Retinal Stimulates ATP Hydrolysis by Purified and Reconstituted ABCR, the Photoreceptor-specific ATP-binding Cassette Transporter Responsible for Stargardt Disease*

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Many substrates for P-glycoprotein, an ABC transporter that mediates multidrug resistance in mammalian cells, have been shown to stimulate its ATPase activity in vitro. In the present study, we used this property as a criterion to search for natural and artificial substrates and/or allosteric regulators of ABCR, the rod photoreceptor-specific ABC transporter responsible for Stargardt disease, an early onset macular degeneration. ABCR was immunoaffinity purified to apparent homogeneity from bovine rod outer segments and reconstituted into liposomes. All-trans-retinal, a candidate ligand, stimulates the ATPase activity of ABCR 3–4-fold, with a half-maximal effect at 10–15 μM. 11-cis- and 13-cis-retinal show similar activity. All-trans-retinal stimulates the ATPase activity of ABCR with Michaelis-Menten behavior indicative of simple noncooperative binding that is associated with a rate-limiting enzyme-substrate intermediate in the pathway of ATP hydrolysis. Among 37 structurally diverse non-retinoid compounds, including nine previously characterized substrates or sensitizers of P-glycoprotein, only four show significant ATPase stimulation when tested at 20 μM. The dose-response curves of these four compounds are indicative of multiple binding sites and/or modes of interaction with ABCR. Two of these compounds, amiodarone and digitonin, can act synergistically with all-trans-retinal, implying that they interact with a site or sites on ABCR different from the one with which all-trans-retinal interacts. Unlike retinal, amiodarone appears to interact with both free and ATP-bound ABCR. Together with clinical observations on Stargardt disease and the localization of ABCR to rod outer segment disc membranes, these data suggest that retinoids, and most likely retinal, are the natural substrates for transport by ABCR in rod outer segments. These observations have significant implications for understanding the visual cycle and the pathogenesis of Stargardt disease and for the identification of compounds that could modify the natural history of Stargardt disease or other retinopathies associated with impaired ABCR function.

Stargardt disease (Mendelian Inheritance in Man number 248200; also called fundus flavimaculatus) is an autosomal recessive disorder that affects approximately one person in 10,000 (1). Affected individuals experience central vision loss and a progressive bilateral atrophy of the macular region of the retina and retinal pigment epithelium (RPE).† A distinctive feature of Stargardt disease is the accumulation of fluorescent, shortwave absorbing material in the RPE, which appears in postmortem samples as lipofuscin-like deposits (2–4). These clinical and histopathologic features resemble those seen in the more common age-related macular degeneration, suggesting that investigation of the pathophysiology of Stargardt disease may reveal mechanisms relevant to age-related macular degeneration (5–8).

The gene responsible for Stargardt disease codes for a member of the ABC transporter family, ABCR (9–11). The spectrum of mutations identified thus far in Stargardt disease patients suggests that these individuals carry at least one partially functional allele. By contrast, some cases of autosomal recessive retinitis pigmentosa, a disease characterized by a progressive degeneration of the rod-rich peripheral retina, are associated with homozygosity of frameshift or splice site mutations in the ABCR gene which are likely to result in complete loss of ABCR function (12, 13). An association between a subset of subjects with age-related macular degeneration and variant alleles of the ABCR gene has been reported (14), but interpretation of the data has been controversial (15).

Expression of the ABCR gene is confined exclusively to retinal rod photoreceptors, and the ABCR protein is localized to the rim and incisures of rod outer segment discs, where it is present at a molar ratio of approximately 1:120 with rhodopsin (16–19). This highly specific localization suggests that ABCR may transport a molecule or ion that plays a specialized role in photoreceptor homeostasis. Although small quantities of ABCR may also be present in the rod outer segment plasma membrane, the localization of the majority of ABCR to the internal disc membranes suggests that the relevant transport event is between the luminal and cytosolic faces of the disc membrane. Because outer segment turnover occurs via RPE phagocytosis, a derangement in this transport mechanism (with the resulting accumulation of one or more compounds within the outer segment) could plausibly explain the progressive accumulation of material within the RPE in Stargardt disease.

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‡‡‡ The abbreviations used are: RPE, retinal pigment epithelium; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ROS, rod outer segments.

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From the preceding discussion it is clear that identifying the molecule or ion transported by ABCR is a prerequisite to understanding the normal role of ABCR and the pathogenesis of Stargardt disease. To date, substrates have been identified for only a few of the over 30 known mammalian ABC transporters. In these few cases, critical information regarding the identity of the substrate(s) has often come from genetic studies. For example, the defect in chloride resorption in humans with cystic fibrosis provided the first indication that CFTR mediates chloride transport (20), and mutations in the human canalicular organic anion transporter, SPGP, have been found in a subset of patients with progressive familial intrahepatic cholestasis, an inherited disorder of hepatobiliary excretion (21). For P-glycoprotein and its close relatives, overexpression of the corresponding genes or cloned cDNAs in a variety of mammalian cells was found to confer resistance to chemotherapeutic agents, and targeted disruption of these genes in the mouse shows that MDR1A is responsible for extrusion of hydrophobic compounds across the blood-brain barrier (22).

For a number of transporters, including CFTR, SPGP, and P-glycoprotein, these observations have been extended by experiments in vitro. Purified and reconstituted CFTR shows chloride channel activity (23), purified and reconstituted P-glycoprotein shows substrate-dependent ATP hydrolysis (24–27) and ATP-dependent vectorial transport of substrates (28, 29), and partially purified recombinant SPGP mediates taurocholate transport and taurocholate-dependent ATP hydrolysis (30). In the in vitro experiments with P-glycoprotein, ATP hydrolysis is stimulated to different extents by compounds that are extruded by this protein in living cells. A second class of compounds, called chemosensitizers, increases the sensitivity of cells to chemotherapeutic agents and also stimulates ATPase activity in vitro, possibly by uncoupling ATP hydrolysis and substrate extrusion. This work suggests that it may be possible to identify substrates and allosteric regulators of other ABC transporters by searching for compounds that stimulate the ATPase activity of the purified and reconstituted transporters.

To characterize the biochemical properties of ABCR with the goal of identifying its substrate, we report here a procedure for solubilizing and functionally reconstituting the purified protein into lipid membranes. Several compounds, including amiodarone, digitonin, and several geometric isomers of retinal, produce a significant increase in ATPase activity of this preparation. Interestingly, amiodarone and digitonin greatly enhance the stimulatory activity of all-trans-retinal but not of each other, suggesting that they act by a similar mechanism which differs from the mechanism by which all-trans-retinal acts. These data, together with the clinical and histological data summarized above, suggest a model in which ABCR is involved in the recycling of retinoids that attends photoactivation of rhodopsin (the visual cycle).

MATERIALS AND METHODS

Reagents—Reagents and their sources are as follows: Extracti-Gel detergent removal resin (Pierce); CNBr-activated Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech); dark adapted bovine retinas (Schenk Packing Co., Stanwood, WA); (R)-(+)-1-octyl-3-ol, 5-methoxy-1,3,3-trimethylisopiperindle-2,3-(3H)naphtho[2,1-b](pyran), metergoline, Meldola’s blue, lasalocid, (±)-isoseroxepine, hydroquinine 4-methyl-2-quinolyl ether, (±)-hexochinacetate, coumarin 334, coynanthine hydrochloride hydrate, 2-tert-butylanthraquinone, Brilliant Cyanine MOO, and Brilliant Green (Aldrich); digitoxin, digoxigenin, and digoxin (Fluka); activated charcoal, caprylic acid, crude sheep brain PE, all-trans-retinol, all-trans-retinol, all-trans-retinoic acid, β-ionone, actinomycin D, amiodarone, colchicine, nifedipine, trifluoperazine, verapamil, vinblastine, vincristine, CHAPS, cholic acid, quercetin, progesterone, ethidium bromide, and dehydroabietinylamine acetate (Sigma); bromophenol blue and bromocresol green (Bio-Rad); digitonin (Eastman Kodak Co.); β-octyl glucose and n-dodecyl-β-maltoside (Calbiochem); brain polar lipids and egg PC (Avanti Polar Lipids); [γ-32P]ATP (NEN Life Science Products). 11-cis-retinal was a gift of Dr. Lubert Stryer. Lipid sonication was performed using the G12SP1 Specific Ultrasonic Cleaner from Laboratory Supplies Co.

Preparation of ROS—Bovine ROS were prepared as described adapted and frozen bovine retinas under dim red light as described (31). Aliquots were stored at −80 °C in the dark.

Conjugation of Rim 3F4 Antibody to Sepharose Beads—Monoclonal antibody Rim 3F4, which recognizes a linear epitope near the C terminus of bovine ABCR (16), was purified from ascites fluid by caprylic acid precipitation (32) and conjugated to CNBr-activated Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech) at a ratio of 1 mg of antibody per ml of resin. The resin was discarded after each use to improve reproducibility in the purification of ABCR.

Purification of ABCR from Bovine ROS—All purification procedures prior to the elution step were performed under red dim light at 4 °C to minimize aggregation of photobleached rhodopsin. Purified ROS from 4–8 bovine retinas were diluted into greater than 10 volumes 1× phosphate-buffered saline, pelleted, and solubilized in 1.4 ml of ROS/CHAPS buffer (10% glycerol, 0.75% CHAPS, 50 mM HEPES, pH 7.0, 0.5 mg/ml crude brain PE, and 0.5 mg/ml egg PC, 140 mM NaCl, 3 mM MgCl2, 5 mM β-mercaptoethanol). The sample was rotated at 4 °C in the dark for 1 h to completely solubilize the ROS membranes. Insoluble material was removed by microcentrifugation at 16,000 rpm for 10 min, and the supernatant was added to 200 μl of Rim 3F4-Sepharose and rotated in the dark at 4 °C for 3 h. The resin was recovered by microcentrifugation at 3000 rpm and washed three times for 1 min and then twice for 20 min (with gentle rotation) with 1 ml of ice-cold ROS/CHAPS buffer. Three successive elutions were performed at 4 °C using 80μl each of 0.2 mg/ml of a peptide (H2N-NEFYDLPLHPTAG-COOH) containing the Rim 3F4 epitope (16) in ROS/CHAPS buffer for a total of 45 min. An irrelevant peptide, JN50 (H2N-MLRNNL-GNSSDC-CONH2), was used as a control for the specificity of elution.

Reconstitution of ABCR into Proteoliposomes—240 μl of immunoaffinity purified ABCR in ROS/CHAPS buffer was added to a premixed solution containing 30 μl of 15% octyl glucoside and 90 μl 50 mg/ml sonicated lipid in 25 mM HEPES, 140 mM NaCl, 10% glycerol. The sample was mixed, incubated on ice for 30 min, and then rapidly diluted into 2 ml of reconstitution buffer (25 mM HEPES, pH 7.0, 140 mM NaCl, 1 mM EDTA, 10% glycerol) at room temperature and incubated at room temperature for an additional 2 min. To remove detergent, the sample was passed (at room temperature) over a 2-ml Extracti-Gel (Pierce) column that had been thoroughly prewashed with the reconstitution buffer. Light microscopic examination of the reconstituted preparation stained with 1,1'-dioctadecyl-3,3',3'-tetraethyldicycyanocarbonylamine shows a heterogeneous distribution of vesicle sizes. Unless noted otherwise all reconstitution experiments described here were performed with swine brain polar lipids.

ATPase Assays—ATPase assays were performed using ABCR that had been purified from ROS and reconstituted into liposomes on the same day. ATPase assays were performed in 300 μl of reconstitution buffer supplemented with MgCl2 to a final concentration of 3 mM. Appropriate dilutions and/or mixtures of test compounds were prepared in ethanol and were added to 0.1% of the volume of the ATPase reaction; control reactions received the same volume of ethanol alone. The reactions were started by the addition of 30 μl of 10× ATP mix containing 50 μM β-mercaptoethanol, 500 μM ATP with 50 μCi of 3000 Ci/mmol of [γ-32P]ATP, and incubated at 37 °C for 2 h. For experiments in which the ATP concentration varied, the [γ-32P]ATP was held constant at 5 μCi/ml. At the starting and final time points, triplicate samples of 50 μl were added to 200 μl of 10% activated charcoal in 10 mM HCl, vortexed vigorously for 2 min, and microcentrifuged to pellet the charcoal. 50 μl of the resulting supernatant was counted. For experiments to differentiate the effects of retinal isomers, the ATPase assay was manipulated under dim red light and incubated in the dark. All other ATPase assays are performed in room light. Data points in Lineweaver-Burk plots were fitted to a straight line by a least squares criterion; no attempt was made to fit the data to more complex curves.

Handling and Storage of Retinoids—All procedures were performed under dim red light or in the dark. Retinal, retinol, and retinoic acid solutions were stored in ethanol, and each compound was monitored by determining an absorption spectrum. Retinoids were handled in glass vials to minimize absorption to plastic. For storage, aliquots were dried under argon and maintained in the dark at −80 °C. Oxidized all-trans-retinal was prepared by exposing a sample of all-trans-retinal in ethanol to air at 37 °C overnight in a sealed glass vial.

SDS-PAGE, Western Blots, and Determination of ABCR Concentra
acrylamide) and stained with Coomassie Blue. The ROS lane and probed with affinity purified rabbit anti-ABCR antibodies. These were performed as described (18). Western blots were derived contaminants in the final immunoaffinity purified ABCR polypeptide, with an apparent molecular mass of approximately 250 kDa, is eluted with the Rim 3F4 peptide but is excluded in brain lipid possesses considerable ATPase activity (Fig. 1B and see Fig. 7 below). We ascribe this activity to ABCR because it is far lower in the mock-eluted sample. In the experiments described below in which various compounds were used to stimulate ATPase activity, we observed variation in ATPase stimulation relative to the basal ATPase activity of up to 1.5-fold for identical experimental protocols carried out on different ABCR preparations. This variation may reflect differences between ABCR preparations in the level of minor contaminating ATPases, which would presumably contribute to the basal activity but not the stimulated activity, and/or differences in the absolute amount of lipid present in the sample which therefore changes the ratio of lipid to the added compounds, most of which are hydrophobic. To avoid normalizing and averaging data from different ABCR preparations, all of the data presented in the figures show the results of single representative experiments.

**Stimulation of ABCR ATPase Activity by Low Molecular Weight Compounds**—As one approach to identifying substrates or allosteric regulators of ABCR, we surveyed a group of structurally diverse compounds for their ability to stimulate or inhibit the ATPase activity of purified and reconstituted ABCR. Our earlier localization of ABCR to ROS disc membranes suggested the possibility that it might be involved in the movement of retinal or retinol (9, 16, 18), and therefore the collection includes a variety of retinoids. The structures of these compounds are shown in Fig. 2, and they fall into four groups as follows: retinoids (compounds 1–5), known P-glycoprotein substrates or sensitizers (compounds 6–14), detergents and digitonin-related (compounds 15–22), and compounds selected for their structural diversity (compounds 23–43). The compounds were initially tested at 20 μM (Fig. 3), a concentration at which many P-glycoprotein substrates and sensitizers enhance ATP hydrolysis by P-glycoprotein (24–27). Most of the compounds had little or no effect on ATPase activity, and none significantly inhibited ATPase activity. However, seven compounds, three retinal isomers (all-trans-retinal, 11-cis-retinal,
FIG. 2. Chemical structures of compounds tested for stimulation or inhibition of reconstituted ATPase activity. A, retinoids: 1, 11-cis-retinal; 2, 13-cis-retinal; 3, all-trans-retinal; 4, all-trans-retinoic acid; and 5, all-trans-retinol. B, P-glycoprotein substrates and sensitizers.

A Retinoids

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4

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B P-glycoprotein substrates and sensitizers

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C Detergents and digitonin related compounds

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D Other diverse compounds

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FIG. 2. Chemical structures of compounds tested for stimulation or inhibition of reconstituted ATPase activity. A, retinoids: 1, 11-cis-retinal; 2, 13-cis-retinal; 3, all-trans-retinal; 4, all-trans-retinoic acid; and 5, all-trans-retinol. B, P-glycoprotein substrates and sensitizers.
Fig. 3. Effect of various compounds at 20 μM on the ATPase activity of purified and reconstituted ABCR. Each panel includes a basal reaction and an all-trans-retinal-stimulated reaction, and ATPase activity is shown as a percent of basal activity. In this figure and in all subsequent figures, the basal ATPase is the ATPase level observed in the absence of activators for Rim 3F4-eluted and reconstituted ABCR or, for these panels showing Rim 3F4-eluted ABCR in ROS/CHAPS buffer, the basal ATPase is the level in ROS/CHAPS buffer. A, nine P-glycoprotein substrates and sensitizers. B, eight detergents and compounds related to digitonin and digoxigenin. C, 20 chemically diverse compounds. See Fig. 2 for chemical structures.

6, nifedipine; 7, actinomycin D (Sar, sarcosine; Meval, N-methylvaline); 8, vinblastine; 9, trifluoperazine; 10, vinceristine; 11, colchicine; 12, progesterone; 13, amiodarone; and 14, verapamil. C, detergents and digitonin-related compounds: 15, (−)-hecogenin acetate; 16, CHAPS; 17, cholic acid; 18, n-octyl-D-glucopyranoside; 19, digitonin; 20, digoxin; 21, digoxin; and 22, digoxigenin. D, other structurally diverse compounds: 23, ethidium bromide; 24, metergoline; 25, brilliant green; 26, hydroquinone 4-methyl-2-quinolyl ether; 27, brom cresol green; 28, 6-bromo-1′,3′,3′-trihydroxy-8-nitrrespiro[2H]benzopyran-2,2′,2′-indole; 29, Medola’s blue; 30, coumanin 334; 31, dehydroabietylamine acetate; 32, 2,6-bis-(2,2-dimethyl-1,3-cyclohexadiene-1-carboxylmethyloxy)-2,6-bis-(2,2-dimethyl-1,3-cyclohexadiene-1-carboxylmethyloxy)benzene; 33, iso-proterenol; 34, lasalocid; 35, (−)-isoreserpine; 36, brilliant crocein MOO; 37, beta-ionone; 38, bromphenol blue; 39, 1-octyn-3-ol; 40, hexanal; 41, cornyanthine; 42, 5-methoxy-1,3,3-trimethylspiro[indoline-2,3′-2H]naphtho[2,1-b]pyran]; and 43, xylene cyanol FF.
of the basal and all-trans-retinal-stimulated ATPase activity to azide or ouabain, and (d) the lack of stimulation of other membrane ATPases by all-trans-retinal, we believe that both the basal and all-trans-retinal-stimulated ATPase activities observed here are derived from ABCR and not from contaminating ATPases. In the text below we will assume that this is the case.

**Factors Affecting the All-trans-retinal-dependent Stimulation of ATP Hydrolysis**—The ATPase activity of purified and reconstituted ABCR in the presence or absence of all-trans-retinal exhibits a broad pH optimum, and the reaction continues linearly for at least 180 min at 37 °C (Fig. 6, A and B). The basal and all-trans-retinal-stimulated ATPase activities of ABCR are sensitive to lipid environment, a property shared with P-glycoprotein (40). Crude sheep brain PE, brain polar lipid, and *Escherichia coli* polar lipid support all-trans-retinal-stimulated ATPase, but egg PC and soybean PC do not (Fig. 6C and data not shown). In contrast to the reconstituted sample, purified ABCR solubilized in ROS/CHAPS buffer shows no stimulation by all-trans-retinal (data not shown), suggesting that the precise arrangement of protein-lipid contacts is a critical factor in the ability of retinal to activate the ATPase activity. The lack of all-trans-retinal stimulation may be related to the high basal V$_\text{max}$ observed for ABCR in a mixed CHAPS/lipid environment relative to that in lipid. A similar difference has been observed between detergent solubilized and reconstituted P-glycoprotein in ATPase stimulation by both substrates and sensitizers (26).

An analysis of the ATPase activity of ABCR in the presence of varying ATP concentration shows simple Michaelis-Menten behavior for ATP regardless of whether ABCR is solubilized in ROS/CHAPS buffer (Fig. 7, A and C) or reconstituted in brain lipid (Fig. 7, B and D). In ROS/CHAPS buffer, the $K_m$ for ATP is 278 μM, and the $V_{\text{max}}$ is 27 nmol of ATP/min/mg ABCR, assuming that all of the ABCR is functional. For comparison, highly purified P-glycoprotein in detergent has a $K_m$ for ATP of 940 μM and a $V_{\text{max}}$ of 321 nmol of ATP/min/mg (26). Reconstituted ABCR in the absence or presence of 80 μM all-trans-retinal shows a $K_m$ for ATP of 33 or 725 μM and a $V_{\text{max}}$ of 1.3 or 29 nmol of ATP/min/mg of ABCR, respectively. These data suggest that one effect of a lipid environment is to constrain the ATP hydrolytic activity of ABCR and that this constraint is lost when the protein is solubilized in a CHAPS/lipid mixture. In these calculations we assume that all of the ABCR present in the reaction (and quantitated by Western blotting) is functionally reconstituted and has equivalent access to ATP. As these assumptions are unlikely to apply, the calculated $V_{\text{max}}$ represents a lower limit of the true $V_{\text{max}}$, a consideration that makes any quantitative comparison between solubilized and reconstituted preparations problematic. In the Lineweaver-Burk plot shown in Fig. 7D the parallel shift of the curve of $1/V$ versus 1/[ATP] upon addition of all-trans-retinal indicates that all-trans-retinal acts as an “uncompetitive activator” analogous in effect but opposite in sign to the action of a classical uncompetitive enzyme inhibitor (41, 42). The simplest interpretation of these kinetic data is that all-trans-retinal binds to and alters the ABCR-ATP intermediate at the rate-limiting step in the ATP hydrolytic pathway.

**Comparison of the Stimulatory Activities of Different Retinoids**—To explore the stereoechemical specificity of ABCR for all-trans-retinal, we compared the stimulatory activity of various retinoids (Fig. 8). The three geometric isomers of retinal and a photoisomeric mixture of retinal isomers (containing all-trans-, 7-cis-, 9-cis-, 11-cis-, 13-cis-, and various di-cis-retinal isomers) are all equally active in ATPase stimulation (Fig. 8A and data not shown). All-trans-retinol, all-trans-retinoic

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**Fig. 5.** Effect of vanadate, azide, and ouabain on the ATPase activity of purified and reconstituted ABCR. *A,* titration curve of basal and all-trans-retinal-stimulated ATPase activity at sodium vanadate concentrations up to 250 μM. *B,* effect of 1 mM sodium azide or 1 mM ouabain on basal and all-trans-retinal-stimulated ATPase activity. Both basal and stimulated ATPase activities are relatively insensitive to the three inhibitors.

trans-retinal produces a dose-dependent ATPase activation that is half-maximal at 10–15 μM and shows a simple Michaelis-Menten dose dependence (Fig. 4, A and F; and see Figs. 7, 10, and 11 below); amiodarone, dehydroabietylamine, and 2-tert-butylanthroquinone activate the ATPase beginning at approximately 10–20 μM and show no evidence of saturation up to 50 μM (Fig. 4, B, D, and E); and digitonin activates the ATPase with a sharp optimum at approximately 20 μM, indicative of a high affinity stimulatory interaction and a lower affinity inhibitory interaction (Fig. 4C). The dose-response curves suggest a mechanistic distinction between all-trans-retinal, which appears to act through a cooperative mechanism that involves two or more sites.

As an additional method for assessing the origin of the ATPase activity in the reconstituted ABCR preparation, we examined the sensitivity of basal and all-trans-retinal-stimulated ATPase activity to 1 mM sodium azide, 1 mM ouabain, and a range of sodium vanadate concentrations. Sodium azide and ouabain at 1 mM had no effect, and sodium vanadate showed a very modest concentration-dependent inhibition (Fig. 5). These experiments rule out contamination by two of the most abundant membrane ATPases, the mitochondrial F$_1$F$_\text{0}$-ATPase which is sensitive to azide, and the Na$^+$K-ATPase which is sensitive to ouabain. Moreover, all-trans-retinal does not appear to be a general activator of membrane ATPases as it had no effect on the ATPase activity of crude mouse brain or liver membranes at concentrations of 100, 50, 25, 12, or 6 μM. Based on (a) the high purity of the ABCR protein in this preparation, (b) the low ATPase activity and the lack of ATPase stimulation by all-trans-retinal in mock-eluted controls, (c) the insensitivity
**ATPase Activity of ABCR**

**Fig. 6.** Factors affecting basal and retinal-stimulated ATPase activity of purified and reconstituted ABCR. A, pH dependence of ATPase in the presence or absence of 80 μM all-trans-retinal. Sheep brain PE rather than brain polar lipid was used in the pH titration. ABCR has a shallow pH dependence in the range pH = 6.0–8.0. B, time course of ATP hydrolysis in the presence or absence of 40 μM all-trans-retinal at 37 °C. The linear time course of ATP hydrolysis over a 3-h incubation implies that the standard 2-h reaction time used to quantitate ATPase activity is within the linear range. C, comparison of retinal stimulated ATPase following ABCR reconstitution with brain polar lipid, brain PE, or egg PC.

**Fig. 7.** $K_M$ for ATP and $V_{max}$ for ATP hydrolysis determined for purified ABCR in ROS/CHAPS buffer or following reconstitution in the presence or absence of all-trans-retinal. A and C, the ATP concentration dependence of ATP hydrolysis by purified ABCR in ROS/CHAPS buffer shows simple Michaelis-Menten behavior. B and D, the ATP concentration dependence of ATP hydrolysis by purified and reconstituted ABCR in the absence or presence of 80 μM all-trans-retinal. C and D show the Lineweaver-Burk plots derived from the data shown in A and B, respectively. Based on only the intercepts of the fitted lines, the $K_M$ for ATP and the $V_{max}$ of ATP hydrolysis are as follows: 278 μM and 27 nmol/min/mg (ROS/CHAPS buffer), 33 μM and 1.3 nmol/min/mg (reconstituted, no addition), and 725 μM and 29 nmol/min/mg (reconstituted, plus 80 μM all-trans-retinal). All-trans-retinal addition therefore increases both the $K_M$ for ATP and the $V_{max}$ of ATP hydrolysis more than 20-fold. In these calculations of reaction velocity, we assume that all of the ABCR present in the reaction is functional and has access to ATP. As this assumption is unlikely to apply, any quantitative comparison between solubilized and reconstituted preparations is problematic. The calculated $V_{max}$ values therefore represent a lower limit of the true $V_{max}$. At the 1–3 mM ATP concentration present in the outer segment the reaction velocity will be close to $V_{max}$.

that is indistinguishable from that of pure all-trans-retinal (Fig. 8B), suggesting that ABCR does not specifically recognize oxidized retinoids preferentially over all-trans-retinal, and therefore the activity of all-trans-retinal in this assay is not due to small amounts of contaminating oxidation products. We note that ABCR is not efficiently stimulated by the two hydrophobic nonretinoid aldehydes tested, β-ionone and hexanal (compounds 37 and 40 in Fig. 2), which show little or no activity at 20 μM.

**Synergistic Activation of ABCR by All-trans-retinal, Amiodarone, and Digitonin**—The different shapes of the dose-response curves for all-trans-retinal, amiodarone, and digitonin suggest that these compounds may interact with ABCR in different ways. To examine this possibility we asked whether mixtures of these compounds activate ABCR in an additive or nonadditive manner. Fig. 9 shows an experiment in which these and various other compounds, each at a fixed concentration, were assayed singly or in combination for their effects on ABCR ATPase. In this experiment, verapamil and progesterone were included as negative controls. β-Ionone was included because it produced significant ATPase activation when present at concentrations equal to or greater than 50 μM (Fig. 8C).

As described above and shown in Figs. 3, 4, and 8, Fig. 9 shows that 30 μM all-trans-retinal, 20 μM digitonin, and 40 μM amiodarone each activate the ABCR ATPase 2–5-fold over the basal level, 50 μM β-ionone induces an activation of less than 1.5-fold, and 30 μM progesterone or 30 μM verapamil neither activates nor inhibits the ATPase. Incubation with a combination of 30 μM all-trans-retinal and either 30 μM progesterone or 30 μM verapamil results in a slight reduction in the ATPase activity relative to retinal alone, whereas incubation with a combination of 30 μM all-trans-retinal and 50 μM β-ionone results in a level of activation that is slightly greater than that predicted by adding the two individual levels of activation. Interestingly, incubation with 30 μM all-trans-retinal with either 40 μM amiodarone or 20 μM digitonin or with a combination of 40 μM amiodarone and 20 μM digitonin produces an increase in ATPase activity significantly above that predicted for simple additivity. The synergy obtained with 30 μM all-trans-retinal and a combination of 40 μM amiodarone and 20 μM digitonin is no greater than that produced by either 40 μM amiodarone or 20 μM digitonin alone, suggesting that the latter two compounds synergize with all-trans-retinal by the same mechanism and that this synergistic effect is saturated when either amiodarone or digitonin is present at 40 or 20 μM, respectively. In this experiment, β-ionone at 50 μM is unable to substitute for all-trans-retinal and produces, at most, a small
Fig. 8. Concentration dependence of stimulation by various retinoids of the ATPase activity of purified and reconstituted ABCR. A, all-trans-, 11-cis-, and 13-cis-retinal are equally effective stimulators of ATPase activity. B, all-trans-retinal and partially oxidized all-trans-retinal are equally effective stimulators of ATPase activity. The inset shows the absorption spectra of all-trans-retinal before and after overnight oxidation, a treatment that oxidizes an estimated 10–30% of the all-trans-retinal. C, all-trans-retinal, all-trans-retinol, and all-trans-retinoic acid. D, all-trans-retinal and β-ionone. The various isomers of retinal are significantly more potent stimulators of ATPase than are retinol, retinoic acid, or β-ionone.

Fig. 9. Synergistic stimulation ATPase activity of purified and reconstituted ABCR by all-trans-retinal and a subset of low molecular weight compounds. The bar graph shows the relative ATPase activity in the absence of added compounds (basal) or in the presence of 30 μM all-trans-retinal, 40 μM amiodarone, 50 μM β-ionone, 20 μM digitonin, 30 μM progesterone, 30 μM verapamil either singly or in the indicated combinations. Progesterone and verapamil, two stimulators of P-glycoprotein ATPase, serve as controls. The filled regions indicate that part of the ATPase activity that is in excess of the sum of the activities of the individual compounds.

Enhancement in the ATPase activation when added to reactions containing 30 μM all-trans-retinal or a combination of 30 μM all-trans-retinal and either 40 μM amiodarone or 20 μM digitonin or both. The combination of 40 μM amiodarone, 20 μM digitonin, and 50 μM β-ionone corresponds approximately to the sum of the activation levels obtained for the three compounds individually. These data are reminiscent of the synergistic effects on transport recently reported for some pairs of P-glycoprotein substrates (43, 44).

To quantitate the concentration dependence of the synergistic interaction between all-trans-retinal and amiodarone, digitonin, or β-ionone, experiments were conducted in which one compound was added at a fixed concentration, and a second compound was added at a variety of concentrations (Fig. 10). In Fig. 10, the degree of synergy can be assessed by comparing the experimental curve in the presence of both compounds with the curve calculated for simple additivity. In one set of experiments the dose-response curve for ATPase activation by all-trans-retinal was determined in the presence or absence of 40 μM amiodarone (Fig. 10A), 20 μM digitonin (Fig. 10B), or 50 μM β-ionone (Fig. 10C). In this experiment, a somewhat greater synergy was observed with all-trans-retinal and β-ionone compared with the experiment shown in Fig. 9. In a second set of experiments, the dose-response curve for ATPase activation by amiodarone was determined in the presence or absence of 100 μM all-trans-retinal (Fig. 10D) or 20 μM digitonin (Fig. 10E). At this concentration, all-trans-retinal shows appreciable synergy with 40 μM amiodarone (Fig. 10A). This experiment shows that the synergy between all-trans-retinal and amiodarone is minimal below 20 μM amiodarone and then rises steeply at amiodarone concentrations greater than 30 μM (Fig. 10D). In Fig. 10E, the combination of amiodarone and digitonin shows greater than additive activation of ATPase below 40 μM amiodarone, but this effect is absent above 40–50 μM amiodarone. The greater than additive behavior of digitonin and amiodarone at low amiodarone concentration may simply reflect the nonlinear concentration dependence of ATPase stimulation by amiodarone alone, as can be seen from the shape of the amiodarone dose-response curve in Fig. 10E (e.g., the degree of stimulation by 50 μM amiodarone is greater than the sum of that produced individually by 20 and 30 μM amiodarone). If the stimulatory mechanism(s) of digitonin and amiodarone are similar, as suggested by their ability to synergize at higher concentrations with all-trans-retinal but not with each other, then this could account for the greater than additive behavior of these two compounds at low amiodarone concentration.

Kinetic Analysis of ATPase Stimulation by All-trans-retinal and Amiodarone—To explore the mechanism by which amio-
The effect of all-trans-retinal on the apparent affinity for all-trans-retinal and amiodarone individually and in combination on the $K_m$ for ATP and the $V_{max}$ for ATP hydrolysis (Figs. 10A and 11). A Lineweaver-Burk plot of the relationship between the rate of ATP hydrolysis at 50 $\mu$M ATP and the concentration of all-trans-retinal (Fig. 10F) shows that its fit to a straight line without cooperation at a single class of binding site. The 1.7-fold decrease in the ratio $K_{app}$ from 9.6 to 4.6 $\mu$M all-trans-retinal raises the $V_{max}$ for ATP by 1.7-fold and decreases the $K_m$ for all-trans-retinal from 9.6 to 4.6 $\mu$M all-trans-retinal with only a modest change in $K_m$. Amiodarone addition in the absence of all-trans-retinal lowers the $K_m$ for ATP by approximately 2-fold (Fig. 11B), whereas amiodarone addition in the presence of 100 $\mu$M all-trans-retinal raises the $K_m$ for ATP approximately 2-fold (Fig. 11C). Importantly, these changes are accompanied by a 2-fold decrease in the ratio $K_{app}/V_{max}$ (the slope of the best fitting straight line), an effect that is analogous, but opposite in sign, to that of classical noncompetitive enzyme inhibitors. We will therefore refer to amiodarone as a "noncompetitive activator".

The simplest model to account for the kinetic behavior of amiodarone is one in which ABCR is assumed to exist in an equilibrium mixture of enzymatically active and inactive species. By analogy with the diminution in the number of active enzymes resulting from the action of a noncompetitive inhibitor, we propose that amiodarone binding shifts the equilibrium between active and inactive enzymes in favor of the active species.

In contrast to amiodarone, the effect of all-trans-retinal, either alone or in combination with amiodarone, is to increase the $V_{max}$ for ATP hydrolysis and the $K_m$ for ATP by the same factor, thereby leaving the ratio $K_{app}/V_{max}$ (the slope of the best fitting straight line) unchanged (Figs. 7D and 11D). This effect on kinetic parameters is analogous but opposite in sign to that displayed by classical uncompetitive enzyme inhibitors, and we will therefore refer to all-trans-retinal as an uncompetitive activator. The simplest model that accounts for this kinetic behavior is one in which all-trans-retinal interacts specifically.
with an intermediate in the ATPase reaction pathway. By analogy with the specific trapping of enzyme-substrate intermediates produced by the action of a classical uncompetitive enzyme inhibitor, we propose that the binding of all-trans-retinal creates an accelerated pathway for ATP hydrolysis. The data can also be formalized along the lines of a double-displacement (“ping pong”) kinetic scheme in which the progress of one substrate, ATP, through the reaction pathway is accelerated by the binding of the second component, all-trans-retinal, at an intermediate point in the pathway (41, 42). The two different modes of interaction between ABCR and amiodarone or all-trans-retinal predicts that their combined effects will by multiplicative rather than additive, and therefore consists in a simple way for their ability to act simultaneously and synergistically on ABCR. The simple Michaelis-Menten behavior and uncompetitive mode of ATPase activation exhibited by amiodarone implicates this compound as an allosteric effector. These observations suggest that the division of ATPase activators into noncompetitive and uncompetitive classes may prove to be generally useful in the study of other ABC transporters.

**DISCUSSION**

**Purification and Reconstitution of ABCR**—The experiments reported here define a purification procedure and a set of lipid, detergent, and buffer conditions that permit the isolation of nearly homogenous ABCR and its reconstitution into lipid membranes in functional form. The purified and reconstituted preparation has ATPase activity that appears to derive from ABCR rather than from a contaminating ATPase. The stimulated ATPase activity of ABCR is highly sensitive to its local membrane environment, as has been observed for P-glycoprotein (40). PE supports functional reconstitution as do lipid mixtures containing PE but PC does not. When ABCR is solubilized in a mixture of PE, PC, and CHAPS (ROS/CHAPS buffer) it retains ATPase activity, but this ATPase is not stimulated by all-trans-retinal. This effect of CHAPS is unlikely to reflect denaturation of ABCR, since sensitivity to all-trans-retinal appears upon transfer of ABCR from this CHAPS/lipid mixture to brain lipid alone. These observations suggest instead that the mixed CHAPS/lipid micelle permits cycles of ATP hydrolysis that are uncoupled from conformational events at the site to which all-trans-retinal binds.

**Retinal Stimulation of ABCR ATPase**—The finding that all-trans-retinal (and other geometric isomers of retinal) stimulates the ATPase activity of ABCR can be explained by any of three mechanisms as follows: (a) all-trans-retinal exerts a general effect on the lipid environment that alters the conformation of ABCR; (b) all-trans-retinal binds to ABCR as an allosteric effector of the ATPase cycle but is not itself a substrate for transport; and (c) all-trans-retinal is a substrate for transport, and its presence accelerates ATP hydrolysis by a conformational coupling between the transport domain and one or both ATP binding domains.

The first mechanism is unlikely because (a) it does not account for the specificity of ATPase stimulation by all-trans-retinal relative to the other compounds tested, (b) it seems implausible that retinal should exert an effect on bulk membrane properties in a reaction in which lipid is present at 1–1.5 mg/ml and retinal is present at 10 μM (equivalent to 3.3 μg/ml), and (c) a quantitative analysis of the concentration dependence of all-trans-retinal stimulation indicates that it acts with simple Michaelis-Menten behavior.

The second mechanism, if correct, would imply a novel strategy for modulating the activity of a rod outer segment protein, i.e. sensing the concentration of free retinal or a related retinoid. Under this assumption, ABCR activity might be modulated by the level of all-trans-retinal released by photoactivated rhodopsin or of free 11-cis-retinal imported from the RPE for rhodopsin regeneration. This mechanism would be in contrast to the regulatory mechanisms identified thus far among outer segment proteins that involve phosphorylation or binding to calcium, GTP, or cGMP. Arguing against a simple allosteric mechanism is the effect of all-trans-retinal on the Km for ATP and the Vmax for ATP hydrolysis which indicates that all-trans-retinal acts via an uncompetitive interaction with ABCR.

The third possible mechanism, that various geometric isomers of retinal and/or other retinoids are transported by ABCR in a reaction that is coupled to ATP hydrolysis, can only be definitively distinguished from an allosteric mechanism by directly demonstrating ATP-dependent vectorial transport of these compounds in a reconstituted membrane system. At present, the strongest evidence in favor of this hypothesis is the observation that all-trans-retinal acts without cooperativity at a single class of binding site(s) on ABCR and exhibits uncompetitive stimulation of ATPase activity. This kinetic behavior
implies that all-trans-retinal binds to an intermediate in the ATPase reaction pathway and that this binding accelerates a rate-limiting step in ATP hydrolysis and/or release of the hydrolysis products. This behavior is precisely that predicted for a transported substrate, and it was not observed with any of the 37 nonretinoid compounds tested in this study. The localization of ABCR to the rod disc membrane also favors retinal (and/or retinol) as the natural ligand since it predicts that the endogenous ligand should be present within ROS, a property fulfilled by all-trans-retinal, 11-cis-retinal, and all-trans-retinol. Given the relatively simple chemical composition of ROS (45) and the absence, at present, of any data implicating other transport processes involving the photoreceptor disc membrane, there are few competing candidate substrates for ABCR-mediated transport. Finally, we also favor this hypothesis because, as described below, various pieces of circumstantial evidence suggest that retinal may require active transport across or extraction from the disc membrane and that Stargardt disease is associated with an accumulation of retinal or its derivatives in ROS.

**Retinoid Metabolism in the Rod Outer Segment**—Photoactivation of rhodopsin results in the hydrolysis and release of all-trans-retinal which is reduced within the outer segment to all-trans-retinol by all-trans-retinol dehydrogenase (named for the reverse reaction). All-trans-retinol is transported to the RPE where it is esterified, isomerized to the 11-cis-configuration, released from the ester linkage, and returned to the outer segment (46). Many of the steps in this process are still poorly understood. In particular, most of the enzymes involved have yet to be purified, and it is not known which steps involve active transport or passive diffusion. The flux of retinal through this pathway can be extremely high under physiological conditions: viewing the blue sky on a cloudless day produces 20,000 photoisomerizations per rod/s in the human retina (47).

Analysis of mice that were exposed to physiological light levels indicates that reduction of all-trans-retinal is the rate-limiting step in the visual cycle (48); following a light flash or under constant illumination, all-trans-retinal accumulates in the retina without a concomitant accumulation of other retinoids. In vitro experiments suggest that this accumulation may increase photoreceptor noise because the combination of free all-trans-retinal and opsin produces a partially active species (49–52). By contrast, electrophysiological analyses of single photoreceptor cells have failed to reveal a desensitizing effect of exogenously applied all-trans-retinal (reviewed in 53), suggesting either that the in vitro effect is of little physiological relevance, that exogenous all-trans-retinal cannot readily gain access to opsin, or that there are mechanisms in the intact cell that minimize the desensitizing effect of all-trans-retinal. If ABCR facilitates the reduction of all-trans-retinal by all-trans-retinol dehydrogenase, then one might predict that ABCR dysfunction would lead to an increase in photoreceptor noise. In this regard, it is interesting that individuals with Stargardt disease show an elevated rod threshold and a delayed recovery of rod sensitivity following light exposure (54).

When all-trans-retinal is released from photactivated rhodopsin, it presumably enters the hydrophobic environment of the disc membrane. In the disc membrane, free all-trans-retinal can react to form a Schiff base with the amine present in PE (55, 56), which constitutes 40% of ROS lipid (45). If we assume that Schiff base adducts between all-trans-retinal and PE can form on either the luminal or the cytosolic face of the disc membrane then it seems plausible to suppose that these adducts on the cytosolic face will be available for reduction by all-trans-retinol dehydrogenase, whereas those on the luminal face will be sequestered from the enzyme active site. This line of reasoning assumes that the active site of all-trans-retinol dehydrogenase has access to substrates associated with only one side of the disc membrane, consistent with the known asymmetric disposition of membrane-associated proteins. In the absence of an active mechanism for transmembrane flipping of the PE-all-trans-retinal Schiff base adduct, we would predict that this adduct would accumulate on the luminal face of the disc membrane. Its abundance there would presumably reflect the rate of spontaneous hydrolysis of the PE-all-trans-retinal Schiff base and subsequent equilibration of all-trans-retinol to the cytosolic leaflet of the disc membrane.

**A Model for ABCR in the Visual Cycle**—The considerations outlined in the preceding three paragraphs, together with the localization of ABCR to the disc membrane and the retinal-dependent stimulation of ATP hydrolysis by ABCR, suggest that ABCR acts in the visual cycle to flip PE-all-trans-retinal adducts from the luminal to the cytosolic face of the disc membrane, move free all-trans-retinal from the lipid phase of the
disc membrane to a juxtamembrane location, or possibly reori-
ent all-trans-retinal in the bilayer (Fig. 12A). In the first case,
ABC activity would resemble the PC flipase activity of
mouse MDR-2 (57) and human MDR3 (58), whereas in the
latter cases, ABC activity would resemble the drug extrusion
activity of P-glycoprotein, especially as envisioned by the “hy-
drophobic vacuum cleaner model” (59) in which P-glycoprotein
is proposed to extract hydrophobic compounds from the lipid
phase and deliver them to the extracellular space. In each case,
we hypothesize that the effect of ABCR activity would be to
deliver more efficiently all-trans-retinal to all-trans-retinol de-
hydrogenase, leading to lower photoreceptor noise, more rapid
recovery following illumination, and decreased accumulation of
all-trans-retinal or its adducts within the disc membrane.
In these models of ABCR action, the proposed vectorial movement
is topologically equivalent to importing a substrate from the
extracellular space, a direction of transport opposite to the
direction of lipid flipase activity demonstrated for mouse
MDR2 (57) and the human MDR1 and MDR3 P-glycoproteins
(58) and opposite to the direction of drug extrusion mediated by
P-glycoprotein. Substrate import by ABC transporters is com-
monly observed in bacteria but has thus far not been reported
in multicellular organisms (60).

These models may account for the presence of ABCR in rods
but not cones, a difference that may be related to differences in
the visual cycle between these cell types (61). Since the luminal
face of the cone disc membrane remains contiguous with the
plasma membrane, free all-trans-retinal within the cone disc
membrane or all-trans-retinal-PE adducts formed on the luminal
face of the cone disc could react with other amines in the
extracellular space and/or bind to extracellular proteins such as
interphotoreceptor retinol-binding protein.

The stimulation of ATPase activity by 11-cis-retinal and to a
lesser extent by all-trans-retinol suggests that ABCR may also
play a role in the movement of these compounds (Fig. 12A). The
facile reaction of 11-cis-retinal and opsin in vitro has generally
been interpreted to mean that this reaction does not require
additional cofactors or catalysts in vivo. However, it is possible
that in vivo the selective pressure for rapid and complete re-
generation, which is thought to be one of the rate-limiting steps
in dark adaptation, has led to the evolution of enzymes that
accelerate this reaction. The relative abundance of ABCR to-
gether with the rapid lateral diffusion of opsin within the disc
membrane (62) should permit frequent encounters between
these proteins. Whereas any models for the role of ABCR in this
reaction are necessarily speculative, if ABCR facilitates the
binding of 11-cis-retinal to opsin, an ABCR-dependent acceler-
ation in regeneration rate should be demonstrable in vitro.

Implications for the Pathogenesis of Stargardt Disease and
Other Retinopathies—The work presented here supports the
idea that Stargardt disease and other retinopathies caused by
mutations in ABCR arise from defects in retinoid metabolism
(Fig. 12B). This idea was originally proposed as an explanation
for the delayed recovery of rod sensitivity observed in Stargardt
disease patients (54). The persistence of this delay in rod re-
covery despite high levels of dietary vitamin A (63) distin-
guishes it from classical night blindness of dietary origin (64),
and more specifically suggests a model in which the defect in
Stargardt disease is not related to the availability of 11-cis-
retinal but rather to a defect in removing the product of pho-
tobleaching, all-trans-retinal. The model outlined above may also be relevant to recent structural analyses of the fluorescent, shortwave absorbing compounds that accumulate with age in the human RPE. These analyses show that the major component is a direrinal-etha-
nolamine derivative, A2-E (65, 66). A2-E has been hypothe-
sized to form from the condensation of all-trans-retinal and PE
in the outer segment and then to accumulate over time in the
RPE as a residual product of ROS digestion (65). Although it is
not known definitively whether A2-E comprises the fluores-
cent, shortwave absorbing material that accumulates to high
levels in the RPE of Stargardt disease patients, one in vivo
study suggests that this compound or a similar one is present
at high concentration in these patients (67, 68).

The severe defect in rod vision characterized by homozogos-
ity for ABCR null mutations is in contrast to the milder im-
pairment of rod vision experienced by many patients with
Stargardt disease. If ABCR is important only as a transporter
and not as a structural protein, then the clinical data would
suggest that a complete absence of ABCR-mediated transport
leads to the death of rod photoreceptors, the final common
event in retinitis pigmentosa.

Implications for Pharmacologic Intervention in Retinopa-
thies—The in vitro assay described here may lend itself to the
identification of compounds that either enhance or diminish
the activity of ABCR. Those that enhance ATPase activity
might represent lead compounds for drug development with
the goal of enhancing ABCR activity in patients with Stargardt
disease or in a subset of individuals at risk for age-related
macular degeneration. In vitro, such compounds might in-
crease the ATPase activity of ABCR when added alone or in
combination with a transported compound. We note, however,
that this assay represents only a screening tool; any interpre-
tation of altered ATPase activity will require a detailed under-
standing of the mechanism of action of the test compound
because, in theory, ATPase activity could be increased by either
promoting or circumventing the normal transport cycle. Al-
though we cannot make any firm predictions regarding the in
vivo effect on ABCR function of compounds like amiodarone
and digitonin, the existence of such compounds suggests that
environmental or drug effects may be relevant to degenerative
retinal diseases. In this context, it may be of interest to study
visual function in patients receiving amiodarone, a drug that
has been widely used in the treatment of cardiac arrhythmias
at tissue concentrations at or above 40 μM (69).

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