Sodium–potassium adenosine triphosphatases (Na/K-ATPases) are plasma membrane-spanning ion pumps that play a fundamental role in tissue homeostasis and development (reviewed by Kaplan,4 Aperia et al.,2 and Krupinski and Beitel3). Their minimal functional unit consists of an α and a β subunit. Four different α isoforms (ATP1A1–4) and three β isoforms (ATP1B1–3) are known in higher eukaryotes, all of which are expressed in a tissue-specific manner.4,5 In the retina, the Na/K-ATPases consist largely of ATP1A3 (85%) and ATP1B2 (80%) subunits6 and consequently are referred to as retinal Na/K-ATPases. Na/K-ATPases are tightly regulated at various levels, such as by cardiac glycosides, which halt its ion pump function and have been linked to disturbances in retinal homeostasis. In this study, we investigated the crosstalk between retinoschisin and cardiac glycosides at the retinal Na/K-ATPase and the consequences of this interplay on retinal integrity.

**METHODS.** The effect of cardiac glycosides (ouabain and digoxin) on the binding of retinoschisin to the retinal Na/K-ATPase was investigated via western blot and immunocytochemistry. Also, the influence of retinoschisin on the binding of cardiac glycosides was analyzed via enzymatic assays, which quantified cardiac glycoside-sensitive Na/K-ATPase pump activity. Moreover, retinoschisin-dependent binding of tritium-labeled ouabain to the Na/K-ATPase was determined. Finally, a reciprocal effect of retinoschisin and cardiac glycosides on Na/K-ATPase localization and photoreceptor degeneration was addressed using immunohistochemistry in retinoschisin-deficient murine retinal explants.

**RESULTS.** Cardiac glycosides displaced retinoschisin from the retinal Na/K-ATPase; however, retinoschisin did not affect cardiac glycoside binding. Notably, cardiac glycosides reduced the capacity of retinoschisin to regulate Na/K-ATPase localization and to protect against photoreceptor degeneration.

**CONCLUSIONS.** Our findings reveal opposing effects of retinoschisin and cardiac glycosides on retinal Na/K-ATPase binding and on retinal integrity, suggesting that a fine-tuned interplay between both components is required to maintain retinal homeostasis. This observation provides new insight into the mechanisms underlying the pathological effects of cardiac glycoside treatment on retinal integrity.

Keywords: X-linked juvenile retinoschisis, retinoschisin, retina, Na/K-ATPase, cardiac glycosides
MATERIALS AND METHODS

Animal Models

The study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rts1b-knockout (retinoschisin-deficient) mice were kept on a C57BL/6 background for more than 20 generations. Mice were housed under specific pathogen-free barrier conditions at the Central Animal Facility of the University of Regensburg, in strict compliance with National Institutes of Health guidelines. Mice were sacrificed 16 or 18 days after birth by cervical dislocation.

Cell Culture

Y-79 cells (ATCC; LGC Standards GmbH, Wesel, Germany) and Hek293 cells (Hek293 EBNA cells; Invitrogen, Carlsbad, CA, USA) were grown as described in Plössl et al.

Media and Media Supplies

All media and cell culture supplies were purchased from Life Technologies (Carlsbad, CA, USA).

Primary Antibodies

Primary antibodies against ATP1A3 and ATP1B2 (Thermo Fisher Scientific, Waltham, MA, USA) and against ACTB (Sigma Aldrich, St. Louis, MO, USA) were used according to each manufacturer’s recommendations. Primary antibodies against retinoschisin were kindly provided by Robert Molday (University of British Columbia, Vancouver, Canada) and diluted as described previously.

Expression Constructs

The generation of expression constructs for non-tagged and Myc-tagged retinoschisin (NM_000330.4) is described in Plössl et al.; for ATP1A3 (NM_152296.4) and ATP1B2 (NM_001678.4), in Friedrich et al.; for bicistronic expression of ATP1A3 and ATP1B2, in Plössl et al.; and for the ouabain-insensitive ATP1A3 mutant, in Plössl et al.

Transfection

Hek293 cells subjected to ³H-labeled ouabain assays or to immunocytochemistry were transfected with Bio Mirus TransIT-LT1 Transfection Reagent (Thermo Fisher Scientific). All other transfections were performed using the calcium-phosphate method.

Purification of Recombinant Retinoschisin

Purification of recombinant retinoschisin from the supernatant of Hek293 cells, heterologously expressing Myc-tagged retinoschisin, was performed as described by Plössl and colleagues. The supernatant of Hek293 cells transfected with pCDNA3.1 (Thermo Fisher Scientific) was subjected to the same purification procedure, and eluates served as control treatment.

Retinoschisin Binding to Hek293 and Y-79 Cells

Retinoschisin binding to Hek293 cells was assessed as described, with supernatant of Hek293 cells stably secreting recombinant retinoschisin as input, ouabain and digoxin (for concentrations, see Figs. 1 and 2) were added to the input and incubated for 1 hour (western blot) or 2 hours (immunocytochemistry). Retinoschisin binding to Y-79 cells was performed likewise, but with 4 × 10⁶ cells in 2 mL input (for ouabain and digoxin concentrations, see Figs. 1 and 2). Western blot and immunocytochemical analyses were performed as described previously. Fluorescence microscopy was performed with an Axioskop2 mot plus microscope (Carl Zeiss Meditec, Oberkochen, Germany) at 40× magnification.

Analysis of Na/K-ATPase Activity in Murine Retinal Membranes

The Na/K-ATPase-catalyzed release of free phosphate was measured colorimetrically in retinal membranes of wild-type and retinoschisin-deficient mice (postnatal day 16) as a function of ouabain and digoxin (for concentrations, see Fig. 3), as described in Plössl et al. The values obtained at 10⁻³ M ouabain, representing unspecific (not Na/K-ATPase-catalyzed) adenosine triphosphate (ATP) cleavage, were subtracted from all other values. Data were processed by nonlinear regression (SigmaPlot 12.5; Systat Software, San Jose, CA, USA).

Tritium-Labeled Ouabain Binding Assays

Hek293 cells cultivated in 12-well plates were transfected with a bicistronic expression construct for ATP1A3 and ATP1B2 or an empty pCEP4 vector (Thermo Fisher Scientific). Forty-eight hours after transfection, the medium was replaced with 300 μL Dulbecco’s modified Eagle’s medium containing purified retinoschisin (for concentrations, see Fig. 4) or an equal volume of control eluate, followed by a 30-minute incubation at 37°C. Subsequently, tritium (³H)-labeled ouabain, 250 μCi (Perkin Elmer, Rodgau, Germany; for concentrations, see Fig. 4), was added followed by a 1-hour incubation at 37°C. Cells were resuspended in 1 mL PBS, centrifuged, and washed twice with PBS. The pellet was dissolved in 100 μL 5% SDS and transferred to scintillation cups with 5 mL of scintillation cocktail (Rotiszint eco plus; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Bound radioactivity was measured in a scintillation counter for 1 minute. The same protocol was applied to Y-79 cells, with the following modifications: 8.5 × 10⁶ cells were seeded in 24-well plates coated with poly-γ-lysine. After 24 hours, the medium was replaced by 1 mL RPMI 1640 medium (Thermo Fisher Scientific) and retinoschisin (for concentrations, see Fig. 4).

Cultivation of Murine Retinal Explants

Retinas from retinoschisin-deficient mice at postnatal day 18 were dissected and cultivated on filters as described previously. They were cultured in medium containing either purified retinoschisin (333 ng/mL) or control eluate with cardiac glycosides (for concentrations, see Figs. 5 and 6), and the medium was replaced after 48 hours. After 4 days, explants were processed for analysis.
Cryosectioning and immunolabeling of retinal explants were performed as described previously. Sections were counterstained with 4,6-diamidino-2-phenylindol (DAPI, 1:1000; Molecular Probes, Leiden, The Netherlands). TUNEL staining of retinal explants was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche-1168795910, provided by Sigma Aldrich), according to the manufacturer's instructions. Confocal microscopic images were taken with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), at 40× magnification.

**Statistical Analyses**

The normality of the data was assessed by the Shapiro–Wilk normality test. Data not following a Gaussian distribution were analyzed using the Mann–Whitney U test (two experimental groups) or the Kruskall–Wallis test, with post ad hoc Dunn's multiple comparison test and Bonferroni correction (more than two experimental groups). Data following a Gaussian distribution were analyzed using Student's t-test (two experimental groups) or ANOVA with Tukey's multiple comparison test (more than two experimental groups). Statistical analyses were performed using the XLSTAT add-in software (Addinsoft Inc., New York, NY, USA).

**RESULTS**

**Cardiac Glycosides Impair Retinoschisin Binding to the Retinal Na/K-ATPase**

First, the effect of cardiac glycosides on the binding of retinoschisin to the retinal Na/K-ATPase was investigated. As described previously, retinoschisin binds to Hek293 cells heterologously expressing the two subunits of the retinal Na/K-ATPase (ATP1A3 and ATP1B2). We tested whether exposure to ouabain (10−3, 10−5, 10−7 M) or digoxin (10−8, 10−7, 10−6, or 10−5 M) had an influence on the binding capacity of retinoschisin.

Indeed, western blot analyses revealed that increasing concentrations of ouabain (Fig. 1A) or digoxin (Fig. 1B) impaired retinoschisin binding to Hek293 cells heterologously expressing ATP1A3 and ATP1B2. Interestingly, antibodies against retinoschisin (red) and ATP1B2 (green). Scale bars: 25 μm. Retinoschisin signals of 20 ATP1B2-expressing cells per biological replicate were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data represent the mean ± SD of four (D, ouabain treatment) or five (E, digoxin treatment) biological replicates, calibrated against the control. Asterisks show statistically significant differences compared to control ("P < 0.05; Mann–Whitney U test). (F–G) Y-79 cells were subjected to recombinant retinoschisin for 1 hour in the presence of 0 (control), 10−6, 5 × 10−7, 10−5 M ouabain (F) or 0 (control), 10−7, 10−5, or 10−3 M digoxin (G), followed by intensive washing. Retinoschisin binding was investigated by western blot analyses with antibodies against retinoschisin. ACTB staining served as loading control. Densitometric quantification of retinoschisin binding was performed on immunoblots from six (F, ouabain treatment) or four (G, digoxin treatment) individual experiments. Signals were normalized against ACTB and calibrated against the control. Data represent the mean ± SD. Asterisks show statistically significant differences compared to control ("P < 0.05; Mann–Whitney U test). (F–G) Y-79 cells were subjected to recombinant retinoschisin for 1 hour in the presence of 0 (control), 10−6, 10−5, 10−7, 10−3, or 10−2 M ouabain (A) or 0 (control), 10−6, 10−7, 10−5, or 10−3 M digoxin (B), followed by intensive washing. Retinoschisin binding was evaluated by western blot analyses with antibodies against retinoschisin. ACTB staining served as loading control. Densitometric quantification of retinoschisin binding was performed on immunoblots from five (A, ouabain treatment) or seven (B, digoxin treatment) individual experiments. Signals were normalized against ACTB and calibrated against the control. Data represent the mean ± SD. Asterisks represent statistically significant differences compared to control ("P < 0.05; Kruskall–Wallis test followed by Dunn’s multiple comparison test and Bonferroni correction).
As described previously, anti-ATP1B2 staining revealed several used in retinoschisin binding experiments before (Figs. 1A, 1B) insensitive Na/K-ATPases. (Fig. 2) treatment on ATP1B2 levels. Furthermore, we investigated whether cardiac glycosides affect retinoschisin levels in the input. Hek293 cells heterologously expressing the retinal Na/K-ATPase were incubated with cardiac glycosides (0, 10^-3, or 10^-2 M ouabain in Fig. 2C; 0, digoxin showed a much stronger effect than ouabain (reduction to 33.0 ± 16.3% at 10^-2 M ouabain and to 46.9 ± 21.0% at 10^-3 M digoxin; \( P < 0.05 \) compared to control).

Compared to the western blot analyses, the displacement of retinoschisin by cardiac glycosides was even more pronounced in the immunocytochemical analyses (Figs. 1C–1E, Supplementary Fig. S1A), as 10^-3 M ouabain or 10^-6 M digoxin reduced retinoschisin binding to Hek293 cells heterologously expressing the retinal Na/K-ATPase to 53.9 ± 5.1% and 24.8 ± 19.1%, respectively, compared to control (\( P < 0.05 \)).

These results were corroborated in Y-79 cells, a human retinoblastoma cell line that endogenously expresses the retinal Na/K-ATPase. Y-79 cells exhibited a reduction in retinoschisin binding, which was inversely proportional to increasing cardiac glycoside concentrations (Figs. 1F, 1G). Again, digoxin exerted a stronger effect than ouabain (41.3 ± 43.6% at 10^-2 M ouabain and 42.4 ± 7.2% at 10^-6 M digoxin; \( P < 0.05 \) compared to control).

Next, we examined whether the inhibitory effect of cardiac glycosides on retinoschisin binding is caused by competition at the Na/K-ATPase or is the result of other cellular processes. We used the transfected Hek293 cells from the binding experiments described before (Figs. 1A, 1B) and quantified the expression level of ATP1B2, the Na/K-ATPase subunit to which retinoschisin binds. Our analyses revealed no effect of ouabain (Fig. 2A) or digoxin (Fig. 2B) treatment on ATP1B2 levels. Furthermore, we investigated whether cardiac glycosides affect retinoschisin levels in the input. Hek293 cells heterologously expressing the retinal Na/K-ATPase were incubated with cardiac glycosides (0, 10^-3, or 10^-2 M ouabain or 10^-6 M digoxin; \( P < 0.05 \) compared to control).

ACTB and calibrated against the control. Data represent the mean ± SD. (C–D) Hek293 cells were transfected with expression constructs for ATP1A3 and ATP1B2. After 48 hours, they were subjected to recombinant retinoschisin in the presence of 0 (control), 10^-3, or 10^-2 M ouabain (C) or 0 (control), 10^-6, or 10^-5 M digoxin (D). After 1 hour and 2 hours, samples were taken from the supernatant containing recombinant retinoschisin (input) and subjected to western blot analyses with antibodies against retinoschisin. Densitometric quantification of retinoschisin binding was performed on immunoblots from six individual experiments. Signals were calibrated against the control. (E–F) Hek293 cells were transfected with expression constructs for a ouabain-insensitive mutant of ATP1A3 (ATP1A3-OI) and ATP1B2. After 48 hours, they were subjected to recombinant retinoschisin for 2 hours in the presence of 0 (control), 10^-7, 10^-5, 10^-3, or 10^-2 M ouabain (E) or in the presence of (control), 10^-8, 10^-7, 10^-6, or 10^-5 M digoxin (F), followed by intensive washing. Retinoschisin binding was investigated by western blot analyses with antibodies against retinoschisin. ACTB staining served as loading control. Densitometric quantification of retinoschisin binding was performed on immunoblots from each of five individual experiments. Signals were normalized against ACTB and calibrated against the control. Data represent the mean ± SD. (G–I) Hek293 cells were transfected with expression constructs for ATP1B2 and ATP1A3-OI. After 48 hours, they were subjected to recombinant retinoschisin for 2 hours in the presence of 0 M (control) or 10^-3 M ouabain (G) or in the presence of 0 M (control) or 10^-5 M digoxin (see Supplementary Fig. S1B), followed by intensive washing. Subsequently, retinoschisin binding was analyzed via immunocytochemistry with antibodies against retinoschisin (red) and ATP1B2 (green). Scale bars: 25 μm. Retinoschisin signals of 20 ATP1B2 expressing cells per biological replicate were measured using ImageJ. Data represent the mean ± SD of four (H, ouabain treatment) or five (I, digoxin treatment) biological replicates, calibrated against the control.
Retinoschisin and Cardiac Glycoside Crosstalk

Retinoschisin Does Not Affect Ouabain Binding by the Retinal Na/K-ATPase

We next explored an influence of retinoschisin on cardiac glycoside binding by investigating cardiac glycoside-induced inhibition of the active ion transport of the Na/K-ATPase. Thus, we performed the ouabain-sensitive ATP hydrolysis assay to determine Na/K-ATPase activity in murine retinal membranes. Increasing the concentrations of ouabain (Figs. 3A, 3C) or digoxin (Figs. 3B, 3D) decreased the Na/K-ATPase activity in all assays. However, the presence of endogenous (Figs. 3A, 3B) or recombinant (Figs. 3C, 3D) retinoschisin failed to affect the inhibitory capacity of ouabain or digoxin (P > 0.05 compared with retinoschisin-deficient retinae at all concentrations of ouabain or digoxin).

In an alternative approach, we investigated the binding of 3H-labeled ouabain to Hek293 cells heterologously expressing the retinal Na/K-ATPase in the presence of retinoschisin. Our first assay assessed binding of ouabain at different concentrations, in the presence of an excess amount of retinoschisin determined in previous binding assays (Supplementary Fig. S2A). With increasing concentrations of 3H-labeled ouabain, bound radioactivity (measured as counts per minute) increased (Fig. 4A). Compared to control cells (transfected with pCEP4), Hek293 cells transfected with a bicistronic expression vector for ATP1A3 and ATP1B2 showed an approximately twofold increase in bound radioactivity, apparently reflecting additional ouabain binding by the heterologously expressed retinal Na/K-ATPase. However, retinoschisin had no effect on bound radioactivity and thus on ouabain binding (Fig. 4A). We performed additional analyses investigating the binding of 10−7 M ouabain in the presence of increasing amounts of retinoschisin (Fig. 4B). Again, retinoschisin did not affect ouabain binding to Hek293 cells heterologously expressing the retinal Na/K-ATPase.
Retinoschisin and Cardiac Glycoside Crosstalk

**FIGURE 4.** Effect of retinoschisin on ouabain binding. (A, B) Hek293 cells were transfected with pCEP4 or with bicistronic expression constructs for ATP1A3 and ATP1B2. (A) After 48 hours, cells were subjected to 1.5 μg/mL recombinant retinoschisin (RS1) or the same volume of control eluate (ctr) for 30 minutes, followed by the addition of 0, 0.5 × 10^{-9}, 10^{-8}, 2.5 × 10^{-8}, 5 × 10^{-8}, or 10^{-7} M 3H-labeled ouabain for 30 minutes. (B) Alternatively, cells were subjected to 0, 0.5, 1, 1.5, or 2 μg/mL RS1 or the same volume of control eluate (ctr) for 30 minutes, followed by the addition of 10^{-7} M 3H-labeled ouabain for 30 minutes (#, without 3H-labeled ouabain). (C) Y-79 cells were subjected to 120 ng/mL recombinant RS1 or the same volume of control eluate (ctr) for 30 minutes, followed by the addition of 0, 0.5 × 10^{-9}, 10^{-8}, 2.5 × 10^{-8}, 5 × 10^{-8}, or 10^{-7} M 3H-labeled ouabain. (D) Alternatively, Y-79 cells were subjected to 0, 75, 150, 225, or 300 ng/mL RS1 or the same volume of control eluate (ctr) for 30 minutes, followed by the addition of 10^{-7} M 3H-labeled ouabain and 30 minutes of incubation (#, without 3H-labeled ouabain). (A–D) After incubation with retinoschisin and ouabain, cells were washed and lysed, and bound radioactivity was determined in a scintillation counter. (A, C) Data represent the mean ± SD of measured counts/minute of five (A) or nine (C) biological replicates. (B, D) Data represent the mean ± SD of five (B) or seven (D) biological replicates, calibrated against signals of cells incubated with 3H-labeled ouabain but without retinoschisin or control eluate (gray bar).

Binding of 3H-labeled ouabain was also investigated in Y-79 cells. In general, an excess amount of retinoschisin (as determined in Supplementary Fig. S2B) had no effect on the binding of 3H-labeled ouabain (in concentrations from 5 × 10^{-9} to 10^{-7} M) by Y-79 cells (Fig. 4C). Furthermore, increasing concentrations of retinoschisin did not affect binding of 10^{-7} M ouabain (Fig. 4D).

Cardiac Glycosides Hamper Regulation of Retinal Na/K-ATPase Localization by Retinoschisin

Previous analyses have suggested that retinoschisin is required for proper Na/K-ATPase localization in the retina, as the presence of retinoschisin leads to a pronounced enrichment of the retinal Na/K-ATPase at the inner segments, compared to the outer nuclear layer, in murine retinae. In contrast, retinoschisin deficiency results in a diffuse distribution of the retinal Na/K-ATPase across inner segments and the outer nuclear layer. We investigated the interplay of retinoschisin and cardiac glycosides on retinal Na/K-ATPase localization in retinoschisin-deficient retinal explants. Consistent with our previous data, incubation of retinoschisin-deficient retinae with retinoschisin led to Na/K-ATPase enrichment at the inner segments (Fig. 5A). The ratio of Na/K-ATPase signal intensity from the inner segments to the outer nuclear layer, r(IS/ONL), was 2.76 ± 0.45 in retinoschisin-treated explants compared with 1.57 ± 0.29 in control explants (P < 0.01) (Figs. 5A, 5B). In explants treated with 10^{-5} M ouabain, the r(IS/ONL) was similar to that of control explants (1.73 ± 0.56; P < 0.01 compared with retinoschisin-
FIGURE 5. Effect of ouabain and retinoschisin on Na/K-ATPase localization. (A) Retinoschisin-deficient retinas explanted at postnatal day 18 were cultured for 4 days in medium containing retinoschisin (RS1) or the same volume of control eluate (ctr) in the presence of 0 or 10⁻⁵ M ouabain (O) or in the presence of 0 or 10⁻⁶ M digoxin (D) (see Supplementary Fig. S3). After washing and embedding, cryosections of the explants were subjected to staining with anti-Atp1a3-antibody (green), anti-retinoschisin antibody (red), and DAPI (blue, depicting nuclei). Scale bar: 10 μm. (B, C) Na/K-ATPase signals in the inner segments and outer nuclear layer were measured using ImageJ. Data represent the mean ± SD of five biological replicates for ouabain treatment (B) and of six biological replicates for digoxin treatment (C), calibrated against the control. Asterisks represent statistically significant differences (*P < 0.05; ANOVA test followed by Tukey's multiple comparison test). (D, E) Retinoschisin signals in the inner segments were measured using ImageJ. Data represent the mean ± SD of five biological replicates for ouabain treatment (D) and of six biological replicates for digoxin treatment (E), calibrated against the control. Asterisks represent statistically significant differences (*P < 0.05; two-sided Student’s t-test).

Antagonistic Effects of Cardiac Glycosides and Retinoschisin on Retinal Integrity

Previous publications have reported that Rs1h-deficient mice exhibit retinal degeneration, with a major burst of apoptotic nuclei occurring in the outer nuclear layer, around postnatal day 18.33,34 In our final assay, we aimed to investigate how the interplay of cardiac glycosides and retinoschisin affects retinal integrity. We therefore incubated Rs1h-deficient murine retinas explanted at postnatal day 18, with recombinant retinoschisin or a control eluate with or without cardiac glycosides (Fig. 6, Supplementary Fig. S4).

After 4 days of incubation, retinoschisin-deficient retinal explants incubated with retinoschisin showed fewer apoptotic nuclei in the outer nuclear layer (9.1 ± 5.3%) than did explants treated with the control eluate (23.3 ± 19.9% apoptotic nuclei) (Figs. 6A, 6B). In explants treated with 10⁻⁵ M ouabain, the number of apoptotic nuclei increased to around 54.3 ± 31.1% (Figs. 6A, 6B). In explants subjected to retinoschisin and 10⁻⁵ M ouabain, 40.3 ± 17.9% apoptotic nuclei were observed. The difference in the percentages of apoptotic nuclei between either retinoschisin- or ouabain-treated explants was statistically significant (P < 0.05).

Digoxin treatment showed similar effects (Fig. 6C, Supplementary Fig. S4). Although retinoschisin exerted a protective effect against apoptosis (13.9 ± 16.7% apoptotic nuclei in retinoschisin treated explants compared with 24.0 ± 19.9% in control explants), treatment with 10⁻⁶ M digoxin strongly increased apoptosis (55.4 ± 27.6% apoptotic nuclei; P < 0.05 compared with retinoschisin-treated explants).
In this study, cardiac glycosides reduced retinoschisin binding to the heterologously expressed retinal Na/K-ATPase. This reduction was not accompanied by a decrease of retinoschisin in the input or by a decrease of ATP1B2, the retinoschisin binding partner of the retinal Na/K-ATPase. Moreover, cardiac glycosides did not impair retinoschisin binding to a ouabain-insensitive ATP1A3 mutant, excluding a Na/K-ATPase-independent interaction between retinoschisin and cardiac glycosides. Consistent with these in vitro results, retinoschisin binding to Y-79 cells and marine retinae was also decreased by cardiac glycosides. Taken together, these findings suggest a displacement of retinoschisin from the Na/K-ATPase by cardiac glycosides.

In contrast, cardiac glycoside binding was not affected by retinoschisin. This raises the question of how the two components interact at the Na/K-ATPase interface. The binding site for cardiac glycosides is formed by transmembrane helices 1 to 6 of the Na/K-ATPase α subunit, whereas retinoschisin binds to the extracellular domain of the β subunit. One explanation for the observed effect of cardiac glycosides on retinoschisin binding could lie in conformational alterations of the Na/K-ATPase. During ion transport, the Na/K-ATPase switches between the so-called E1 (Na\(^{+}\)-bound state) and E2 (K\(^{-}\)-bound state) conformations. This conformational change affects the entire Na/K-ATPase, including distances between the α and β subunits. Ouabain binding induces the conformational switch from E1 to E2 and stabilizes the E2 conformation. If retinoschisin has a higher affinity for E1, stabilization of E2 by cardiac glycosides could decrease retinoschisin binding to the Na/K-ATPase. Alternatively, bound cardiac glycosides could partially overlap with, and potentially block, the binding region of retinoschisin. Interestingly, the suggested retinoschisin binding patch on ATP1B2 lies very close to the outer cardiac glycoside interface of the Na/K-ATPase. In crystal structure analyses of ATP1A1 and ATP1B1 Na/K-ATPases, the sugar moieties of bound cardiac glycosides were located in a cavity enclosed by polar residues of the α and β subunits (i.e., Gln84 of the ATP1B1-ectodomain). The homologous amino acid of Gln84 in ATP1B1 is Glu89 in ATP1B2, which is directly adjacent to the putative retinoschisin binding patch (composed of four hydrophobic stretches, amino acids 83–88, 108–121, 181–184, and 240, on ATP1B2). Accessibility to the ATP1B2 binding patch by retinoschisin might thus be blocked by the protruding cardiac glycoside sugar moieties. Notably, digoxin has three sugar moieties and is bulkier than ouabain, which has only one sugar residue. This could explain the stronger effect of digoxin compared to ouabain on retinoschisin displacement.

Cardiac glycosides decrease the capacity of retinoschisin to regulate correct Na/K-ATPase localization at the photoreceptor inner segments, likely due to displacement of retinoschisin at the Na/K-ATPase. Also, cardiac glycosides and retinoschisin revealed opposing effects on retinal integrity. Retinoschisin has a known protective effect on retinal Na/K-ATPase-independent interaction between retinoschisin and cardiac glycosides. Consistent with these in vitro results, retinoschisin binding to Y-79 cells and marine retinae was also decreased by cardiac glycosides. Taken together, these findings suggest a displacement of retinoschisin from the Na/K-ATPase by cardiac glycosides.

**DISCUSSION**

In this study, we investigated the interplay between retinoschisin and cardiac glycosides at the retinal Na/K-ATPase, as well as their effect on Na/K-ATPase localization and retinal integrity. Although cardiac glycosides displaced retinoschisin from Hek293 cells heterologously expressing the retinal Na/K-ATPase, from Y-79 cells, and from murine retinal membranes, retinoschisin did not affect cardiac glycoside binding by the retinal Na/K-ATPase. Cardiac glycosides impede retinoschisin-induced enrichment of the retinal Na/K-ATPase at the inner segments and lessen the protective effect of retinoschisin against photoreceptor degeneration. This suggests that a fine-tuned interplay between cardiac glycosides and retinoschisin is required to maintain retinal homeostasis.
Retinoschisin and Cardiac Glycoside Crosstalk

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