The Effects of Acetazolamide on the Electroretinographic Responses in Rats

Oliver Findl, Ronald M. Hansen, and Anne B. Fulton

Purpose. To study the mechanisms and sites of action of the carbonic anhydrase inhibitor, acetazolamide (AZM), on the rod- and cone-mediated electroretinogram (ERG) of the dark-adapted rat.

Methods. After a within-subjects design, ERG responses to brief, full-field flashes were recorded from adult (60 to 90 days old) albino rats, with and without AZM (5 mg/100 g, intraperitoneally). Flickering stimuli (6 and 26 Hz) were used to study rod- and cone-mediated responses. Aspartate-isolated responses of the isolated retina were recorded with and without AZM in the superfusate. The a-wave and PIH responses were fitted with a model of the rod’s response by estimating the maximum response \( R_mF \), sensitivity \( S \), and delay \( \tau_c \). The b-wave response amplitude and implicit time were examined as a function of stimulus energy. The parameters obtained in the AZM-treated and untreated conditions were compared.

Results. Acetazolamide causes a significant decrease in saturated rod response, b-wave amplitude, aspartate-isolated PIH, and the rod- and cone-mediated responses to flickering light. The estimated sensitivity of the rod response \( S \), b-wave sensitivity, and b-wave implicit time are not altered significantly by AZM.


Carbonic anhydrase (CA), a ubiquitous enzyme, catalyses the reaction of carbon dioxide and water to carbonic acid, which then dissociates to bicarbonate and hydrogen ions. The enzyme thus increases the effectiveness of the bicarbonate buffer system and is, therefore, of great importance in regulating tissue pH. In the posterior segment of the eye, CA is found in retinal pigment epithelial (RPE) cells, Müller cells, and the majority of cones, but not in rods.1,2 Carbonic anhydrase inhibitors (CAIs), such as acetazolamide (AZM), acidify the neural retina primarily in the vicinity of the photoreceptors by inhibiting CA in RPE and Müller cells.3,4 When CA is inhibited, the acid efflux from retinal cells, especially the photoreceptors, is buffered less efficiently, and acidification results.4 Thus, AZM’s effect on the rod photosensitivity appears to be mediated by RPE and Müller cells. Acetazolamide does not affect the photoreceptor response.5 To our knowledge, the effect of AZM on isolated cones has not been studied.

Because hydrogen ions suppress the dark current of rods,6 acidification predicts attenuation of the photoreceptor response. Indeed, acidification attenuates the photoreceptor response and b-wave response of the ERG in some studies.7,8,9 Therefore, one would expect the receptors’ responses and the b-wave response of the ERG to be attenuated by AZM. Despite this, in the intact ERG, CAIs are reported both to decrease10 and to increase11–13 ERG b-wave amplitude. The effect of CAIs on the ERG a-wave, generated by the photoreceptor’s response to flashes of light, has been studied less extensively.12

The effects of AZM on the rod photosensitivity, as assessed by the electroretinographic (ERG) a-wave in the intact rat and the aspartate-isolated rod response from the retina isolated from its RPE, are examined. The
Effect of AZM on the intact scotopic ERG b-wave also is examined. Acetazolamide's effect on cone- and rod-mediated responses are assessed with flickering light.

METHODS

Experiment 1: ERG Responses of Rats

Adult (60 to 90 days old) Sprague–Dawley albino rats were tested using a repeated measures design. They were randomly assigned to acetazolamide (AZM; 5 mg/100 g, intraperitoneally [Sigma, St. Louis, MO]) or no AZM (control) 30 minutes before anesthesia. Three days later, they were tested in the complementary condition—no AZM or AZM.

All rats were maintained on a 12-hour light/12-hour dark cycle. Before the experiments, each rat was dark-adapted overnight. This investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Under dim red illumination, each rat was lightly anesthetized (sodium pentobarbital 4 mg/100 g, intraperitoneally), and the left pupil was dilated with 1% cyclopentolate. The cornea was anesthetized with 0.5% proparacaine. A bipolar Burian-Allen-type electrode was placed on the cornea, and a ground electrode was placed on the tail. Electroretinographic responses to flashes were recorded using a Nicolet (Madison, WI) Compact 4 (AC coupled 1 to 1000 Hz; 1000 gain).

Thirteen rats were tested with white stimuli (Nova-tron [Dallas, TX] strobe, series 2100; <1 msec duration) delivered through a 41-cm diameter integrating sphere, controlled in intensity by calibrated neutral-density filters. The stimuli ranged from those that evoked a small b-wave (<15 μV) to those that saturated the a-wave amplitude. Intervals between flashes were at least 15 seconds for low-intensity flashes and 1 minute for bright flashes, and they were selected so that successive flashes did not attenuate the amplitudes of a- or b-wave responses. The unattenuated white flash, measured with a detector (S-350; United Detector Technology, Orlando, FL) placed at the position of the rat's eye, was 3.7 log μW/cm²·flash at the cornea and was estimated to produce ~10⁴OT isomerizations per rod per flash. Responses to every other flash in the intensity series are shown. Calibration bar = 100 μV. (lower panel) The amplitudes of the b-waves, shown in the records above, are plotted as a function of log relative stimulus energy. For the control condition, Vₘₐₓ (equation 2) is 151 μV, log σ is −4.53 log units, and n is 0.71. For the acetazolamide condition, the values of these parameters are 128 μV, −4.49 log units, and 0.74. In both conditions, the Naka-Rushton equation (equation 2) provides a good fit (r² = 0.99).

Derivation of Rod Photoresponse Characteristics

The photoresponse characteristics were calculated using, in a fashion similar to that of Hood and Birch,¹⁴ the formulation

\[ P₃(i, t) = \left[ 1 - \exp\left( -i \cdot S \cdot (t - t_d)^2 \right) \right] \cdot Rm_{p3} \text{ for } t > t_d. \]  

This formulation has similarities with the quantitative model of the processes involved in the activation of phototransduction described by Lamb and Pugh.¹⁵
In equation 1, $i$ is the number of photoisomerizations caused by the stimulus, and $t$ the time after flash onset. $S$ is a sensitivity parameter in units sec$^{-1}$, $Rm_{\text{ref}}$ is the estimated saturated rod response amplitude, and is $t_d$ a brief delay.

To estimate the number of isomerizations for this calculation, the following were assumed: the prerreceptor absorbance, a specific axial rhodopsin density of 0.016 mm$^{-1}$, an average rod outer segment diameter of 1.52 $\mu$m, an average rod outer segment length of 20 $\mu$m, and quantum efficiency of isomerization of 0.67. Thus, log relative stimulus energy (503 nm) = 0 was estimated to produce $\sim 10^{4.96}$ isomerizations per flash in the rods of the intact eye. We assumed that the responses of the dark-adapted retina are rod dominated because a-wave stimulus–response functions obtained with white, red, blue, and 503-nm stimuli were superimposable after horizontal shifting. The a-waveforms obtained with each of these stimuli were similar.

A least squares minimization (fmin) procedure based on the simplex algorithm in the Math (The Math Works, Natick, MA) package was used to estimate $Rm_{\text{ref}}$, $S$, and $t_d$. Fitting was restricted to the leading edge of the a-wave response before obvious intrusion of the b-wave. Sample a-wave records and fits of the model are shown in Figures 2A and 2B. The Naka–Rushton function

$$V/V_{\text{max}} = i^n/(i^n + \sigma^n)$$

was fitted to the b-wave amplitudes of each rat with an iterative procedure that minimized the mean square deviation of the data from equation 2. Each parameter was free to vary. In this equation, $V$ is the b-wave amplitude, $V_{\text{max}}$ the saturated amplitude, $i$ the number of photoisomerizations caused by the stimulus, and $\sigma$ the value of $i$ that evoked a half-maximum b-wave amplitude. The exponent, $n$, indicates the slope of the function at $\sigma$.

**Experiment 2: Flicker**

A preliminary experiment was performed on three rats to determine the critical flicker fusion response over a range of stimuli produced using a Grass PS-22 strobe (Grass Instruments, Quincy, MA). At each intensity, the frequency at which the response could no longer be detected ($<3$ $\mu$V) was noted. Based on the critical flicker fusion results, 6-Hz flicker was selected to test the rods and 26 Hz to test the cones.

The responses of eight dark-adapted rats to both 6- and 26-Hz flicker were recorded in the control and AZM conditions. At 6 Hz, responses were recorded over a 500-msec epoch, and six sweeps were averaged. At 26 Hz, the sampling time was 160 msec, and 10 sweeps were averaged. The trough-to-peak amplitude of the wavelets was measured, and the mean was calculated.

**Experiment 3: Isolated Retina**

After anesthesia (sodium pentobarbital, 5 mg/100 g, intraperitoneally), the retina was removed under dim red illumination and placed on a tantalum grid, photoreceptor side down, in a chamber. The grid effectively divided the chamber into two compartments, each containing a silver ring electrode. The retina was superfused at a rate of 10 ml/minute, with the following solution: NaCl, 116; KCl, 4.7; NaHCO3, 25; glucose, 7; CaCl2, 2.5; KH2PO4, 1.2; MgSO4, 1.2; NaAsp, 2.5 mmol/l; pH 7.4 (±0.01). This solution isolates PII, the photoreceptor component of the ERG. The solution was bubbled with 95% O2 and 5% CO2. Electroretinographic responses were recorded (AC coupled 1 to 1000 Hz; 1000 gain) before, during, and after AZM (2 mmol/l; pH readjusted after addition) was added to the superfusate, one solution completely replacing the other about 1 minute after a switch. All experiments were performed at room temperature (21°C) rather than body temperature.

The unattenuated white flash at the position of the isolated retina produced 3.9 log $\mu$W/cm$^2$-flash. For derivation of the rod photoreceptor characteristics, equation 1 was fitted to the responses. The unattenuated flash (503 nm) was estimated to produce $\sim 10^{4.96}$ isomerizations per rod per flash. Sample responses and fits of the model (equation 1) are shown in Figures 2C and 2D.

**RESULTS**

For the intact ERG, AZM decreased $Rm_{\text{ref}}$, the saturated amplitude of the rod response, in every rat. The mean amplitude was significantly smaller under AZM than in the control condition (Fig. 3). $Rm_{\text{ref}}$ in the control condition was 113 $\mu$V but only 89 $\mu$V under AZM (paired $t$-test; $t$ = 4.6; $df$ = 12; $P < 0.01$). On average the amplitude under AZM decreased approximately 25% (median decrease, 24.0%; range, 0.9% to 45.3%). The sensitivity parameter, $S$, showed no significant change with the drug (mean ± SE: control, 12.5 ± 1.1 sec$^{-2}$; AZM, 11.6 ± 1.2 sec$^{-2}$; not significant). Also, the estimated delay, $t_d$, did not change with AZM (control, 3.52 ± 0.07 msec; AZM, 3.57 ± 0.12 msec; not significant).

The saturated b-wave amplitude ($V_{\text{max}}$) of every rat was decreased by AZM. The mean amplitudes in the control and AZM conditions were 202 $\mu$V and 150 $\mu$V, respectively ($t$ = 5.6; $df$ = 12; $P < 0.01$). The
FIGURE 2. Sample records and model fits. (A) Intact electroretinographic a-wave responses, in the control condition, to flashes estimated to produce $10^4$ to $10^5$ isomerizations per rod per flash are shown. (B) The model (equation 1) fitted to the a-waves in panel A is shown by the dashed lines. (C) Aspartate-isolated responses, in the control condition, to flashes estimated to produce $10^6$ to $10^7$ isomerizations per rod per flash are shown. (D) The model (equation 1) fitted to the aspartate-isolated responses in panel C is shown by the dashed lines.

The mean critical flicker fusion function is shown in Figure 4A. Sample rod-mediated (6 Hz) and cone-mediated (26 Hz) flicker responses in the control and AZM conditions are shown in Figure 4B. Acetazolamide decreased the amplitude of the rod-mediated response to flicker (6 Hz) in every rat. The mean amplitude (Fig. 5) was 47 $\mu$V in the control condition and 33 $\mu$V under AZM. This decrease was significant ($t = 4.2; df = 7; P < 0.01$). The median decrease was 22.7% (range, 6.1% to 48.0%), similar to the decrease of the saturated rod photoreceptor sensitivity. The percent of AZM-induced decrease in $R_{n0}$ and $V_{n0}$ did not differ significantly.

The mean critical flicker fusion function is shown in Figure 4A. Sample rod-mediated (6 Hz) and cone-mediated (26 Hz) flicker responses in the control and AZM conditions are shown in Figure 4B. Acetazolamide decreased the amplitude of the rod-mediated response to flicker (6 Hz) in every rat. The mean amplitude (Fig. 5) was 47 $\mu$V in the control condition and 33 $\mu$V under AZM. This decrease was significant ($t = 4.2; df = 7; P < 0.01$). The median decrease was 22.7% (range, 6.1% to 48.0%), similar to the decrease of the saturated rod photoreceptor sensitivity. The percent of AZM-induced decrease in $R_{n0}$ and $V_{n0}$ did not differ significantly.

The mean critical flicker fusion function is shown in Figure 4A. Sample rod-mediated (6 Hz) and cone-mediated (26 Hz) flicker responses in the control and AZM conditions are shown in Figure 4B. Acetazolamide decreased the amplitude of the rod-mediated response to flicker (6 Hz) in every rat. The mean amplitude (Fig. 5) was 47 $\mu$V in the control condition and 33 $\mu$V under AZM. This decrease was significant ($t = 4.2; df = 7; P < 0.01$). The median decrease was 22.7% (range, 6.1% to 48.0%), similar to the decrease of the saturated rod photoreceptor sensitivity. The percent of AZM-induced decrease in $R_{n0}$ and $V_{n0}$ did not differ significantly.
Effects of Acetazolamide

**Rod Response**

**Saturated Amplitude**

- Mean (±SE) saturated amplitude ($R_{mP3}$ in equation 1) and sensitivity ($S$ in equation 1) of the rod response, derived from the electretinographic a-waves, are shown.

**Sensitivity $S$**

- The mean (±SE) saturated amplitude ($V_{max}$ in equation 2) and log photoisomerizations at log $\sigma$ (equation 2) of the b-wave are shown.

![Comparison of electroretinographic responses in the control (solid bars) and acetazolamide (open bars) conditions.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933186/)

**B-wave Response**

- The mean (±SE) saturated amplitude ($V_{max}$ in equation 2) and log photoisomerizations at log $\sigma$ (equation 2) of the b-wave are shown.

Acetazolamide decreased the saturated amplitude ($R_{mP3}$ in equation 1) of the aspartate-isolated response of every retina. The mean saturated amplitude in the control condition was 150 μV; in the AZM condition, it was 96 μV ($t = 3.6; df = 3; P < 0.05$). The median decrease was 36.4% (range, 25.4% to 48.5%). Neither the sensitivity parameter, $S$, nor the delay, $t_d$, were significantly altered by AZM. In the control condition, $S$ was 2.6 ± 0.7 sec$^{-2}$, and $t_d$ was 8.61 ± 0.35 msec; under AZM, $S$ was 1.2 ± 0.2 sec$^{-2}$, and $t_d$ was 8.98 ± 0.94 msec (mean ± SE).

**DISCUSSION**

Acetazolamide attenuated the saturated amplitude of the rod photoresponse of every rat and every isolated retina. The saturated b-wave amplitude of the intact ERG was also attenuated in all rats. On average, the attenuation was approximately 25% as it is for both the cone- and rod-mediated responses to flicker. There was, however, no significant change in $S$, rod sensitivity, or b-wave sensitivity, log $\sigma$.

Previous investigations of the effects of CAIs on the intact ERG have yielded discrepant results. Broeders et al. injected the CAI methazolamide (50 mg/kg) into the vitreous of rabbits and found, in accord with our results, that rod- and cone-mediated a- and b-wave amplitudes decreased significantly. Odom et al., on the other hand, found a significant increase in the rabbit b-wave amplitude after only 15 minutes of dark adaptation. They also tested human subjects ($n = 6$) under AZM (8 mg/kg; per os). In the dark-adapted condition, they found a significant increase (8%) in b-wave amplitude and a trend toward an a-wave decrease, whereas under light-adapted conditions, both a- and b-wave amplitudes were reduced. Stanescu and Michaelis reported an increase in the human b-wave amplitude 10 minutes after AZM injection but a return to baseline 5 minutes later. Perhaps unstable conditions of adaptation contribute to the discrepancies.

The lack of an effect on rod sensitivity ($S$) suggested that the gain of the processes involved in the activation of phototransduction was not altered. Thus, AZM's attenuation of the photoresponse by approximately one quarter to one third caused no detectable effect on the activation of phototransduction. The attenuation of response amplitude was probably caused by a decrease in the dark current, which was modified by alterations in retinal pH. This could have resulted from an indirect effect of retinal pH on intracellular Ca++ concentration or by direct interaction of hydrogen ions with the cation channels, causing the photoreceptor's resting membrane potential to hyperpolarize and attenuating the saturated photoresponse. Such a change in dark current would not have affected the receptor's sensitivity, as our results indicate.

As mentioned, in the intact cat eye, AZM applied systemically has been shown to acidify the retina as assessed by intraretinal pH measurements. This pH change is thought to originate in the drug's effect on CA primarily in the RPE and subsequently the membrane bicarbonate ion transport. This resulted in less effective buffering of the acid load produced by rod glycolysis that maintained the dark current of the rod. Such acidification, or increase in protons, is known to suppress the dark current in isolated rods and, in general, to attenuate the ERG. Protons possibly do this by decreasing Na$^+$/Ca++ exchange in the rods or by competing with Ca++ for binding sites. Both processes increase Ca++ levels, which in turn...
Flicker Response

FIGURE 4. Results of the flicker experiments. (A) Mean (±SE) flicker fusion frequency is shown as a function of log relative stimulus intensity. The solid arrow indicates the flicker rate used to obtain rod-dominated responses, and the dashed arrow indicates that used to obtain cone-dominated responses. Log relative stimulus intensity = 0 corresponds to Grass 16. (B) Representative flicker responses in the control (left) and acetazolamide (right) conditions. The top panels show rod-mediated flicker (6 Hz) response in the control and acetazolamide conditions. The lower panels show the cone-mediated flicker (26 Hz) response in the control and acetazolamide conditions. Calibration bars are shown for each panel.

downregulate cGMP,\textsuperscript{31} to decrease the dark current—that is, the light-sensitive Na\textsuperscript{+} conductance in the rod outer segment.

Acetazolamide’s other site of action is the Müller cell. Throughout the nervous system, glial cells are involved in extracellular pH regulation, and Müller cells have been shown to do so as well.\textsuperscript{4} As with inhibition of CA in the RPE, the AZM effect mediated by Müller cells is to decrease the buffering capacity of the retina’s extracellular fluid compartment.\textsuperscript{32} This results in acidification and a subsequent decrease in dark current. Our results in the isolated retina, which lacked RPE, are consistent with inhibition of CA in Müller cells. In the intact eye, in which RPE and Müller cells might have mediated the CAI effect, the relative contribution of the RPE and Müller cells was not assessed. We did note that the change in saturated response was slightly larger in the isolated retina than in the intact ERG (36% versus 24%). The effect of AZM on the b-wave response might have been secondary to the effects on the rods, mediated by pH effects on the b-wave generators, or by a direct effect on Müller glial cells.

Acidification of the retina also inhibits energy metabolism in the inner segment of the rod.\textsuperscript{33,34} A decrease or even a depletion of guanosine triphosphate, adenosine triphosphate, or both, in the rod affects the activation steps in the phototransduction cascade, slowing down closing of the light-sensitive cation channels in the ROS.\textsuperscript{34} Such a change in activation altered the sensitivity parameter, $S$, which estimated the gain of the processes involved in phototransduction. In the isolated retina, $S$ was smaller under AZM than under control conditions, although this difference was not statistically significant. For the intact ERG responses, the values of $S$ in the control and AZM conditions were similar. Possibly, CA inhibition by AZM was more complete in the isolated retina than in the intact eye, causing more depression of inner segment metabolism. Alternatively, in the intact eye, compensatory
mechanisms might have counteracted the metabolic depression that accompanied the acidification of the retina.

The photoreceptor’s dark current accounts for approximately 40% of the retina’s O₂ consumption and sets high energy demands on the cell. Slight reductions in the dark current are likely to have substantial effects on reducing the metabolic needs of photoreceptors. This might be a way of “resting” photoreceptor cells. Creating slight acidity in the extracellular space surrounding the photoreceptors, with little change in plasma pH, could perhaps achieve this goal. Possibly, this is the mechanism underlying the beneficial effect of AZM treatment in a patient with retinitis pigmentosa.

Key Words
acetazolamide, photoreceptor, phototransduction, rats, electroretinogram

Acknowledgments
The authors thank Dr. J. Chunguang for help with some of the experiments.

References
28. Yamamoto F, Borgula GA, Steinberg RH. Effects of


