

Analysis of Na,K-ATPase by Western Blot

Proteins were separated electrophoretically on a 7.5% sodium dodecyl sulfate-polyacrylamide gel using the Laemmli buffer system.13 Prestained molecular weight markers, brain membrane protein material (60 μg), and lens membrane protein material (80 μg) from epithelial cells or from lens fiber cells were applied to specified lanes of the gel. The separated proteins were transferred electrophoretically to nitrocellulose at 100 V for 60 minutes in transfer buffer containing Tris (25 mM), glycine (192 mM), and 10% methanol at pH 8.3. The nitrocellulose was then blocked with 2% ovalbumin in TBS (0.03 M Tris buffer, pH 7.5, 0.15 M NaCl, and 0.5% Tween 20) for 1 hour and incubated for another hour with the primary Na,K-ATPase antibody (rabbit anti-rat Na,K-ATPase). Three polyclonal antibodies were used in this study. They were directed against the α1, α2, and α3 isoforms of rat Na,K-ATPase. These antibodies, purchased from Upstate Biotechnology (Lake Placid, NY), were prepared based on the method by Shyjan and Levenson12 in which portions of cDNA encoding each of the rat Na,K-ATPase isoforms were expressed in Escherichia coli.

Brain Membrane Preparation

Membrane material was isolated from rat brain, which is known to express both Na,K-ATPase α1, α2, and α3 isoforms.9 Brain membrane material was used as a positive control for each Na,K-ATPase immunoblot. Brains were obtained from adult rats immediately after death. Whole brains (25 g) were homogenized in 50 ml of ice-cold buffer containing NaCl (150 mM), EDTA (2.6 mM), PMSF (40 μM), and aprotinin (0.027 trypsin inhibitor U/ml) at pH 7. The homogenate was centrifuged at 3000g for 10 minutes, the pellet discarded, and the supernatant collected and centrifuged at 10,000g for 20 minutes. The supernatant was then collected and centrifuged at 100,000g for 20 minutes, and the pellet, enriched in membrane material, was resuspended in 3 ml of 0.01 M TES buffer, pH 7.5, containing 20% (wt/vol) glycerol, KCl (100 mM), pepstatin A (14 μM), antipain (16.5 μM), leupeptin (21 μM), EDTA (1 mM), dithiothreitol (1 mM), PMSF (40 μM), and aprotinin (0.027 trypsin inhibitor U/ml). This mixture was divided into aliquots, frozen in liquid nitrogen, and stored at −70°C. The protein concentration of the enriched membrane material was determined by the bicinchoninic acid assay using bovine serum albumin as a standard.

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Figure 1. Western blot to confirm Na,K-ATPase antibody specificity, showing Na,K-ATPase immunoreactive polypeptides detected in membrane material isolated from rat skeletal muscle (M), kidney medulla (K), and brain (B). After sodium dodecyl sulfate gel electrophoresis, blotted proteins were probed for α1, α2, and α3 Na,K-ATPase polypeptides.

coli as fusion proteins with the E. coli trpE gene, and antibodies were raised against these proteins in rabbits. After incubation with the primary antibody, the nitrocellulose was washed with TTBS once for 10 minutes and then twice for 5 minutes before incubation for 1 hour with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Richmond, CA). The nitrocellulose was then washed with TTBS buffer once for 15 minutes and four times for 5 minutes each. Visualization of the Na,K-ATPase polypeptide immunoblots was made possible by incubating the nitrocellulose sheet with chemiluminescence substrates (DuPont, Wilmington, DE) for 1 minute and then exposing the sheet to x-ray film.

Following a procedure detailed by Delamere et al.,8 membrane material was isolated from rat kidney medulla and skeletal muscle. We used these membrane preparations, together with membrane material isolated from rat brain, to verify that the Na,K-ATPase antisera are specific. As shown in Figure 1, Western blots confirmed the appearance of Na,K-ATPase α1 in kidney medulla, Na,K-ATPase α1 and α2 in skeletal muscle, and Na,K-ATPase α1, α2, and α3 in brain. This tissue-specific distribution of Na,K-ATPase α isoforms has been described by Young and Lingrel.9

Because some Western blot experiments were conducted to examine differences between epithelial and fiber cell membrane material, we routinely checked epithelial membrane preparations for contamination with fibers. Electrophoretically separated proteins were stained as described by Delamere et al.,8 and it was confirmed that some fiber protein bands were absent from epithelial membrane preparations. Similarly, contamination of fiber cell membrane preparations by epithelium was ruled out by the absence of detectable Na,K-ATPase α2 and α3 immunoreactive polypeptide in fiber material. Many protein bands appeared with similar density in fiber and epithelial preparations, confirming that the two pools of material contained roughly similar amounts of membrane protein.

Analysis of Na,K-ATPase mRNA by Northern Blot

RNA was isolated from samples of frozen lens capsule–epithelium or fiber cell material following the procedure of Chomczynski et al.13 Total RNA (25 μg) was separated electrophoretically on a 1% agarose gel containing formaldehyde as described by Lehrach et al.14 The RNA was transferred to a nylon membrane (Hybond; Amersham, Arlington Heights, IL) and prehybridized, hybridized, and then washed according to the manufacturer's protocol and probed with isoform-specific (approximately 300 bp) probes for rat Na,K-ATPase α1, α2, and α3 subunits.9 Hybridization was performed with 2 x 10⁶ cpm/ml random hexanucleotide-primed 32P-dCTP-labeled probes. Bands were visualized by autoradiography with a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). The isoform-specific probes used in this study for Na,K-ATPase α1, α2, and α3 were kindly provided by Dr. Jerry Lingrel (University of Cincinnati).

RESULTS

Detection of Na,K-ATPase by Western Blot

Membrane preparations were obtained from freshly isolated adult rat lenses. As described in Methods, epithelial and cortical fiber cell material was prepared separately, and membrane material was isolated from each cell type. For lens epithelium material, a sharply defined, dense immunoblot reaction for the Na,K-ATPase α1 isoform was observed at 104 kDa (Fig. 2). Dense immunoblot reactions for the Na,K-ATPase α2 isoform (106 kDa) and the Na,K-ATPase α3 isoform

Figure 2. A Western blot showing Na,K-ATPase immunoreactive polypeptides detected in epithelial (E) and fiber (F) cell membrane material isolated from adult rat lenses and membrane material isolated from adult rat brain (B). After sodium dodecyl sulfate gel electrophoresis, blotted proteins were probed for α1, α2, and α3 Na,K-ATPase polypeptides.
and Na,K-ATPase a3 polypeptides were not detected by Northern Blot. Detection of Na,K-ATPase mRNA by Northern Blot revealed a distinct band for Na,K-ATPase a1 mRNA detected in lens epithelial material. The Na,K-ATPase a1 immunoreactive band was identical to that observed for Na,K-ATPase a1 immunoreactive polypeptide in epithelial material. The Na,K-ATPase a1 immunoreactive band density was also similar in epithelial material isolated from epithelial and fiber cells. Immunoreactive bands for Na,K-ATPase a2 and Na,K-ATPase a3 polypeptides were not detected in membrane material isolated from lens fiber cells.

Detection of Na,K-ATPase mRNA by Northern Blot

Total RNA was obtained from freshly isolated adult rat lenses. As described in Methods, epithelial and fiber cell material was prepared separately. Northern analysis for Na,K-ATPase a subunit mRNA in lens epithelial material revealed a distinct band for Na,K-ATPase a1 isoform mRNA, as well as two distinct bands for Na,K-ATPase a2 isoform mRNA and a slightly less dense single band for Na,K-ATPase a3 isoform mRNA (Fig. 3). The electrophoretic mobility of the Na,K-ATPase a1, a2, and a3 mRNA detected in lens epithelial material was identical to that observed for Na,K-ATPase a1, a2, and a3 mRNA detected in rat brain. It was shown earlier that different rat tissues display two mRNA transcripts for the Na,K-ATPase a2 isoform. In the current study, the two transcripts were observed in both brain and lens epithelium.

Lens fiber cells differed sharply from the epithelial cells in that Na,K-ATPase a subunit mRNA from lens fibers could not be detected by Northern analysis. Neither Na,K-ATPase a1, a2, nor a3 mRNA was observed. To confirm that fiber cell mRNA was not degraded, we stripped the blots that had been used to probe for Na,K-ATPase a subunit mRNA, then re-probed the same lanes for β-actin mRNA. Distinct β-actin mRNA bands were observed in both epithelium and fiber cell material (Fig. 3).

Fiber Cells in the Neonatal Lens

In the adult rat lens, Na,K-ATPase a1 immunoreactive polypeptide was observed in fiber cell membrane material, but a1 mRNA could not be detected in RNA isolated from fiber cells. This suggests that for most lens fiber cells, Na,K-ATPase a1 polypeptide could have been expressed earlier, possibly during the process of fiber cell elongation and growth. To test this idea, experiments were conducted using neonatal rat lenses freshly isolated from 3-day-old rats. The 3-day neonatal lens is at a stage at which growth is rapid, which means that there is a relatively high proportion of fiber cells in the process of elongation. Fiber cell material was obtained from 3-day-old neonatal rat lenses and total RNA was isolated from pooled tissue samples. Northern analysis of Na,K-ATPase a subunit mRNA revealed a distinct band for Na,K-ATPase a1 mRNA in material isolated from neonatal (3-day) lens fiber cells (Fig. 4). The electrophoretic mobility of the Na,K-ATPase a1 mRNA detected in the neonatal lens fiber cells was identical to that observed for a1 mRNA detected in material isolated from adult lens epithelium and from rat brain. Neither Na,K-ATPase a2 nor a3 mRNA could be detected in material isolated from neonatal lens fiber cells. As described above, β-actin mRNA was probed to confirm that the amount of RNA run in parallel lanes was roughly equivalent for adult and neonatal lens fiber samples and to confirm that the RNA was not degraded (Fig. 4). By Western blot, Na,K-ATPase a1 polypeptide was detected in membrane material isolated from neonatal lens fiber cells (Fig. 4). The electrophoretic mobility of Na,K-ATPase a1 (104 kDa) was the same in the neonatal lens fibers, adult lens fibers, and brain. As in the adult lens fibers, Na,K-ATPase a2 and a3 immunoreactive polypeptides could not be detected in neonatal lens fiber membrane material.

DISCUSSION

In one of the earliest studies on Na,K-ATPase, Bonting and coworkers determined that lens epithelial cells have a much greater Na,K-ATPase activity than lens fibers. This has since been confirmed by others, and the unequal distribution of Na,K-ATPase activity between epithelium and fiber cells underpins a theoretical model in which active ion transport by the anterior surface epithelial cell monolayer is predicted to contribute significantly to the regulation of sodium and potassium for the entire lens cell mass. This model was first described by Kinsey and Reddy. The special-
FIGURE 4. Na,K-ATPase in the neonatal (3-day-old) lens fiber cells. (A) Northern blot: Na,K-ATPase α subunit mRNA and β-actin mRNA detected in neonatal (3-day-old) rat lens fiber cells (N), adult rat lens fiber cells (A), and adult rat brain (B). Total RNA was isolated from each tissue, and 25 μg samples were separated on an agarose–formaldehyde gel, transferred to a nylon sheet, and probed for Na,K-ATPase α1, α2, and β-actin mRNA as described in Methods. (B) Western blot: Na,K-ATPase immunoreactive polypeptides detected in fiber cell membrane material isolated from neonatal (3-day-old) rat lenses (N), fiber cell membrane material isolated from adult rat lenses (A), and membrane material isolated from adult rat brain (B). After sodium dodecyl sulfate gel electrophoresis, blotted proteins were probed for α1, α2, and α3 Na,K-ATPase polypeptides.

Judged by Northern analysis and confirmed by Western blot studies, significant amounts of Na,K-ATPase α1, α2, and α3 immunoreactive polypeptide were found to be expressed in adult rat lens epithelium. As stated above, the results of an earlier study suggested that increased lens permeability apparently can cause increased expression of Na,K-ATPase α polypeptide by the porcine lens epithelium. In this earlier study, we were unable to detect new synthesis of Na,K-ATPase polypeptide by lens fiber cells, which suggests that lens fiber cell Na,K-ATPase abundance may not be dynamically regulated. This difference between lens epithelium and fiber cells concurs with the current study, in which mRNA for Na,K-ATPase α subunit was not detectable in material isolated from fiber cells in the adult rat lens. The lack of Na,K-ATPase mRNA is consistent for Na,K-ATPase α2 and α3 isoforms for which no immunoreactive polypeptides were found in fiber cell material. However, the situation for the Na.K-ATPase α1 isoform is that adult rat lens fiber cells contain a substantial amount of α1 immunoreactive polypeptide but an undetectable amount of α1 mRNA. Based on these findings, it seems probable that turnover of fiber cell Na,K-ATPase is slow in mature lens fiber cells. The simplest explanation is that the Na,K-ATPase α1 polypeptide observed in adult lens fibers was synthesized at some earlier time, perhaps during the process of lens fiber cell elongation and growth. Consistent with this notion, we were able to detect Na,K-ATPase α1 mRNA in fiber cell material isolated from neonatal (3-day-old) lenses.

The detection of Na,K-ATPase α1 mRNA, as well as Na,K-ATPase α1 polypeptide, in fiber cells obtained from the neonatal lens suggests that synthesis of Na,K-ATPase protein by lens fiber cells might take place when the lens is in a stage of rapid growth. In the mature lens, the lack of detectable
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Na,K-ATPase α subunit mRNA in the fiber cells suggests that these cells have a low rate of ongoing Na,K-ATPase protein biosynthesis. If this proves to be correct, it must follow that much of the Na,K-ATPase α2 was detected only in lenses fibers is old. It is tempting to speculate that age-related deterioration of the Na,K-ATPase protein contributes to the low Na,K-ATPase activity detected in lens fiber cells. However, it should be noted that electrophoretic mobility of Na,K-ATPase α1 immunoreactive polypeptide was identical in brain, lens epithelium, and lens fiber cell membrane material, arguing against proteolytic degradation of the fiber cell Na,K-ATPase α1 polypeptide.

The detection of multiple Na,K-ATPase α isoforms in rat lens epithelium agrees with multiple isoforms detected in bovine lens epithelium but differs from the situation in the porcine lens in which Na,K-ATPase α2 was detected only in lenses cultured under conditions in which membrane permeability was increased and α3 was not detected under any circumstances. However, the porcine lens fiber cells were similar to the rat lens fiber cells in that only the Na,K-ATPase α1 isoform was detectable. These findings are consistent with the idea that after the process of lens cell differentiation in the pig and rat, fiber cells primarily express the α1 isoform well accepted as the “housekeeper” form of the sodium pump expressed in almost all cells. It has been found that Na,K-ATPase α2 and α3 isoforms can be expressed in response to external stimuli, such as thyroid hormone, aldosterone, and low external potassium concentration. It also has been speculated that the receptor–second-messenger pathway may be able to regulate the activity of Na,K-ATPase α2 or α3 isoform. Interestingly, two groups of investigators recently discovered cholinergic receptor-mediated mechanisms in the lens epithelium. It is possible that Na,K-ATPase in lens epithelium could be upregulated or downregulated in response to hormones, growth factors, or neurotransmitters that occasionally enter the aqueous humor.

Key Words
epithelium, fibers, isoforms, lens, Na,K-ATPase

References
20. Shamraj OI, Melvin D, Lingrel JB. Expression of Na,K-


