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Retinoschisin and Cardiac Glycoside Crosstalk at the Retinal Na/K-ATPase

Verena Schmid, Karolina Plössl, Carina Schmid, Sarah Bernklau, Bernhard H. F. Weber, and Ulrike Friedrich

Institute of Human Genetics, University of Regensburg, Regensburg, Germany

PURPOSE. Mutations in the *RS1* gene, which encodes retinoschisin, cause X-linked juvenile retinoschisis, a retinal dystrophy in males. Retinoschisin specifically interacts with the retinal sodium–potassium adenosine triphosphatase (Na/K-ATPase), a transmembrane ion pump. Na/K-ATPases also bind cardiac glycosides, which control the activity of the pump 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

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,[1] Aperia et al.,[2] and Krupinski and Beitel[3]). 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%) subunits[6] 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 (reviewed by Therien and Blostein[7]). Serum-derived cardiac glycosides can cross the blood–brain barrier and are highly enriched in the retina,[8–11] suggesting a role for endogenous cardiac glycosides in retinal development and integrity. Moreover, patients administered cardiac glycosides for the treatment of chronic heart failure often experience a loss of visual acuity as a negative side effect.[11–14] Retinoschisin,

a protein secreted by photoreceptor and bipolar cells,[15] binds to the retinal Na/K-ATPase.[16,17] Mutations in the *RS1* gene, which encodes retinoschisin, cause X-linked juvenile retinoschisis (XLRS, OMIM #312700), a hereditary degenerative disorder of the macula.[18–20] Previous studies have shown that retinoschisin is required for proper retinal Na/K-ATPase localization and has a protective effect against photoreceptor degeneration.[16,21–23]

In this study, we investigated the interplay between retinoschisin and cardiac glycoside binding at the retinal Na/K-ATPase and its consequences on retinal integrity. We chose to investigate the cardiac glycosides ouabain and digoxin, which are endogenous hormones in humans[24] but are also widely used for the treatment of heart diseases.[25] We show that these cardiac glycosides hamper retinoschisin binding to the retinal Na/K-ATPase, whereas retinoschisin does not affect cardiac glycoside affinity to the retinal Na/K-ATPase. Notably, cardiac glycosides were found to impair the capacity of retinoschisin to regulate Na/K-ATPase localization and to protect against photoreceptor degeneration.

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. *Rs1h*-knockout (retinoschisin-deficient) mice[26] 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.[22]

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.[21]

Expression Constructs

The generation of expression constructs for non-tagged and Myc-tagged retinoschisin (NM_000330.4) is described in Plössl et al.[23]; for ATP1A3 (NM_152296.4) and ATP1B2 (NM_001678.4), in Friedrich et al.[16]; for bicistronic expression of ATP1A3 and ATP1B2, in Plössl et al.[27]; and for the ouabain-insensitive ATP1A3 mutant, in Plössl et al.[22]

Transfection

Hek293 cells subjected to ^{3}H-labeled ouabain assays or to immunocytochemistry were transfected with Bio Mirus *Trans*IT-LTI Transfection Reagent (Thermo Fisher Scientific). All other transfections were performed using the calcium-phosphate method.[28]

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.[21,23] 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.[16,21–23,27] 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 4×10^6 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.[16,22,27] Fluorescence microscopy was performed with an Axioskop2 mot *plus* microscope (Carl Zeiss Meditec, Oberkochen, Germany) at $40\times$ 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.[22] The values obtained at 10^{-3} M ouabain, representing unspecific (not Na/K-ATPase-catalyzed) adenosine triphosphate (ATP) cleavage,[29] 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 (^{3}H)-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: 8.5×10^5 cells were seeded in 24-well plates coated with poly-L-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

Retinae from retinoschisin-deficient mice at postnatal day 18 were dissected and cultivated on filters as described previously.[27,30] 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.
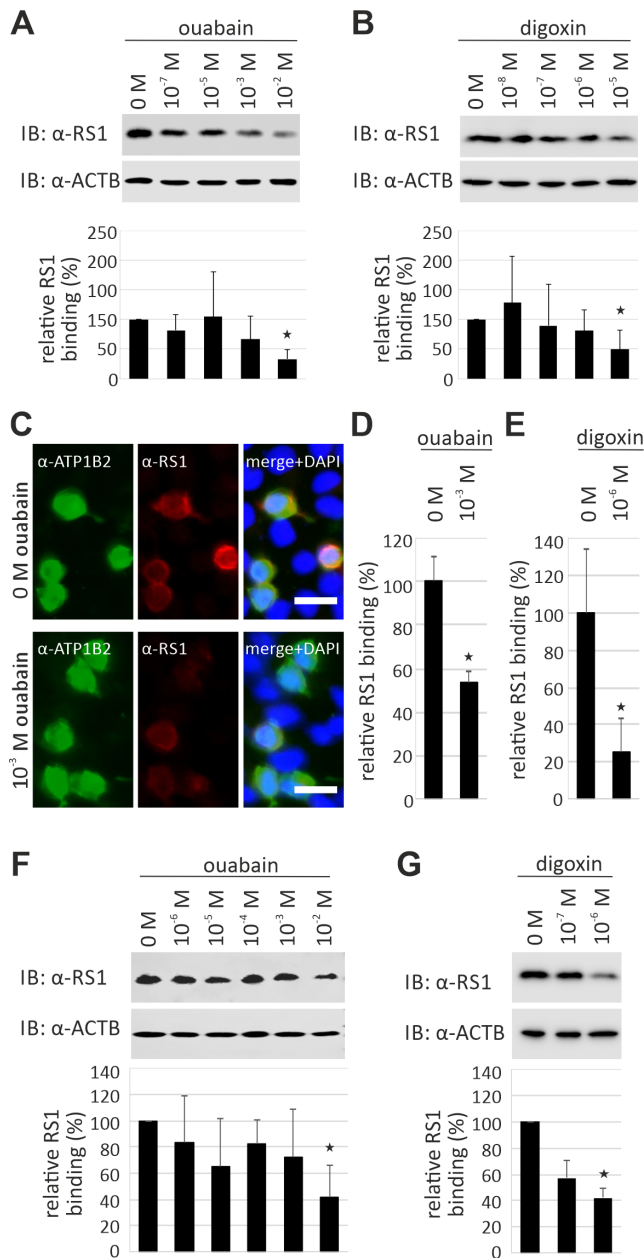
FIGURE 1. Effect of cardiac glycosides on retinoschisin binding to the retinal Na/K-ATPase. (**A, B**) Hek293 cells were transfected with expression constructs for ATP1A3 and ATP1B2. After 48 hours, they were subjected to recombinant retinoschisin for 1 hour in the presence of 0 (control), 10^{-7}, 10^{-5}, 10^{-3}, or 10^{-2} M ouabain (**A**) or 0 (control), 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} 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). (**C–E**) Hek293 cells were transfected with expression constructs for ATP1A3 and ATP1B2. After 48 hours, they were subjected to recombinant retinoschisin for 2 hours in the presence of 0 M (control) or 10^{-3} M ouabain (**C**) or in the presence of 0 M (control) or 10^{-6} M digoxin, respectively (see Supplementary Fig. S1A), followed by intensive washing. Subsequently, retinoschisin binding was analyzed via immunocytochemistry with

Immunohistochemical Analyses of Retinal Explants

Cryosectioning and immunolabeling of retinal explants were performed as described previously.[16] 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-11684795910, 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,[16,21–23] 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^{-7}, 10^{-5}, 10^{-3}, or 10^{-2} 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}, 10^{-5}, 10^{-4}, 10^{-3}, or 10^{-2} M ouabain (**F**) or 0 (control), 10^{-7}, or 10^{-6} 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; Kruskall–Wallis test followed by Dunn's multiple comparison test and Bonferroni correction).
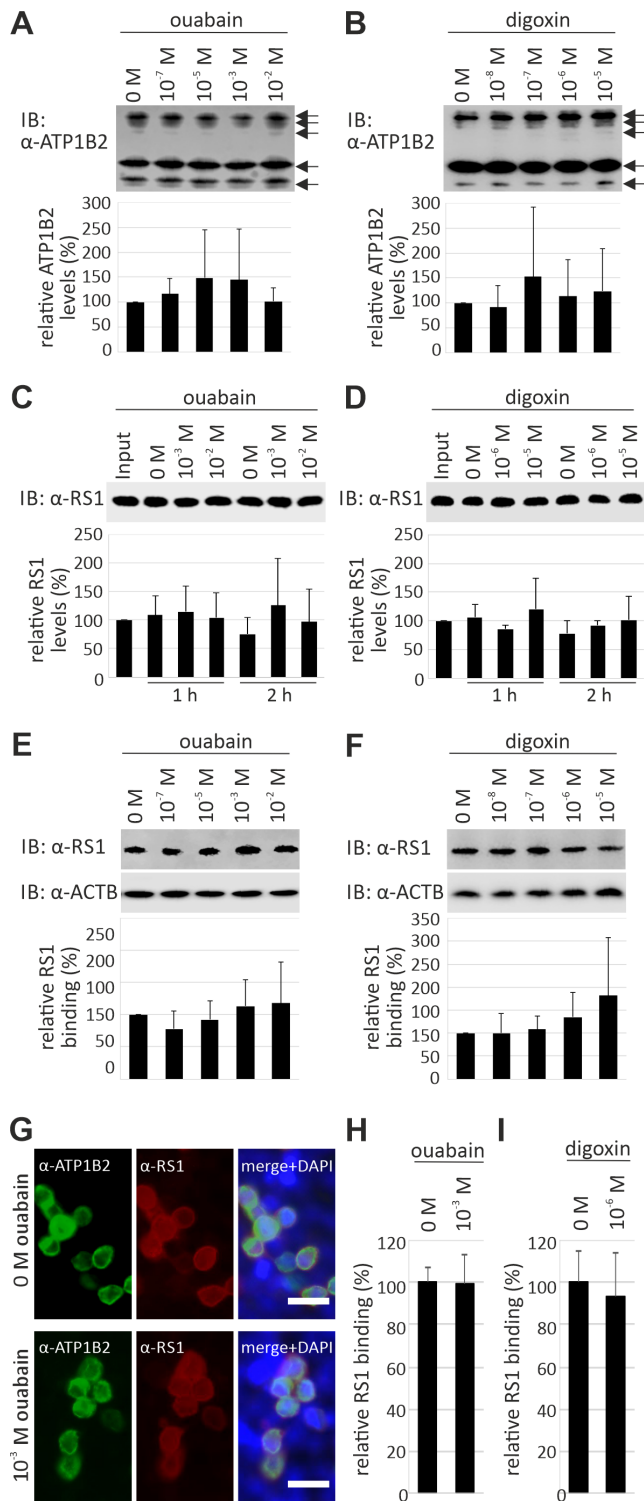
FIGURE 2. Effect of cardiac glycosides on ATP1B2 expression, retinoschisin levels in the supernatant containing recombinant retinoschisin (input), and retinoschisin binding to ouabain-insensitive Na/K-ATPases. (**A, B**) ATP1B2 levels in the Hek293 cells used in retinoschisin binding experiments before (Figs. 1A, 1B) were analyzed by western blot with antibodies against ATP1B2. As described previously,[22] anti-ATP1B2 staining revealed several protein bands with molecular weights between 25 and 55 kDa, reflecting differently *N*-glycosylated ATP1B2 subforms. 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

digoxin showed a much stronger effect than ouabain (reduction to $33.0 \pm 16.3\%$ at 10^{-2} M ouabain and to $46.9 \pm 21.0\%$ at 10^{-5} 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 $53.9 \pm 5.1\%$ and $24.8 \pm 19.1\%$, 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.[23] 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 ($41.3 \pm 43.6\%$ at 10^{-2} M ouabain and $42.4 \pm 7.2\%$ 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.[22] 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 in Fig. 2C; 0,

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^{-6} 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.
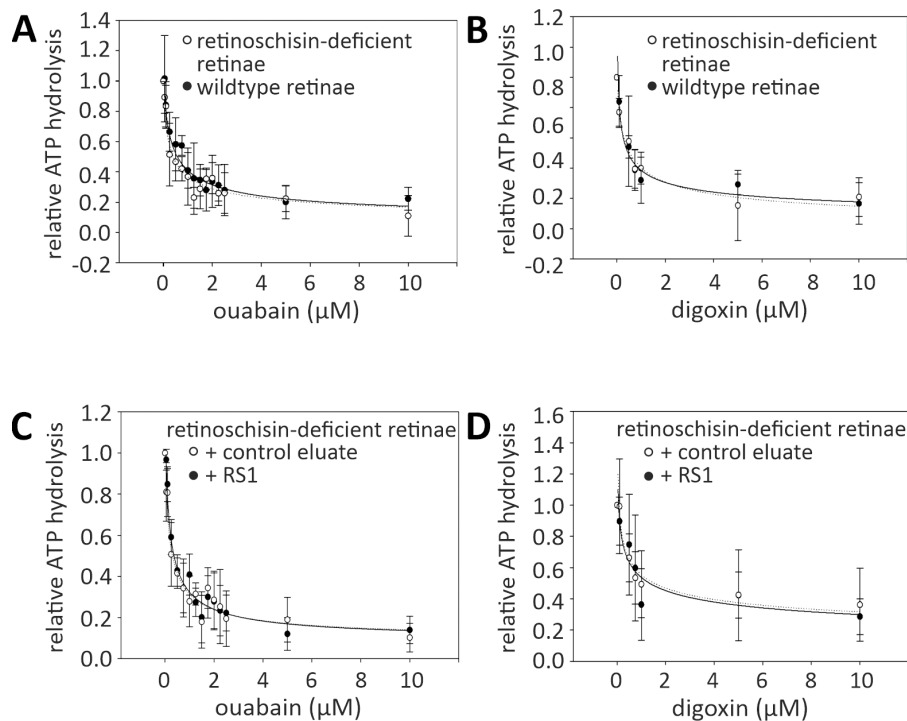
FIGURE 3. Effect of retinoschisin on inhibition of Na/K-ATPase pump activity by cardiac glycosides. Na/K-ATPase catalyzed ATP hydrolysis in murine retinal membrane fractions from (**A, B**) retinoschisin-deficient (open circles) and wild-type mice (closed circles) or from (**C, D**) retinoschisin-deficient mice pre-incubated for 30 minutes with recombinant retinoschisin (1 µg/mL, closed circles) or control eluate (open circles). Assays were performed in the presence of 0, 5×10^{-8}, 10^{-7}, 2.5×10^{-7}, 5×10^{-7}, 7.5×10^{-7}, 10^{-6}, 1.25×10^{-6}, 1.5×10^{-6}, 1.75×10^{-6}, 2×10^{-6}, 2.25×10^{-6}, 2.5×10^{-6}, 5×10^{-6}, and 10^{-5} M ouabain (**A, C**) or in the presence of 0, 10^{-7}, 5×10^{-7}, 7.5×10^{-7}, 10^{-6}, 5×10^{-6}, and 10^{-5} M digoxin (**B, D**). Data represent the mean ± SD of six (**A, B**) or five (**C, D**) biological replicates, calibrated against values obtained for 0 M ouabain.

10^{-6}, or 10^{-5}, M digoxin in Fig. 2D) and retinoschisin, as described before (Fig. 1). After incubation (1 hour for western blot or 2 hours for immunocytochemical analyses), retinoschisin levels in the input were unaffected by the presence of the cardiac glycosides (Figs. 2C, 2D). Finally, we investigated retinoschisin binding to Hek293 cells heterologously expressing ATP1B2 and a ouabain-insensitive ATP1A3 (ATP1A3-OI) mutant. Due to two point mutations at the ouabain binding site (Q108R and N119D), ATP1A3-OI remains fully functional but can no longer bind ouabain.[22,31] It should also fail to bind digoxin, as crystal structures exhibited similar interactions between the steroid cores of ouabain and digoxin and the transmembrane helices of the α subunit.[32] Hek293 cells heterologously expressing ATP1A3-OI and ATP1B2 were subjected to the retinoschisin binding assays, applying the same treatment conditions as before (Figs. 1A–1E). Neither ouabain nor digoxin had an effect on the binding of retinoschisin to the transfected Hek293 cells (Figs. 2E–2I, Supplementary Fig. S1B), in contrast with the results obtained for Hek293 cells transfected with normal ATP1A3 and ATP1B2 (Figs. 1A–1E).

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[22,29] 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.
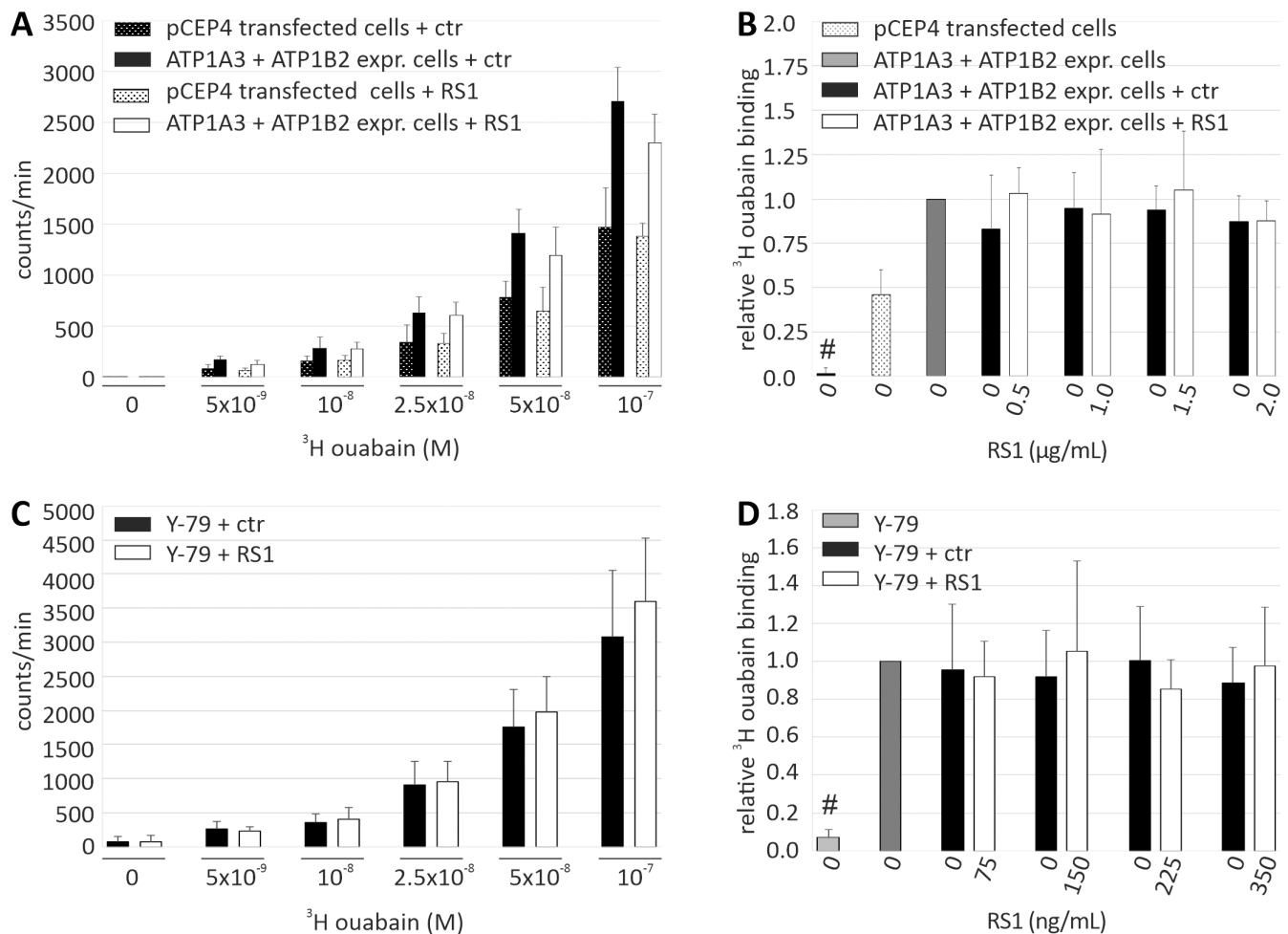
FIGURE 4. Effect of retinoschisin on ouabain binding. (**A, B**) Hek293 cells were transfected with pCEP4 or with bicistronic expression constructs for ATP1A3 and ATP1B2. (**A**) After 48 hours, cells were subjected to 1.5 µg/mL recombinant retinoschisin (RS1) or the same volume of control eluate (ctr) 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 ^{3}H-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 ^{3}H-labeled ouabain for 30 minutes (#, without ^{3}H-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 ^{3}H-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 ^{3}H-labeled ouabain and 30 minutes of incubation (#, without ^{3}H-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 ^{3}H-labeled ouabain but without retinoschisin or control eluate (gray bar).

Binding of ^{3}H-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 ^{3}H-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,[16,21,22] 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.[16,21,22]

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-
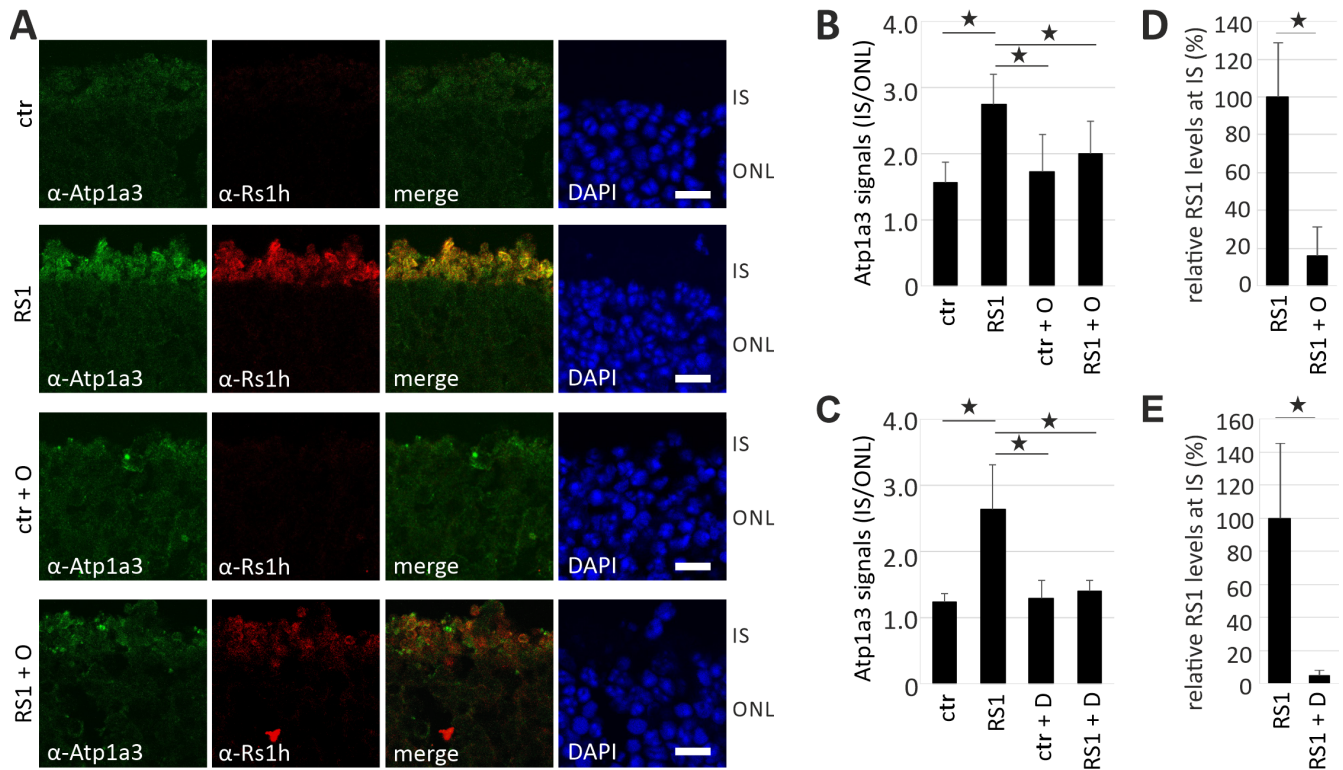
FIGURE 5. Effect of ouabain and retinoschisin on Na/K-ATPase localization. (**A**) Retinoschisin-deficient retinae explanted at postnatal day 18 were cultured for 4 days in medium containing retinoschisin (RS1) or the same volume of control eluate (ctr) in the presence of 0 or 10^{-5} M ouabain (O) or 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 $\pm$ 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 $\pm$ 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).

treated explants), indicating that ouabain alone had no effect on retinal Na/K-ATPase localization. When explants were treated with retinoschisin and 10^{-5} M ouabain, the effect of retinoschisin on retinal Na/K-ATPase enrichment was significantly reduced, with an r(IS/ONL) of 2.00 $\pm$ 0.47 ($P < 0.05$ compared with explants treated only with retinoschisin) (Figs. 5A, 5B). Similar results were obtained with digoxin (10^{-6} M) (Fig. 5C, Supplementary Fig. S3). In controls or explants treated with digoxin or digoxin plus retinoschisin, enrichment of the retinal Na/K-ATPase at the inner segments was significantly reduced to r(IS/ONL) values of around 1.4, compared with explants treated only with retinoschisin, which had an r(IS/ONL) of 2.64 $\pm$ 0.67 ($P > 0.05$).

Consistent with the previous results for Hek293 and Y-79 cells (Fig. 1), ouabain and digoxin treatment also decreased retinoschisin binding to retinoschisin-deficient explants to 16.1 $\pm$ 15.4% or to 4.6 $\pm$ 3.5% (Figs. 5D, 5E), respectively, compared with only retinoschisin-treated explants ($P < 0.01$).

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 retinae 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 $\pm$ 5.3%) than did explants treated with the control eluate (23.3 $\pm$ 19.9% apoptotic nuclei) (Figs. 6 A, 6B). In explants treated with 10^{-5} M ouabain, the number of apoptotic nuclei increased to around 54.3 $\pm$ 31.1% (Figs. 6A, 6B). In explants subjected to retinoschisin and 10^{-5} M ouabain, 40.3 $\pm$ 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 $\pm$ 16.7% apoptotic nuclei in retinoschisin treated explants compared with 24.0 $\pm$ 19.9% in control explants), treatment with 10^{-6} M digoxin strongly increased apoptosis (55.4 $\pm$ 27.6% apoptotic nuclei; $P < 0.05$ compared with retinoschisin-treated explants).
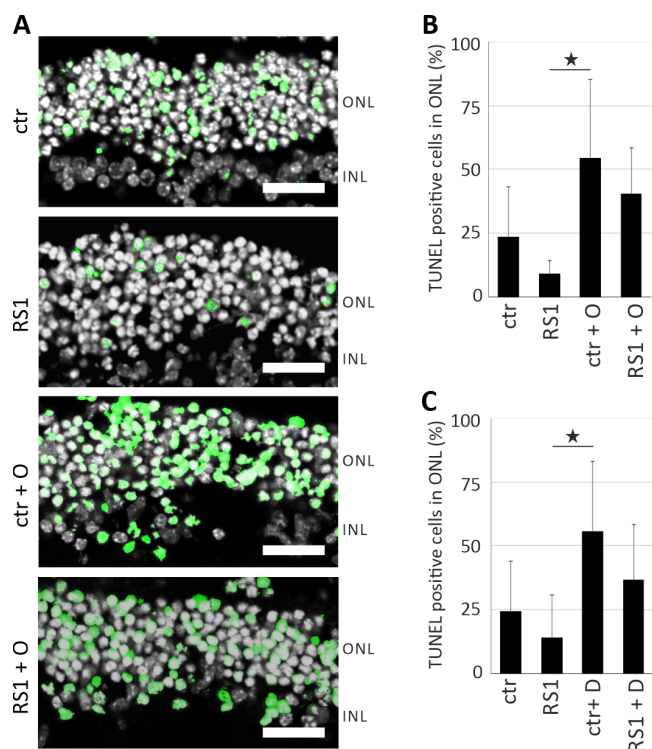
FIGURE 6. Effect of ouabain and retinoschisin on photoreceptor degeneration. (**A**) Retinoschisin-deficient retinae explanted at postnatal day 18 were cultivated for 4 days in medium containing retinoschisin (RS1) or control eluate (ctr) in the presence of 0 or 10^{-5} M ouabain (O), as well as in the presence of 0 or 10^{-6} M digoxin (D) (see Supplementary Fig. S4). After washing and embedding, cryosections of the explants were subjected to TUNEL staining to visualize apoptotic nuclei (green), as well as to DAPI staining to visualize all nuclei (gray). *Scale bars*: 25 μm). INL, inner nuclear layer. (**B**, **C**) After TUNEL staining, TUNEL-positive nuclei and all nuclei were counted in each cryosection with the Cell Counter plugin for ImageJ, and the percentage of TUNEL-positive nuclei was calculated. 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 compared with the control (*P < 0.05; ANOVA test followed by Tukey's multiple comparison test (**B**) and Kruskall-Wallis test followed by Dunn's multiple comparison test and Bonferroni correction (**C**)).

Explants cultivated in the presence of retinoschisin and digoxin exhibited 36.4 ± 22.0% apoptotic nuclei.

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.

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.[22] 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 murine 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,[32,35] whereas retinoschisin binds to the extracellular domain of the β subunit.[21] 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.[36] This conformational change affects the entire Na/K-ATPase, including distances between the α and β subunits.[37] Ouabain binding induces the conformational switch from E1 to E2[38] and stabilizes the E2 conformation.[39] 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).[32] 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).[27] 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.[32] 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 the survival of outer retinal cells,[33,34] but the effect of cardiac glycosides appears to be concentration dependent. Whereas low levels (10^{-11} to 10^{-13} M) of cardiac glycosides were reported to stimulate retinal explant growth,[40] higher concentrations significantly disrupted retinal integrity and functionality.[41–44] Furthermore, treatment of heart diseases with cardiac glycosides can have detrimental repercussions on the patients' vision.[10–12] This evidence suggests that a well-balanced interplay between endogenous cardiac

glycosides and retinoschisin is crucial for proper photoreceptor differentiation and homeostasis.

We have shown that cardiac glycosides displace retinoschisin from the retinal Na/K-ATPase, affecting the capacity of retinoschisin to regulate proper Na/K-ATPase localization and support photoreceptor survival. This observation suggests opposing roles of cardiac glycosides and retinoschisin on photoreceptor development and homeostasis and provides an explanation for the pathological side effects of cardiac glycoside treatment on retinal integrity.

References

1. Kaplan JH. Biochemistry of Na,K-ATPase. *Annu Rev Biochem*. 2002;71:511–535.

2. Aperia A, Akkuratov EE, Fontana JM, Brismar H. Na+-K+-ATPase, a new class of plasma membrane receptors. *Am J Physiol Cell Physiol*. 2016;310:C491–C495.

3. Krupinski T, Beitel GJ. Unexpected roles of the Na-K-ATPase and other ion transporters in cell junctions and tubulogenesis. *Physiology (Bethesda)*. 2009;24:192–201.

4. Wetzel RK, Arystarkhova E, Sweadner KJ. Cellular and subcellular specification of Na,K-ATPase alpha and beta isoforms in the postnatal development of mouse retina. *J Neurosci*. 1999;19:9878–9889.

5. Blanco G, Mercer RW. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol*. 1998;275:F633–F650.

6. Schneider BG, Kraig E. Na+, K(+)-ATPase of the photoreceptor: selective expression of alpha 3 and beta 2 isoforms. *Exp Eye Res*. 1990;51:553–564.

7. Therien AG, Blostein R. Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol*. 2000;279:C541–C566.

8. Lissner W, Greenlee JE, Cameron JD, Goren SB. Localization of tritiated digoxin in the rat eye. *Am J Ophthalmol*. 1971;72:608–614.

9. Binnion PF, Frazer G. [3H]Digoxin in the optic tract in digoxin intoxication. *J Cardiovasc Pharmacol*. 1980;2:699–706.

10. Ritz S, Harding P, Martz W, Schutz HW, Kaatsch HJ. Measurement of digitalis-glycoside levels in ocular tissues: a way to improve postmortem diagnosis of lethal digitalis-glycoside poisoning? I. Digoxin. *Int J Legal Med*. 1992;105:149–154.

11. Fraunfelder FW, Fraunfelder FT, Chambers WA. *Drug-Induced Ocular Side Effects*. 7th ed. Philadelphia, PA: Saunders; 2014.

12. Duncker G. [Ocular side effects of digitalis (author's transl)]. *Klin Monbl Augenheilkd*. 1981;178:397–398.

13. Haustein KO. Digitalis. *Pharmacol Ther*. 1982;18:1–89.

14. Lawrenson JG, Kelly C, Lawrenson AL, Birch J. Acquired colour vision deficiency in patients receiving digoxin maintenance therapy. *Br J Ophthalmol*. 2002;86:1259–1261.

15. Molday LL, Hicks D, Sauer CG, Weber BH, Molday RS. Expression of X-linked retinoschisis protein RS1 in photoreceptor and bipolar cells. *Invest Ophthalmol Vis Sci*. 2001;42:816–825.

16. Friedrich U, Stohr H, Hilfinger D, et al. The Na/K-ATPase is obligatory for membrane anchorage of retinoschisin, the protein involved in the pathogenesis of X-linked juvenile retinoschisis. *Hum Mol Genet*. 2011;20:1132–1142.

17. Molday LL, Wu WW, Molday RS. Retinoschisin (RS1), the protein encoded by the X-linked retinoschisis gene, is anchored to the surface of retinal photoreceptor and bipolar cells through its interactions with a Na/K ATPase-SARM1 complex. *J Biol Chem*. 2007;282:32792–32801.

18. George ND, Yates JR, Bradshaw K, Moore AT. Infantile presentation of X linked retinoschisis. *Br J Ophthalmol*. 1995;79:653–657.

19. Sauer CG, Gehrig A, Warneke-Wittstock R, et al. Positional cloning of the gene associated with X-linked juvenile retinoschisis. *Nat Genet*. 1997;17:164–170.

20. Sikkink SK, Biswas S, Parry NR, Stanga PE, Trump D. X-linked retinoschisis: an update. *J Med Genet*. 2007;44:225–232.

21. Plössl K, Schmid V, Straub K, et al. Pathomechanism of mutated and secreted retinoschisin in X-linked juvenile retinoschisis. *Exp Eye Res*. 2018;177:23–34.

22. Plössl K, Royer M, Bernklau S, et al. Retinoschisin is linked to retinal Na/K-ATPase signaling and localization. *Mol Biol Cell*. 2017;28:2178–2189.

23. Plössl K, Weber BH, Friedrich U. The X-linked juvenile retinoschisis protein retinoschisin is a novel regulator of mitogen-activated protein kinase signalling and apoptosis in the retina. *J Cell Mol Med*. 2017;21:768–780.

24. Schoner W, Scheiner-Bobis G. Endogenous and exogenous cardiac glycosides and their mechanisms of action. *Am J Cardiovasc Drugs*. 2007;7:173–189.

25. Fuerstenwerth H. On the differences between ouabain and digitalis glycosides. *Am J Ther*. 2014;21:35–42.

26. Weber BH, Schrewe H, Molday LL, et al. Inactivation of the murine X-linked juvenile retinoschisis gene, Rs1h, suggests a role of retinoschisin in retinal cell layer organization and synaptic structure. *Proc Natl Acad Sci USA*. 2002;99:6222–6227.

27. Plössl K, Straub K, Schmid V, et al. Identification of the retinoschisin-binding site on the retinal Na/K-ATPase. *PLoS One*. 2019;14:e0216320.

28. Kingston RE, Chen CA, Rose JK. Calcium phosphate transfection. *Curr Protoc Mol Biol*. 2003;Chapter 9:Unit 9.1.

29. Jones DH, Li TY, Arystarkhova E, et al. Na,K-ATPase from mice lacking the gamma subunit (FXYD2) exhibits altered Na+ affinity and decreased thermal stability. *J Biol Chem*. 2005;280:19003–19011.

30. Hsiau TH, Diaconu C, Myers CA, Lee J, Cepko CL, Corbo JC. The *cis*-regulatory logic of the mammalian photoreceptor transcriptional network. *PLoS One*. 2007;2:e643.

31. Price EM, Lingrel JB. Structure-function relationships in the Na,K-ATPase alpha subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry*. 1988;27:8400–8408.

32. Laursen M, Yatime L, Nissen P, Fedosova NU. Crystal structure of the high-affinity Na+K+-ATPase-ouabain complex with Mg2+ bound in the cation binding site. *Proc Natl Acad Sci USA*. 2013;110:10958–10963.

33. Gehrig A, Janssen A, Horling F, Grimm C, Weber BH. The role of caspases in photoreceptor cell death of

the retinoschisin-deficient mouse. *Cytogenet Genome Res.* 2006;115:35–44.

34. Gehrig A, Langmann T, Horling F, et al. Genome-wide expression profiling of the retinoschisin-deficient retina in early postnatal mouse development. *Invest Ophthalmol Vis Sci.* 2007;48:891–900.

35. Yatime L, Laursen M, Morth JP, Esmann M, Nissen P, Fedosova NU. Structural insights into the high affinity binding of cardiotonic steroids to the Na+,K+-ATPase. *J Struct Biol.* 2011;174:296–306.

36. Orlov SN, Klimanova EA, Tverskoi AM, Vladychenskaya EA, Smolyaninova LV, Lopina OD. $Na^+{}_i,K^+{}_i$-dependent and -independent signaling triggered by cardiotonic steroids: facts and artifacts. *Molecules.* 2017;22:635–661.

37. Sanchez-Rodriguez JE, Khalili-Araghi F, Miranda P, Roux B, Holmgren M, Bezanilla F. A structural rearrangement of the Na+/K+-ATPase traps ouabain within the external ion permeation pathway. *J Mol Biol.* 2015;427:1335–1344.

38. Silva E, Soares-da-Silva P. New insights into the regulation of Na+,K+-ATPase by ouabain. *Int Rev Cell Mol Biol.* 2012;294:99–132.

39. Geibel S, Zimmermann D, Zifarelli G, et al. Conformational dynamics of Na+/K+- and H+/K+-ATPase probed by voltage clamp fluorometry. *Ann N Y Acad Sci.* 2003;986:31–38.

40. Lopatina EV, Karetsky AV, Penniyaynen VA, Vinogradova TV. Role of cardiac glycosides in regulation of the growth of retinal tissue explants. *Bull Exp Biol Med.* 2008;146:744–746.

41. de Rezende Correa G, Soares VH, de Araujo-Martins L, Dos Santos AA, Giestal-de-Araujo E. Ouabain and BDNF crosstalk on ganglion cell survival in mixed retinal cell cultures. *Cell Mol Neurobiol.* 2015;35:651–660.

42. Hinshaw SJ, Ogbeifun O, Wandu WS, et al. Digoxin inhibits induction of experimental autoimmune uveitis in mice, but causes severe retinal degeneration. *Invest Ophthalmol Vis Sci.* 2016;57:1441–1447.

43. Kinoshita J, Iwata N, Kimotsuki T, Yasuda M. Digoxin-induced reversible dysfunction of the cone photoreceptors in monkeys. *Invest Ophthalmol Vis Sci.* 2014;55:881–892.

44. Madreperla SA, Johnson MA, Nakatani K. Electrophysiologic and electroretinographic evidence for photoreceptor dysfunction as a toxic effect of digoxin. *Arch Ophthalmol.* 1994;112:807–812.