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

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Purpose. The lens epithelium is thought to conduct Na-K transport for the entire lens cell mass. Lens fibers have a poor ion transport capacity. The authors tested whether different Na,K-ATPase polypeptides are expressed in the two cell types and whether both cells have the machinery needed for ongoing Na,K-ATPase expression as judged by the presence of mRNA for the Na,K-ATPase α subunit.

Methods. Membranes were isolated from adult rat lens epithelium or fibers, and Western blot experiments were conducted for Na,K-ATPase α1, α2, and α3 polypeptides. Total RNA was isolated from adult rat lens epithelium or fiber cells, and Northern analysis was conducted for Na,K-ATPase α1, α2, and α3 mRNA. Some experiments were conducted using fiber cells from neonatal (3-day-old) rat lenses.

Results. Multiple isoforms of Na,K-ATPase were detected in adult rat lens epithelium. Judged by Northern blot band intensity, mRNA for Na,K-ATPase α1 and α2 was more abundant than for α3 mRNA. By Western blot, Na,K-ATPase α1, α2, and α3 polypeptides were observed as sharp bands at 100 to 108 kDa. In fiber cells, only Na,K-ATPase α1 immunoreactive polypeptide was detected. Judged by immunoblot density, the amount of α1 polypeptide was similar in both epithelium and fiber cell material. However, Na,K-ATPase α subunit mRNA was not found in adult lens fibers. To test whether Na,K-ATPase synthesis takes place during fiber cell growth, Northern blot analysis was conducted with RNA from neonatal (3-day-old) lens fibers; Na,K-ATPase α1 mRNA was clearly visible.

Conclusions. Adult rat lens epithelium expresses more than one isoform of Na,K-ATPase catalytic subunit, whereas only the α1 isoform can be detected in fiber cells. In adult rat lens fiber cells, the observation of α1 polypeptide, but no α1 mRNA, suggests that ongoing α1 synthesis is low. Based on the detection of α1 mRNA in neonatal lens fibers, Na,K-ATPase synthesis by lens fibers may be higher during cell elongation and growth. Invest Ophthalmol Vis Sci. 1996;37:1502-1508.

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.3,4 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
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. 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. 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, 5 mM Heps, 0.9 mM dithiothreitol, 0.03 mM EGTA) containing the protease inhibitors antipain (16.5 μM), leupeptin (21 μM), pepstatin A (14 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), EGTA (1 mM), dithiothreitol (1 mM), PMSF (40 μM), and aprotinin (0.027 trypsin inhibitor U/ml) at pH 7. The homogenate was centrifuged at 115,000g for 60 minutes. The final pellet enriched in membrane material was resuspended in buffer A, frozen in liquid nitrogen, and stored at —70°C. The protein content of the partially purified membrane material was determined by the bicinchoninic acid assay using bovine serum albumin as the standard.

Brain Membrane Preparation

Membrane material was isolated from rat brain, which is known to express both Na,K-ATPase α1, α2, and α3 isoforms. 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 100,000g for 20 minutes, the supernatant 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), EGTA (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.

Analysis of Na,K-ATPase by Western Blot

Proteins were separated by electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel using the Laemmli buffer system. 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 TTBS (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 Levenson in which portions of cDNA encoding each of the rat Na,K-ATPase isoforms were expressed in Escherichia coli.

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**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 \( \alpha_1 \) isoform was observed at 104 kDa (Fig. 2). Dense immunoblot reactions for the Na,K-ATPase \( \alpha_2 \) isoform (106 kDa) and the Na,K-ATPase \( \alpha_3 \) isoform 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. Total RNA (25 \( \mu \)g) was separated electrophoretically on a 1% agarose gel containing formaldehyde as described by Lehrach et al. 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 \( \alpha_1, \alpha_2, \) and \( \alpha_3 \) subunits. Hybridization was performed with \( 2 \times 10^6 \) cpm/ml random hexanucleotide-primed \( ^{32}P \)-dCTP-labeled probes. Bands were visualized by autoradiography with a Phosphorlmager system (Molecular Dynamics, Sunnyvale, CA). The isoform-specific probes used in this study for Na,K-ATPase \( \alpha_1, \alpha_2, \) and \( \alpha_3 \) were kindly provided by Dr. Jerry Lingrel (University of Cincinnati).
and Na,K-ATPase α3 polypeptides were not detected by Northern Blot.

FIGURE 3. A Northern blot showing Na,K-ATPase α subunit mRNA and β-actin mRNA detected in adult rat lens epithelium (E), fibers (F), and 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, α3, and β-actin mRNA, as described in Methods.

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 α subunit mRNA in lens epithelial material revealed a distinct band for Na,K-ATPase α1 isoform mRNA, as well as two distinct bands for Na,K-ATPase α2 isoform mRNA and a slightly less dense single band for Na,K-ATPase α3 isoform mRNA (Fig. 3). The electrophoretic mobility of the Na,K-ATPase α1, α2, and α3 mRNA detected in lens epithelium was identical to that observed for Na,K-ATPase α1, α2, and α3 mRNA detected in rat brain. It was shown earlier that different rat tissues display two mRNA transcripts for the Na,K-ATPase α3 isoform.8 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 α subunit mRNA from lens fibers could not be detected by Northern analysis. Neither Na,K-ATPase α1, α2, nor α3 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 α 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 α, immunoreactive polypeptide was observed in fiber cell membrane material, but α1 mRNA could not be detected in RNA isolated from fiber cells. This suggests that for most lens fiber cells, Na,K-ATPase α1 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,15 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 α subunit mRNA revealed a distinct band for Na,K-ATPase α1 mRNA in material isolated from neonatal (3-day) lens fiber cells (Fig. 4). The electrophoretic mobility of the Na,K-ATPase α1 mRNA detected in the neonatal lens fiber cells was identical to that observed for α1 mRNA detected in material isolated from adult lens epithelium and from rat brain. Neither Na,K-ATPase α2 nor α3 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 α1 polypeptide was detected in membrane material isolated from neonatal lens fiber cells (Fig. 4). The electrophoretic mobility of Na,K-ATPase α1 (104 kDa) was the same in the neonatal lens fibers, adult lens fibers, and brain. As in the adult lens fibers, Na,K-ATPase α2 and α3 immunoreactive polypeptides could not be detected in neonatal lens fiber membrane material.

DISCUSSION

In one of the earliest studies on Na,K-ATPase, Bonning and coworkers determined that lens epithelial cells have a much greater Na,K-ATPase activity than lens fibers. This has since been confirmed by others16,17 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.18 The special-
A Northern blot

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B Western blot

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**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, α3, 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 lenses 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
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 that the Na,K-ATPase a2 polypeptide we detect in adult lens 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 epithelium7 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.8 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. In other tissues, it has been found that Na,K-ATPase α2 and α3 isoforms can be expressed in response to external stimuli, such as thyroid hormone,22 aldosterone,23 and low external potassium concentration.24 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.25 Interestingly, two groups of investigators recently discovered cholinergic receptor-mediated mechanisms in the lens epithelium.18,26 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
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