Blocking Chloride Channels in the Rat Lens: Localized Changes in Tissue Hydration Support the Existence of a Circulating Chloride Flux

Miriam A. Young,1 Mark J. Tunstall,2 Joerg Kistler,2 and Paul J. Donaldson1

PURPOSE. To investigate the effects of inhibitors of chloride channels on lens volume and tissue architecture under isotonic conditions.

METHODS. Rat lenses were maintained in organ culture under isotonic conditions in the presence of various putative chloride channel inhibitors. The effect of an inhibitor on lens wet mass and tissue morphology was determined by weighing and histologic examination, respectively.

RESULTS. Exposure to 100 μM of either 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) or 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) caused an increase in wet mass and severe tissue disruption in the lens equatorial region. Two distinctly different zones of tissue damage were evident: a peripheral zone of fiber cell swelling and an inner zone of extensive tissue breakdown. Extracellular space dilations caused the extensive tissue damage in the inner zone and preceded the peripheral fiber cell swellings. That the observed effects were a consequence of the inhibition of chloride channels was supported by (1) the effectiveness of NPPB at the lower dose of 10 μM, (2) the absence of any NPPB effect in chloride-free medium, and (3) an identical effect after exposure to tamoxifen, an inhibitor of the chloride channel regulator p-glycoprotein.

CONCLUSIONS. Study results indicate that chloride channels are active in the lens under isotonic conditions. The spatial and temporal pattern of morphologic changes that was observed is consistent with a steady state efflux of chloride ions and water from peripheral fiber cells and a corresponding influx into fiber cells deeper in the lens. These observations may therefore represent the first visualization of the chloride flux postulated by others to be a component of the lens internal circulation system. (Invest Ophthalmol Vis Sci. 2000;41:3049–3055)

The precise control of tissue hydration is essential for the maintenance of lens transparency. Disruption of the crystalline packing of the fiber cells by either cellular swelling or dilation of the normally tight spaces between the cells increases intralenticular light scattering and can result in the formation of opacities. Indeed a variety of animal cataract models exhibit a disruption of equatorial tissue consistent with the formation of opacities.1–3 Such tissue disruption can also be induced experimentally by exposing isolated lenses under isotonic conditions to inhibitors of Na/K ATPase.4 In all cases, the lenses gain water, which accumulates predominantly in the equatorial tissue and is apparent histologically by the localized breakdown and liquefaction of fiber cells.

Recently we have shown that addition of 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) to isolated rat lenses maintained in organ culture under isotonic conditions also produces an increase in lens hydration.5 Although not entirely specific, NPPB is widely regarded as a chloride channel inhibitor, and it is known that the movement of chloride ions and concomitantly of water across cell membranes plays a central role in the regulation of cell volume in a variety of tissues.6,7 Volume regulation in the hypotonically challenged lens has been documented and shown to involve the movement of chloride ions.8,9 However, our recent results are derived from lenses maintained under isotonic conditions, suggesting that chloride movement may also be important in controlling the volume of the lens under steady state conditions.

These findings are relevant to the understanding of lens physiology. A role for chloride ions in the resting lens is predicted by a functional model in which the lens operates an active microcirculation system to ensure that nutrient and electrolyte exchange is facilitated in deeper regions.10 Furthermore, the similarity between equatorial tissue damage that occurs in vivo in the rat diabetic lens and in vitro in the NPPB-treated normal rat lens5 suggests that the latter constitutes a worthwhile model system to investigate the cellular processes that lead to tissue opacification.

Our present report is an extension of preliminary data presented by Tunstall et al.5 and examines in detail the cellular changes that occur in the resting rat lens when treated with NPPB or other putative inhibitors of chloride channels. Using confocal laser scanning microscopy and electron microscopy, we document the temporal and spatial changes in tissue mor-
phology caused by exposure of the resting rat lens to these inhibitors. These changes are neither observed in experiments where chloride has been removed from the bath solution before applying NPPB or when potassium channels are inhibited. This suggests that the inhibition of chloride movement causes the observed histologic effects, consistent with the existence of a circulating chloride flux in the resting rat lens.

**MATERIALS AND METHODS**

**Chemicals**

5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was obtained from Research Biochemicals Inc. (Natick, MA). Other chemicals including: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, disodium salt (DIDS); 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, disodium salt (SITS); tamoxifen, citrate salt; and fluorescein-conjugated wheat germ agglutinin (FITC-conjugated WGA, *Triticum vulgaris*) were obtained from Sigma (St. Louis, MO).

**Preparation of Lenses**

All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three-to-four-week-old Wistar rats were killed by CO₂ asphyxiation, and the eyes extracted. Lenses were carefully removed from the globe and placed immediately in artificial aqueous humor (AAH: 125 mM NaCl, 4.5 mM KCl, 10 mM NaHCO₃, 2 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, 20 mM sucrose, 1% penicillin/streptomycin, 10 mM HEPES, pH 7.4; osmolality 300 mmol/kg) for 6 hours and screened visually for damage. Lenses that developed opacities during this incubation were discarded. Lenses were either left in AAH or transferred to AAH containing NPPB (10 or 100 µM), SITS (100 µM), DIDS (100 µM), SITS (100 µM), tamoxifen (100 µM), or BaCl₂ (10 mM) for 3, 6, 12, or 18 hours at 37°C before either being weighed and/or prepared for histologic analysis. Hypotonic AAH (osmolality 150 mmol/kg) was identical with AAH but contained only 50 mM NaCl. For experiments in which chloride was removed from the bathing media, lenses were preincubated in chloride-free AAH (125 mM Na gluconate, 4.5 mM K gluconate, 10 mM NaHCO₃, 2 mM CaSO₄, 0.5 mM MgSO₄, 5 mM glucose, 20 mM sucrose, 1% penicillin/streptomycin, 10 mM HEPES, pH 7.4; osmolality 300 mmol/kg) for 6 hours before the addition of NPPB. NPPB and tamoxifen were dissolved in DMSO (0.1% v/v) and methanol (0.2% v/v), respectively. Neither agent when added in the absence of drugs had any effect on lens tissue architecture.

**Confocal Microscopy**

Lenses were fixed in 25% Karnovsky’s solution (50 mM Na cacodylate, 1% paraformaldehyde, 1.25% glutaraldehyde) in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 9.2 mM Na₂HPO₄, 1.2 mM KH₂PO₄, pH 7.4; osmolality 300 mmol/kg) for 4 hours at room temperature. Fixed lenses were superglued to the plate of a vibratome (Vibratome 1000; Technical Products International, Inc., St. Louis, MO), and 170-µm-thick equatorial sections were cut. Sections were incubated in FITC-conjugated WGA (1 µg/ml in PBS) overnight in the dark at room temperature. Sections were then given four 10-minute washes in PBS. Labeled sections were mounted in Citifluor (Agar Scientific, Stansted, Essex, UK) to reduce fading and examined using a Leica TCS 4D Confocal Microscope (Leica Lasertechnik, Heidelberg, Germany) fitted with an argon–krypton mixed gas laser.

**Volume Rendering of Damaged Tissue Regions**

Peripheral fiber cell measurements were acquired from confocal images of equatorial sections using the measurement tool of the public domain program NIH Image (available at http://rsb.info.nih.gov/nih-image/). A line was drawn from the epithelial/fiber cell border at right angles to the broad sides of the fiber cells for a distance of 50 µm, and the number of cells contained within this distance was counted. Equatorial images of control and NPPB-treated lenses at 3, 6, 12, and 18 hours were analyzed. Three counts were collected for each section, and the results were averaged.

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**Quantification of Peripheral Fiber Cell Swelling**

Peripheral fiber cell measurements were acquired from confocal images of equatorial sections using the measurement tool of the public domain program NIH Image (available at http://rsb.info.nih.gov/nih-image/). A line was drawn from the epithelial/fiber cell border at right angles to the broad sides of the fiber cells for a distance of 50 µm, and the number of cells contained within this distance was counted. Equatorial images of control and NPPB-treated lenses at 3, 6, 12, and 18 hours were analyzed. Three counts were collected for each section, and the results were averaged.

**Electron Microscopy**

NPPB-treated lenses were fixed in 25% Karnovsky’s solution for 1 week. Fixed lenses were cut into small segments and then fixed for a further 24 hours. Lens segments were placed in FITC-conjugated WGA (1 µg/ml PBS) for 1 week and subsequently prepared for confocal imaging using a protocol modified from Young et al. Brieﬂy, tissue segments were rinsed in PBS, dehydrated in graded ethanol (50%, 75%, 95%, 100%) and propylene oxide, and infiltrated with Agar 100 resin. The blocks were secured in a Reichert-Jung ultramicrotome chuck and excess resin trimmed from the surface using a glass knife. The chuck was supported in a custom-made Perspex holder designed to fit into the recess on the microscope stage of the confocal microscope. Confocal images were acquired by imaging directly onto the resin embedded tissue using a 100× oil-immersion lens with numerical aperture 0.75. Seventy-two optical sections were acquired to a depth of 14 µm. Each optical section had a 100 × 100 µm field of view and a 512 × 512 image matrix. The overall dimensions of the stack provided cubic voxels. Visualization of the 3D volume was performed using custom software on a Silicon Graphics (Mountain View, CA) workstation.
RESULTS

Treatment with Putative Chloride Channel Inhibitors: Survey of Effects

Previously we have shown that the putative chloride channel blocker NPPB caused a significant increase in rat lens wet weight after 18 hours. In the present study, in addition to NPPB, we also tested two other putative inhibitors of chloride transport for their ability to induce water uptake in rat lenses incubated under isotonic conditions. “Putative” is used here to indicate that these inhibitors may also have other activities, although they are most often used to block chloride transport. DIDS caused a gain in lens wet weight similar to that of NPPB, but no such effect was evident with SITS (Fig. 1). For comparison, incubation in hypotonic AAH without inhibitors produces a larger uptake of water than that induced by NPPB or DIDS under isotonic conditions. Next we investigated whether this uptake of additional water into the lens results in visible tissue damage. The equatorial region is known to be the most active portion of the normal lens and in rat sugar cataracts the most severely affected. Hence, we focused our investigation on detecting signs of tissue disruption in this region. Confocal microscopy of membrane-labeled equatorial sections allowed the documentation of cellular changes over large areas with high definition. Images of control lenses incubated for 18 hours in AAH without inhibitors consistently showed a perfect crystalline packing of fiber cells without any evidence of disruption, as did lenses treated with SITS (Fig. 2A). In contrast, both NPPB (Fig. 2B) and DIDS-treated lenses (Fig. 2C) revealed large areas of liquefaction, which were concentrated in a distinct zone approximately 100 μm from the capsule. Fiber cells outward from this grossly damaged zone appeared intact, but are significantly swollen when compared to the same area in control lenses. Hence, the application of NPPB or DIDS affects predominantly the differentiating fiber cells in the lens equator, and the pattern of damage induced by either reagent differs significantly with the depth from the lens surface. Such differential damage was not apparent in hypotonically swollen lenses, which instead reproducibly exhibited severe damage uniformly over the entire equatorial region (Fig. 2D).

Detailed Morphologic Analysis of NPPB-Induced Damage

The tissue disruption caused by the putative channel inhibitor NPPB was examined in greater detail. In the first instance, we investigated the development of tissue damage caused by 100 μM NPPB over a time course of 18 hours. After 3 hours, disruption of the crystalline tissue architecture was evident in a zone approximately 100 μm from the capsule (Fig. 3A). At 6 hours, these disruptions were further enlarged and occurred together with a swelling of the peripheral fiber cells (Fig. 3B). At 12 and 18 hours, a liquefaction zone had formed in the area where disruption to tissue order first became evident, and peripheral fiber cells appeared more swollen but otherwise appeared intact (Figs. 3C, 3D). To verify that this cell swelling was reproducible and significantly different from control lenses, we quantified this phenomenon by counting the number of individual cells contained within the first 50 μm of a fiber cell column (Fig. 4A) in equatorial sections taken from control and NPPB-treated lenses. The resulting graph shows that a statistically significant reduction in the number of cells contained within a column occurs after 6 hours of treatment with NPPB (Fig. 4B). Hence, peripheral fiber cell swelling becomes significant after 6 hours of exposure to NPPB.

The formation of the zone of liquefaction deeper in the equatorial region was investigated by analyzing the disruptions of tissue architecture that became evident in the same region after 6 hours of treatment with 100 μM NPPB. At higher

FIGURE 1. Percentage increase in rat lens wet mass. A comparison of control lenses versus lenses incubated in hypotonic AAH (150 mmol/kg) or isotonic AAH containing either 100 μM NPPB, DIDS, or SITS for 18 hours. *Significantly different from control (P < 0.05).

FIGURE 2. Localization of water uptake in the rat lens cortex. Equatorial sections of lenses labeled with FITC-conjugated WGA. Lenses were incubated in the following solutions for 18 hours: (A) AAH + SITS (100 μM), (B) AAH + NPPB (100 μM), (C) AAH + DIDS (100 μM), and (D) hypotonic AAH (150 mmol/kg).
magnification, it became obvious that these disruptions were
due to a dilation of the normally tight extracellular space and
this predominantly occurred between the broad sides of adja-
cent fiber cells (Fig. 5A). These dilations were further charac-
terized in lenses optimized for optical sectioning and subse-
quent volume rendering. In these specimens, the cytoplasm of
the fiber cells exhibited a light background staining due to
autofluorescence caused by the extended time in fixative. This
had the advantage of greatly enhancing the visibility of the
extracellular space dilations (Fig. 5B). A volume-rendered
three-dimensional image obtained by merging a series of opti-
cal sections from an NPPB-treated lens shows that these dila-
tions are in fact cystlike in shape and have similar dimensions
in the equatorial and longitudinal planes (Fig. 5C).

The extracellular origin of the cystlike structures was
confirmed by electron microscopy. Again the extracellular
dilations were found predominantly on the broad sides of the
fiber cells, but also showed evidence of enlarged spaces on the
narrow sides (Fig. 6A). At higher magnification, micrographs
revealed that cysts were often limited by junctional domains
that held opposing fiber cell membranes tightly together
(Fig. 6B).

**Evidence Supporting a Role for Chloride Channels**

We now examine whether the effects on lens tissue caused by
NPPB or DIDS can really be attributed to the inhibition of
chloride channels. As pointed out earlier, there are some reservations regarding the specificity of these inhibitors, especially since the effective local concentrations deeper in the lens are unknown. For example, although NPPB has been extensively used to block chloride channels in a variety of systems, at a concentration of 100 μM, it is known to block other anion transporters and to affect potassium channels. DIDS preferentially affects the Cl\(^-\)/HCO\(_3\)^- exchanger, but when used in the 100 μM range, may also have effects on anion channels.

To address the issue of inhibitor specificity, several approaches were used. First, we lowered the concentration of NPPB 10-fold to a level at which NPPB is considered selective for chloride channels over anion transporters. At 10 μM NPPB and after 18 hours of treatment, we still observed a significant disruption of tissue architecture (Fig. 7A) that was equivalent in location and magnitude to the damage caused by a 6-hour exposure to 100 μM NPPB (Fig. 3B). Second, we determined if the observed effects of NPPB were dependent on the presence of extracellular chloride. The replacement of chloride with the impermeant anion gluconate in the absence of NPPB caused the fiber cells to shrink because of a loss of intracellular chloride (data not shown). After a 6-hour preincubation in chloride-free AAH, lenses exposed to 10 μM NPPB for 18 hours exhibited an extended region of peripheral cell swelling, but with no evidence of extracellular space dilations (Fig. 7B).

Third, we used an additional inhibitor, tamoxifen, which has been shown to block chloride channels in patch-clamp studies on isolated lens fiber cells. As with NPPB-treated lenses, tamoxifen lenses gained water (data not shown), and the earliest detectable sign of tissue disruption was the development of a distinct inner zone of extracellular space dilations (Fig. 7C). Fourth, to exclude the possibility that NPPB was exerting its effects via potassium channels, we exposed lenses to 10 mM barium for 18 hours and found that this had no disruptive effect on tissue architecture (Fig. 7D). Therefore, the changes in tissue morphology can be attributed to the inhibition of chloride channels, a process that blocks the movement of chloride ions and produces a localized accumulation of water.

**DISCUSSION**

In this study, we have used confocal and electron microscopy to characterize, both spatially and temporally, the changes in equatorial tissue architecture induced by incubating lenses in the presence of reagents known to block chloride channels. Unfortunately, chloride channel inhibitors are notoriously non-specific and have been shown in other tissues to block a variety of channels and transporters. However, in the present study, we believe that the effects we have observed can be attributed predominately to the blockage of chloride channels for the following reasons. First, three different inhibitors of chloride channel activity all produced the same effects. NPPB and DIDS presumably acted via direct interaction with the channel, and tamoxifen via the chloride channel regulator p-glycoprotein. Second, SITS had no effect on fiber cell architecture. SITS and DIDS are potent inhibitors of the Cl\(^-\)/HCO\(_3\)^- exchanger, and...
inhibited a chloride channel. This would cause an accumulation of chloride ions and inhibition of chloride channels in the inner lens would block release osmolytes and resultant fiber cell swelling. Furthermore, removal of chloride from the extracellular solution would change $E_{Cl}$ and increase in a radial direction the number of peripheral fiber cells that have an outwardly directed chloride flux. Blocking chloride channels under these conditions would not cause extracellular space dilations, but would produce an extended region of peripheral fiber cell swelling such as that observed in Figure 7B.

Although the primary focus of this report is on the contribution of chloride fluxes to the maintenance of lens hydration and tissue architecture, we should not neglect the role played by the cations that accompany chloride movement. In this regard, our observation that exposure of lenses to barium was without detectable effect on tissue architecture (Fig. 7D) requires comment. In our study, barium was used to exclude the possibility that the NPPB-induced tissue damage was caused by a nonspecific inhibition of epithelial potassium channels, which would in turn cause a depolarization of the lens membrane potential. Depolarization of the lens would be expected to reduce the difference in electromotive potential between the surface and inner fiber cells that is responsible for driving the internal circulation. Furthermore, blocking potassium loss from the lens with barium would be expected to reduce the rate of the Na-K pump, which others have shown can give rise to extensive cortical tissue damage. The fact that no changes to tissue architecture were observed after incubating lenses for 18 hours in barium suggests that any reduction of the internal circulation or the Na-K pump rate was insufficient to produce detectable tissue damage. The barium-induced depolarization would further be expected to change $E_{Cl}$ and the resultant direction of chloride fluxes in the lens. However, this would not necessarily lead to detectable tissue damage. Indeed, removing chloride from the extracellular space, which would similarly affect $E_{Cl}$, did not cause significant tissue damage (data not shown). We conclude that although cations undoubtedly play a role in lens circulation, the tissue changes that we have reported after exposure to NPPB can be mainly attributed to the interference with chloride fluxes in the lens.

Does the blockage of chloride channels in the lens constitute a model for osmotic cataract? In an earlier study, the damage caused by NPPB in the resting rat lens was initially considered similar to that caused by hyperhydration in the diabetic rat lens. In both cases, tissue disruption and subsequent liquefaction occur in an equatorial zone approximately 100 μm in from the capsule. However, the detailed aspects of cellular changes are different in the two models. In the diabetic rat lens, fiber cell swelling was demonstrated to be the earliest detectable change, whereas in the NPPB-treated lens extracellular space dilations were the predominant feature. Despite this difference our results suggests that the two phenomena are related and in fact enable us to formulate a hypothesis that explains the distinct localization of the liquefaction zone in the diabetic rat lens. The sorbitol loading of fiber cells in the diabetic rat lens causes an osmotic insult that would normally be compensated by opening chloride and cation channels to release osmolytes and water. Our results show that only the peripheral fiber cells have an $E_{Cl}$ which favors the release of chloride ions, thereby enabling them to regulate their volume.

Figure 8. Model of chloride movement in control and NPPB-treated lenses. Radial differences in the membrane potential (−65 to −45 mV) of fiber cells relative to the extracellular space means that the direction of chloride flux ($J_{Cl}$) changes from an influx into fiber cells deeper in the lens to an efflux from fiber cells at the lens periphery. Blocking chloride channels is thus expected to cause peripheral cell swelling and the formation of extracellular dilations deeper in the lens.

Although this exchanger is present in the lens, it is thought to be quiescent at normal pH. The fact that DIDS but not SITS induced damage in the fiber cells indicates that the actions of DIDS were mediated via the inhibition of chloride channels and not the Cl$^-$/HCO$_3$ exchanger. Third, in patch-clamp experiments on isolated fiber cells, NPPB and tamoxifen both inhibited a chloride channel.

Our findings that water uptake and consequential tissue damage can be induced by blockage of chloride channels in the resting lens constitute evidence that the movement of chloride ions and water normally occurs under steady state conditions and not only when the lens is challenged osmotically. In fact, the existence of a steady state chloride flux is entirely consistent with the lens circulation model. In this model differences in the electromotive potential of surface versus interior membranes are thought to drive the flow of ions and water into and out of the lens. This flux is directed inward via the extracellular spaces at the poles and outward at the lens equator via an intracellular pathway mediated by gap junction channels. By measuring radial differences in membrane potential and the ionic concentration of chloride in the whole lens, one can calculate the electrochemical gradient for chloride ion movement, $E_{Cl}$, as a function of radial distance. One would predict that chloride will move from the extracellular space into fiber cells in the inner lens, but will move from the cytoplasm of fiber cells to the extracellular space in the lens periphery (Fig. 8A). Therefore, one would expect that an inhibition of chloride channels in the inner lens would block the uptake of chloride from the extracellular space by fiber cells. This would cause an accumulation of chloride ions and water in the tortuous extracellular space and the subsequent formation of extracellular space dilations (Fig. 8B). In the lens periphery the efflux of chloride ions from fiber cells would be blocked, thereby causing an intracellular accumulation of osmolytes and resultant fiber cell swelling.

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Deeper in the equatorial region, the osmotic insult would lead to the opening of volume-regulated chloride channels, which because of the \( E_{\text{Cl}} \) in this region would cause an influx rather than an efflux of chloride ions and water. This would lead to an increased rate of fiber cell swelling and ultimately tissue liquefaction in this region. An important step toward testing this hypothesis is the molecular identification and localization of chloride channels in the equatorial fiber cells.

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**References**