Published in final edited form as:

Exp Eye Res. 2007 September ; 85(3): 413–422.

Streptozotocin-induced Diabetes Modulates GABA Receptor Activity in Rat Retinal Neurons

David J. Ramsey^{a,b,c}, Harris Ripps^{a,b,d}, and Haohua Qian^{a,b}

*a*Department of Ophthalmology and Visual Sciences, University of Illinois College of Medicine, 1855 West Taylor Street, Chicago, Illinois 60612, USA.

*b*Department of Physiology and Biophysics, University of Illinois College of Medicine, 1855 West Taylor Street, Chicago, Illinois 60612, USA.

*c*Department of Health Policy and Administration, University of Illinois College of Medicine, 1855 West Taylor Street, Chicago, Illinois 60612, USA.

*d*Department of Anatomy and Cell Biology, University of Illinois College of Medicine, 1855 West Taylor Street, Chicago, Illinois 60612, USA.

Abstract

Neural deficits suggestive of involvement of the GABA signaling pathway can often be detected early in the course of diabetic retinopathy, a leading cause of blindness in the United States. To examine in greater detail the nature of the neuronal changes associated with hyperglycemia, we investigated GABA receptor activity on retinal bipolar cells in streptozotocin-induced diabetic rats; cells from age-matched normal rats served as controls. Patch-clamp recordings from isolated rod-bipolar cells revealed that diabetes enhanced the whole cell currents elicited by GABA. Responses of the GABA_C receptor, the predominant GABA receptor on rat rod bipolar cells, exhibited a greater sensitivity to GABA, larger maximum current responses, slower response kinetics, and a smaller single channel conductance among diabetic cells relative to those recorded from normal controls. Compared with the properties of homomeric $\rho 1$ and heteromeric $\rho 1\rho 2$ receptors formed in a heterologous expression system, these results suggested that there was a greater contribution from the $\rho 1$ subunit in the GABA_C receptor-mediated response of diabetic cells. The levels of mRNA, measured with real-time RT-PCR, were consistent with this finding. There was a significant enhancement in the ratio of $\rho 1/\rho 2$ subunit expression in the retina of diabetic animals, although the levels of GABA $\rho 1$ subunit expression were comparable in diabetic and normal retinas. Taken together, the results suggest that diabetes modifies the subunit composition of the GABA_C receptor on retinal neurons, most likely through its effect on the efficacy of gene transcription.

Keywords

diabetic rat; GABA_C receptor; hyperglycemia; rod-bipolar cell; real-time PCR; patch clamp

Corresponding author: Dr. Haohua Qian, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W. Taylor Street, Chicago, IL 60612, USA, Phone: 1-312-413-7347, Fax: 1-312-996-7773, Email: hqian@uic.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Diabetic retinopathy is a common complication of diabetes and is a leading cause of blindness in the United States (EDIC, 1999). Although diabetic retinopathy is typically diagnosed by abnormalities in the retinal microvasculature after prolonged hyperglycemia, there is well-documented evidence to indicate that neural deficits occur early in the course of the disease (see Fletcher et al, 2005, Barber, 2003 for review). Particular relevant to this study is that diabetes alters the GABA signaling pathway of the inner retina. For example, electroretinographic recordings from diabetic patients often exhibit reductions in the amplitude and implicit time of the oscillatory potentials (Tzekov and Arden, 1999), a GABA-sensitive component of the flash ERG (Wachtmeister, 1998;Tzekov and Arden, 1999;Wachtmeister, 2001). An altered GABA inhibitory signal in the diabetic retina is also supported by evidence of disrupted processing of lateral inhibition (Davies and Morland, 2002), and the fact that GABA levels are elevated in the vitreous of patients with proliferative diabetic retinopathy (Ambati et al., 1997).

Studies of diabetic animal models also reveal aberrations in GABA activity. Changes in the content and localization of GABA are evident in the diabetic retina, with both amacrine and Müller cells accumulating high concentration of GABA (Ishikawa et al., 1996a,b;Takeo-Goto et al., 2002). In addition, the activities of metabolic enzymes for GABA are also modified (Ishikawa et al., 1996a;Vilchis and Salceda, 1996;Honda et al., 1998), and the diabetic rat retina exhibits an enhanced capacity to transport GABA (Vilchis and Salceda, 1996). However, despite the substantial evidence indicating that GABA signaling within the inner retina is affected in diabetes, there is little information about the activity of GABA receptors in response to hyperglycemia.

In a previous study (Ramsey et al., 2006), we established a streptozotocin-induced diabetes model in pigmented (Long Evans) rats, and characterized the global changes in neuronal activity in the retina by means of electroretinography. The results indicated that pathways mediating inhibitory signaling in the inner retina are affected by the disease. In addition, our results provided preliminary evidence that the GABA responses of rod-bipolar cells were altered in diabetes. In the present study, we investigated the modulatory effects of hyperglycemia on the GABA_C receptors present on retinal neurons, with a focus on the GABA_C receptor activity of rod-driven bipolar cells. The GABA_C receptors on retinal bipolar cells have a simple molecular composition that primarily consists of GABA ρ 1 and ρ 2 subunits (Qian and Ripps, 2001;Zhang et al., 2001). These receptors are expressed predominately on the axon terminals of retinal bipolar cells to control signal outflow from the second order neuron in the retina. Patch-clamp recordings from isolated retinal neurons revealed a number of changes in GABA_C receptor activity in the diabetic retina; in particular, an enhanced sensitivity to GABA, slower response kinetics, and a smaller single channel conductance. These results, and those obtained by real time RT-PCR, suggest that hyperglycemia affects the GABA ρ subunit composition of GABA_C receptors on retinal neurons.

Materials and Methods

All experimental procedures conformed to the statement on animal care of the Association for Research in Vision and Ophthalmology, and adhered to the guidelines for the Care and Use of Laboratory Animals formulated by the Animal Committee of the University of Illinois, College of Medicine.

Animals

The procedures for creating STZ-induced diabetic rats were described previously (Ramsey et al., 2006). Briefly, young rats (72 ± 10 days of age, weighing 219 ± 7 g) were injected

intravenously with streptozotocin (60 mg/kg, Sigma, St. Louis, MO) dissolved in 0.9% Na citrate buffer (pH 4.5). Blood glucose levels were measured 48-72 hrs after STZ administration, and a value >350 mg/dl was considered to be diabetic. For simplicity, cells derived from the retinas of these hyperglycemic animals are referred to throughout the text as “diabetic”. Experiments were performed between 8 and 25 weeks following the injection of STZ; age-matched normal animals served as controls.

Cell isolation and patch-clamp recording

Solitary bipolar cells were isolated from the rat retina according to published protocols (Qian and Dowling, 1995). Rats were euthanized in a CO₂ chamber, eyes were enucleated and hemisected, and the retinas gently removed from the posterior eyecup and immersed for 40 min in a modified Ames media (supplemented with 0.88 g/l NaCl, 2.36 g/l HEPES, and 10,000 units/l penicillin/streptomycin, pH 7.4) containing 2 mg/ml papain (EMD Biosciences, San Diego, CA) and 1 mg/ml of L-cysteine (Sigma, St. Louis, MO). After several brief washes, the tissue was triturated through a sterile pipette, and aliquots of the supernatant containing dissociated cells were placed in culture dishes containing the modified Ames medium. The cells were maintained at room temperature for up to 8 hours, and prior to recording, the culture medium was replaced with an extracellular solution consisting (in mM) of NaCl (120), KCl (5), NaHCO₃ (25), CaCl₂ (2), MgCl₂ (1), HEPES (10), dextrose (10), pH 7.4. Whole-cell membrane currents were obtained with a patch pipette filled with an intracellular solution containing (in mM): CsCl (130), KCl (4), CaCl₂ (1), MgCl₂ (2), EGTA (11), HEPES (10), MgATP (1), NaGTP (0.2), pH 7.4. A Perfusion Fast-Step system (Warner Instrument Co., Hamden, CT) was used to deliver drugs onto the cell. The solution changing rate, measured by liquid junction potential, was 25 ms (Qian et al., 2005). Rod bipolar cells were identified by their characteristic morphology. Cells were held at -60 mV, and the GABA-elicited responses were recorded with an Axopatch 200B amplifier controlled by pCLAMP software (Axon instrument, Union City, CA); data were analyzed and plotted using Origin software (Microcal, Northampton, MA). The dose-response data derived from the GABA-elicited current responses of bipolar cells from diabetic and control animals were fit to the Hill equation:

$$\frac{I}{I_{\max}} = \frac{[C]^{\eta}}{[C]^{\eta} + EC_{50}^{\eta}}$$

where I is the membrane current elicited by GABA at concentration [C], I_{max} is the maximum amplitude at response saturation, η is the Hill coefficient (an index of cooperativity), and EC₅₀ is the GABA concentration that gives rise to a half-maximal response.

Noise analysis

The procedure for noise analysis of GABA_C receptor-mediated current responses recorded both from isolated rat rod bipolar cells and HEK cells expressing rat GABA_ρ subunits followed a modified protocol described previously (Qian and Dowling, 1995). Specifically, a 2 Hz zero-phase digital filter (Matlab, The Math Works, Inc, Natick, MA) was used to better separate noise associated with channel activity from variation of baseline and decay of mean response for GABA-elicited currents. Since GABA_C receptors exhibit slow kinetics (Chang and Weiss, 1999; Qian et al., 2005), the responses were divided into several one-second segments, from which the mean (I) and variance (σ²) were calculated. As shown by Palmer (Palmer, 2006), the relation between the mean and the variance can be described by a parabolic function. Since the maximum of variance depends upon the channel open probability, the parabolic function is most useful when the channel open probability is high. When the open probability is low, as is the case for the GABA_C receptor activity on mammalian bipolar cells, a linear fit is more appropriate as it introduces fewer variables. Thus, the derived I and σ² values could be fitted with a linear equation of the form:

$$\sigma^2 = iI + \sigma_0^2$$

where σ_0^2 is baseline variance, and i (the single channel current) is given by the slope of the function.

Heterologous expression of rat GABA_C receptors

HEK293 cells (ATCC, Manassas, VA) seeded at a density of 5×10^5 cells/35 mm dish were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. The cells were transiently transfected with the desired plasmids using Lipofectamine Plus (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol. The rat $\rho 1$ and $\rho 2$ cDNAs, kindly provided by Dr. Stuart Lipton (Burnham Institute for Medical Research) (Zhang et al., 1995), were subcloned into the pcDNA 3.1 eukaryotic expression plasmid (Invitrogen). 1.5 μ g of ρ subunit plasmid (either the $\rho 1$ subunit alone or as a 1:1 mixture of $\rho 1$ and $\rho 2$ subunits) and 0.5 μ g pEGFP (Invitrogen) were used per dish for transfection. The transfected cells were identified under an epifluorescent microscope, and patch clamp experiments were performed 24-48 hours after transfection.

Real time RT-PCR

Approximately 50 ng of total retinal RNA was used for each real-time RT-PCR reaction, performed with a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) using a one-step RT-PCR reaction with the SYBR Green Quantitative RT-PCR Kit (Sigma, St. Louis, MO) according to established protocols (Alden et al., 2002). The following reaction conditions were used: 50°C (30 min) for reverse transcription, 95°C (2 min) for initial denaturing, 50 cycles of 95°C (15 s), 54°C (20 s), 72°C (30 s), and a signal acquisition period of 2 s at 84°C. The specific primers (and respective cDNA position from the starting codon) were: 5'-AGCCAGATGGGAAAGTGCT-3' (526 - 544) and 5'-GTCTTCTGTGTAGGCATAGCTTTC-3' (656 - 633) for the rat $\rho 1$ subunit; 5'-TTCCCCTGTTCTGTTGTTTC-3' (-57 to -38) and 5'-GTCCGGATCTTGGTACATC-3' (154 - 135) for rat $\rho 2$ subunit with expected amplicons of 131 and 211 bp, respectively. Standard curves were generated using known amounts of *in vitro* transcribed mRNA with amplification efficiency of 99.9%. A 60S ribosomal protein L7 was used as a control, and the gene was amplified with the following primers: 5'-GAAGCTCATCTATGAGAAGGC-3' and 5'-AGACGGAGCAGCTGCAGCAC-3' (Alden et al., 2002). Negative controls without templates did not generate any significant signal.

Results

GABA-mediated responses of bipolar cells

To extend our previous observations on the diabetic modification of the GABA_C receptor activity on retinal bipolar cells (Ramsey et al., 2006), we examined the property of GABA-elicited responses from retinal bipolar cells derived from STZ-induced diabetic and normal rats. Rod bipolar cells in the rat retina display a characteristic morphology, i.e., a rounded cell body from which extends several dendrites and a long axon that terminates in a prominent expansion (Fig. 1A). In agreement with earlier reports (Fletcher et al., 1998), these cells constitute the majority of bipolar cells isolated from the rat retina. The morphological criteria for rod bipolar cells were confirmed in separate experiments by positive staining for PKC α , a specific marker for rod bipolar cells (data not shown).

Examples of the GABA (3 μ M)-elicited membrane current recorded from bipolar cells of normal and diabetic retina are illustrated in the left hand pair of traces shown in Fig. 1B. In each case, with the membrane potential held at -60 mV, application of GABA generated a

sustained inward current that deactivated slowly when the drug was withdrawn. Because GABA was delivered by bath superfusion, the whole-cell currents derive from the activation of all the GABA receptors present on the cell membrane, which is dominated, in turn, by the large concentration of receptors at the axon terminal. Before considering the greater response amplitude of the diabetic cell, it is important to recall that although both GABA_A and GABA_C receptors are present on rod-driven retinal bipolar cells, the GABA-elicited currents reflect almost exclusively activation of the GABA_C receptors (Feigenspan et al., 1993; Euler and Wassle, 1998). This feature is evident in the recordings obtained after co-applying bicuculline (100 μ M), a potent and specific blocker of GABA_A receptor channels, with the test solution (right hand pair of traces in Fig. 1B). Note that bicuculline induced a relatively small reduction in response amplitudes, but that the GABA-elicited current from the 'diabetic' bipolar cell is much larger than that elicited from the 'control' cell. The consistency of this finding is evident in Fig. 1C, which summarizes the results from a series of experiments both with and without the addition of bicuculline. The responses recorded from diabetic bipolar cells were significantly larger (-319 ± 9 pA, $n = 47$) than those from control cells (-217 ± 8 pA, $n = 55$) ($p < 0.001$) prior to application of bicuculline, and the difference persisted in the bicuculline-resistant, GABA_C-mediated responses of diabetic (-256 ± 9 pA, $n = 47$) and controls (-171 ± 8 pA, $n = 55$) ($p < 0.001$). Similarly, bicuculline-sensitive (i.e. GABA_A receptor mediated) currents were significantly enhanced in diabetic cells ($p < 0.01$). However, the GABA_A receptor-mediated responses were small and not examined further in this study.

Because of the predominance of the GABA_C receptor on rat bipolar cells (Fletcher et al. 2004; Ramsey et al., 2006), further studies on the effects of hyperglycemia were focused on GABA_C receptor activity, i.e., recorded in the presence of bicuculline. Fig. 2 illustrates the concentration-response amplitude relation for the GABA_C receptor-mediated currents of bipolar cells from normal and diabetic animals. Examples of current traces in response to three different GABA concentrations are shown in Fig. 2A. To compare the GABA sensitivities of the responses elicited from normal ($n = 8$) and diabetic ($n = 7$) cells, the data were normalized to their respective amplitudes in response to 100 μ M GABA (Fig. 2B). In the case of the control animals, the data were fitted to the Hill equation with an EC₅₀ of 4.95 μ M and a slope (η) of 1.41. In contrast, the responses of cells from the diabetic retina were well fit with an EC₅₀ of 1.72 μ M and η of 2.2. A statistical analysis of these data indicated that diabetes significantly increased the sensitivity to GABA ($p < 0.01$) and enhanced the cooperativity ($p < 0.01$) of the GABA_C receptor-mediated responses elicited from rod bipolar cells.

Kinetics of GABA_C receptor currents

Current responses of the GABA_C receptor, whether recorded from neurons or from cells expressing GABA ρ subunits, are characterized by their slow kinetics, a parameter that reflects the subunit composition of the receptor (Qian et al., 1998). This feature is useful when comparing the time course of deactivation (the GABA-offset response) seen in the GABA_C-mediated responses of bipolar cells isolated from normal (Fig. 3A) and diabetic (Fig. 3B) retinas. It is evident that in addition to being of larger amplitude, the responses elicited from diabetic bipolar cells exhibit slower kinetics than those from control cells. This can clearly be seen in Fig. 3C, which plots normalized GABA-deactivation responses of both bipolar cells. Both traces could be fitted by a single exponential decay function (thin line). The average time constant (τ) was 1.57 ± 0.06 sec ($n = 58$) for control cells, and 1.77 ± 0.06 sec ($n = 39$) for diabetic bipolar cells ($p < 0.05$) (Fig. 3D). The slower response kinetics of the GABA_C response recorded from diabetic bipolar cell provides further evidence to suggest that the GABA_C receptors on these cells may have a different subunit composition than that on normal cells.

Single channel properties of the GABA_C receptors on retinal bipolar cells

Noise-analysis techniques were used to probe the single channel properties of the GABA_C receptor on retinal bipolar cells. Fig. 4 shows an example of the fluctuations in the GABA-elicited currents from bipolar cells derived from control (A) and diabetic (B) retinas. A 2 Hz zero-phase digital filter was used to separate the mean current and associated noise (plotted above for clarity). The entire recording was divided into several one-second segments, and the mean and variance of the current response for each segment was calculated. As shown in Fig. 4C there is a linear relation between the mean current and the variance, and the slope of the line equals the single channel current. The averaged results from a number of normal and diabetic cells, summarized in Fig. 4D, show that the single channel conductance was 1.26 ± 0.08 pS ($n=49$) and 0.94 ± 0.09 pS ($p < 0.05$; $n=34$) for control and diabetic cells, respectively. These data indicate that, despite the larger GABA-induced currents of diabetic bipolar cells, hyperglycemia induces a significant reduction in the single channel conductance of the GABA_C receptor. This finding is a further indication that the subunit composition of the rod bipolar cells has been affected by hyperglycemia.

Rat $\rho 2$ subunit alters the properties of the GABA_C receptors formed in HEK cells

The results from patch clamp recording of rat bipolar cells reveal profound changes in the GABA_C receptor properties under diabetes. To investigate whether these changes are indeed an indication that the subunit composition of the GABA_C receptors has been altered, we compared the responses elicited from homomeric rat $\rho 1$ receptor and heteromeric $\rho 1\rho 2$ receptor formed in HEK cells, a commonly used mammalian expression system that lacks endogenous GABA receptors. Examples of the GABA (10 μ M)-elicited current responses of homomeric rat $\rho 1$ receptors and heteromeric $\rho 1\rho 2$ receptor are shown in Figs. 5A and 5B, respectively. The currents from both types of receptor exhibit the slow, sustained response characteristics of the GABA_C receptor. However, co-expressing the $\rho 2$ subunit with the $\rho 1$ subunit accelerated GABA inactivation. As shown in the bar graph (clear bars) of Fig. 5F, the average time constant of GABA inactivation for homomeric rat $\rho 1$ receptors was 17.5 ± 2.8 seconds, whereas the time constant for heteromeric $\rho 1\rho 2$ receptors was 7.0 ± 1.4 seconds.

The large GABA-induced current responses recorded from HEK cells transiently expressing GABA ρ subunits (Figs. 5A and 5B) tend to preclude accurate noise analysis. Accordingly, we used brief applications of GABA to elicit small current responses that generate proportionately greater noise signals. Examples of these responses are shown in Fig. 5C (for the homomeric $\rho 1$ receptor), and Fig. 5D (for the heteromeric $\rho 1\rho 2$ receptor); the associated noise isolated by a 2 Hz zero-phase digital filter is shown above each trace. The mean and variance of the current response for each 1-second segment are shown in Fig. 5E, where the data were fitted with a linear equation (see Methods). The very large differences in the slope of the function indicate that the expressed $\rho 1$ and $\rho 1\rho 2$ receptors exhibit grossly different signal channel currents. The averaged conductances (hatched bar graphs of Fig. 5F) show that the homomeric $\rho 1$ receptor has a much lower single channel conductance (0.25 ± 0.06 pS) than that of the heteromeric $\rho 1\rho 2$ receptor (1.23 ± 0.21 pS).

Transcription levels of GABA_C receptor subunits in diabetic retina

The various changes revealed by patch clamp recordings of GABA_C receptor activity from rod bipolar cells isolated from the diabetic retina suggest that the subunit composition of the receptor has been affected by hyperglycemia. When the response properties of the GABA_C receptor are compared with those elicited from $\rho 1$ and $\rho 1\rho 2$ receptors formed in HEK cells, it appears likely that expression of the $\rho 1$ subunit of the GABA_C receptor relative to that of the $\rho 2$ subunit has been enhanced in rod bipolar cells from the diabetic retina. To directly probe the effects of hyperglycemia on the subunit composition of the GABA_C receptor, we measured the mRNA expression levels of GABA $\rho 1$ and $\rho 2$ subunits using real-time RT-PCR techniques.

The results obtained from samples of diabetic and control retinas are summarized in Fig. 6. The amount of RNA in each sample was determined by the value of Ct, the cycle number for the amount of PCR product to reach the fluorescence threshold; a housekeeping gene, 60S ribosomal protein L7, served as control. When normalized to L7 expression, the expression level of the GABA $\rho 1$ subunit was slightly higher (i.e. ΔCt value lower) in the diabetic than in the normal retina (Fig. 6A), whereas the level of the $\rho 2$ subunit was slightly lower (ΔCt value higher) in the diabetic retina (Fig. 6B). However, when the ratio of the $\rho 1/\rho 2$ subunit expression was calculated, the approximately three-fold difference between the two was significant (Fig. 6C).

Discussion

GABA_C receptors are prominently expressed in the vertebrate retina, and they constitute the majority of the GABA receptor population on rod bipolar cells in the rat retina (Feigenspan et al., 1993; Euler and Wassle, 1998; Lukasiewicz et al., 2004). The GABA_C receptors are thought to be formed by GABA ρ subunits, and each of the three ρ subunits cloned thus far is expressed in retina (Zhang et al., 1995; Ogurusu and Shingai, 1996). The GABA $\rho 1$ and $\rho 2$ subunits have been detected on bipolar cells, whereas the GABA $\rho 3$ subunit has been shown to be expressed on cells located in the ganglion cell layer (Enz et al., 1995; Ogurusu et al., 1997). In this study, we examined a number of pharmacological and physiological properties of the GABA_C receptors on rod bipolar cells isolated from the retinas of STZ-induced diabetic rats and from normal age-matched control animals. Our findings provide strong evidence that diabetes produces a number of significant changes in the response properties of GABA_C receptors. When compared with the results obtained from normal controls, the GABA_C receptor-mediated responses recorded from diabetic bipolar cells exhibit larger response amplitudes, a greater sensitivity to GABA (Figs. 1 and 2), and slower kinetics (Fig. 3). In addition, using noise analysis techniques we estimated the single channel properties of the GABA_C receptors present on rod bipolar cells; the derived values were comparable to those published previously (Feigenspan et al., 1993; Qian and Dowling, 1995; Palmer, 2006; Zhu et al., 2007). Although noise analysis tends to underestimate the value of single channel conductance, the same bias should apply results obtained from normal and diabetic cells. Our study revealed a smaller single channel current for the GABA_C receptors on diabetic bipolar cells (Fig. 4). To our knowledge, this is the first study to demonstrate the extensive diabetes-induced modifications in GABA receptor activity of retinal neurons.

Although the precise subunit composition of the native GABA_C receptor has yet to be determined, it is generally believed that the GABA_C receptors on rat bipolar cells are heteromeric complexes formed, at least in part, by $\rho 1$ and $\rho 2$ subunits (Zhang et al., 1995). Experimental evidence indicates that homomeric receptors formed either by GABA $\rho 1$ or $\rho 2$ subunits exhibit distinct physiological characteristics, and the properties of heteromeric $\rho 1\rho 2$ receptors depend upon the relative expression levels of each subunit gene (Enz and Cutting, 1999; Pan et al., 2006). This is evident, for example, in the *Xenopus* oocyte expression system where the rat $\rho 1$ subunit readily forms functional receptors that give rise to large current response to GABA, whereas the rat $\rho 2$ subunit requires a prolonged expression time to generate relatively small responses (Zhang et al., 1995; Alakuijala et al., 2005). Thus, the electrophysiological data we recorded from the GABA_C receptors on diabetic rod bipolar cells suggest that its subunit composition has been altered, resulting possibly in an enhanced contribution from the GABA $\rho 1$ subunit.

To investigate further the subunit composition of the GABA_C receptor on retinal neurons, we took two approaches: (i) an electrophysiological study of the kinetics and single channel properties of ρ subunits heterologously expressed in HEK cells, and (ii) by measuring the expression levels of GABA $\rho 1$ and $\rho 2$ subunits in the retina using real-time PCR techniques.

When expressed in HEK cells, the receptors formed by the rat $\rho 1$ subunit exhibited very slow response kinetics; incorporating the rat $\rho 2$ subunit accelerated the response (Fig. 5). Similar findings have been reported by (Alakuijala et al., 2005) for rat ρ receptors, and by others (Enz and Cutting, 1999; Pan et al., 2006) for receptors formed by human and perch GABA ρ subunits. In addition, incorporating the rat $\rho 2$ subunit increased the single channel conductance of the GABA_C receptor (Fig. 5). If the GABA ρ subunits in retinal neurons behave similarly to those expressed in HEK cells, these results provide a good indication that any modification in the subunit composition of GABA_C receptors on the bipolar cells of diabetic animals results in an enhanced contribution from the GABA $\rho 1$ subunit, or conversely, a diminished contribution from the GABA $\rho 2$ subunit.

The large current response of cells from diabetic animals is difficult to reconcile with the smaller single channel conductance of their GABA_C receptors. However, a possible mechanism to account for this apparent disparity is the likelihood that the predominant GABA $\rho 1$ subunits exhibit a high open channel probability. Our preliminary results indicate that the receptors formed by GABA $\rho 1$ subunits display a significantly higher probability of channel opening than those formed by GABA $\rho 2$ subunits (data not shown). Other mechanisms may also contribute to the response properties of GABA_C receptors on diabetic bipolar cells. For example, it has been shown that members of the GABA_A receptor family are capable of co-assembling with GABA ρ subunits to form functional receptors (Qian and Ripps, 1999; Qian and Pan, 2002; Milligan et al., 2004; Pan and Qian, 2005). Diabetes could also affect the contribution of these subunits in the formation of GABA_C receptors on retinal neurons. Furthermore, protein modification, such as glycosylation, phosphorylation, and interactions with cytoplasmic proteins could also alter the GABA_C receptor activity on diabetic retinal bipolar cells (Filippova et al., 1999; Hanley et al., 1999; Croci et al., 2003; Song et al., 2005).

Like many other cell types, neurons control their levels of mRNA as an economic way to regulate protein expression (Morris, 1993). Determining the mRNA level of GABA $\rho 1$ and $\rho 2$ subunits in the diabetic retina thus provides a useful means to examine the GABA_C receptor modulation by hyperglycemia. Using real time RT-PCR to determine the expression levels of GABA $\rho 1$ and $\rho 2$ subunits, we found that whereas the levels of the GABA $\rho 1$ subunit in control and diabetic retinas were approximately the same, there was a reduction in the level of the $\rho 2$ subunit in the diabetic retina; i.e., the ratio of expressed subunits ($\rho 1/\rho 2$) was enhanced (Fig. 6). It should be noted, however, that the mRNA for determination of the levels of ρ subunits were extracted from the entire retina, and it is not possible to state with certainty how the ratio in any given cell was altered.

In a previous study (Ramsey et al., 2006), we demonstrated that the distribution pattern of the GABA_C receptor was not significantly altered by hyperglycemia. GABA_C receptor staining was concentrated mainly in the inner plexiform layer and faint labeling was also seen in the outer plexiform layer, representing most likely the receptors present on the axon terminals and dendrites of rod and cone bipolar cells in the rat retina (Enz et al., 1996; Ramsey et al., 2006). Using immunostaining as a semi-quantitative measure of receptor expression in the retina, we observed that IPL staining was slightly weaker (~20%) in diabetic retinal sections than controls, whereas no significant changes were detected for labeling in the OPL (data not shown). Although the results are in agreement with the observed reduction of GABA ρ subunit expression in the diabetic retina, there is, unfortunately, no antibody available that is suitable for Western analysis of the GABA_C receptor.

The present study has focused on the alteration of GABA_C receptor activity in the retina of the STZ-induced diabetic rat, but it is important to note that hyperglycemia also affects other signal transmission pathways in the retina (Vilchis and Salceda, 1996; Kowluru et al., 2001; Li and Puro, 2002; Takeo-Goto et al., 2002; Ng et al., 2004; Ward et al., 2005; Santiago et al.,

2006a;Santiago et al., 2006b). Moreover, diabetes-induced alterations in synaptic transmission and synaptic proteins have been reported in many other parts of the central nervous system. For example, cannabinoid receptor expression is increased (Duarte et al., 2007) and synaptic transmission is altered (Kamal et al., 2006) in hippocampus, NMDA receptors are down regulated in primary sensory cortical neurons (Bean et al., 2006), and postsynaptic density protein 95 and presynaptic proteins (synapsin I and synaptotagmine) are increased in the hypothalamus (Lechuga-Sancho et al., 2006). Therefore, diabetic modulation of synaptic transmission could be a common theme in the central nervous system. Whether the altered gene expression of the GABA_C receptor in the diabetic retina is a direct response to hyperglycemia or a compensatory response to modified synaptic transmission merits further study.

In conclusion, our results have shown that diabetes modulates the properties of GABA_C receptors on retinal bipolar cells, most likely through alterations in the gene expression of GABA receptor subunits. In combination with other changes in GABAergic pathway, these changes could underlie the observed early neurosensory defects detected in diabetic patients. Although both hyperglycemia and reduced insulin signaling have been implicated in many diabetic complications, high blood glucose levels are thought to be the main cause of retinopathy (Davis et al., 1998). Multiple mechanisms of hyperglycemia-induced cell damage have been proposed (Brownlee, 2001), but the events that lead specifically to changes in GABAergic neurons have not been identified. Understanding the molecular mechanism of this modulatory pathway will enhance our understanding of the pathophysiological basis of diabetic retinopathy, provide a foundation for designing novel screening tests, and be of help in developing preventive measures and new therapeutic approaches for the treatment of diabetic patients.

Acknowledgments

The authors would like to thank Dr. Ralph Enz for a generous gift of the GABA_C receptor antibody, Dr. Stuart Lipton for providing rat GABA ρ 1 and ρ 2 subunits and Ms. Ruth Zelkha for her expert assistance with imaging and fluorescence analysis. This work was supported by grants from the National Eye Institute (EY-12028, EY-06516, and EY-01792), an unrestricted departmental award from Research To Prevent Blindness (RPB) to the Department of Ophthalmology and Visual Sciences, and a Medical Student Eye Research Fellowship (DJR), Senior Investigator Award (HR), and Career Development Award (HQ) from RPB. Additional support was provided by grants from the Illinois Society to Prevent Blindness, the Midwest Eye Banks, Sigma Xi (DJR) and by a UIC Provost Award (DJR).

References

- Alakuijala A, TalviOja K, Pasternack A, Pasternack M. Functional characterization of rat ρ 2 subunits expressed in HEK 293 cells. *Eur J Neurosci* 2005;21:692–700. [PubMed: 15733087]
- Alden KJ, Goldspink PH, Ruch SW, Buttrick PM, Garcia J. Enhancement of L- type Ca(2+) current from neonatal mouse ventricular myocytes by constitutively active PKC-betaII. *Am J Physiol Cell Physiol* 2002;282:C768–774. [PubMed: 11880265]
- Ambati J, Chalam KV, Chawla DK, D'Angio CT, Guillet EG, Rose SJ, Vanderlinde RE, Ambati BK. Elevated gamma-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. *Arch Ophthalmol* 1997;115:1161–1166. [PubMed: 9298058]
- Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. *Prog Neuropsychopharmacol Biol Psychiatry* 2003;27:283–290. [PubMed: 12657367]
- Bean L, Zheng H, Patel KP, Monaghan DT. Regional variations in NMDA receptor downregulation in streptozotocin-diabetic rat brain. *Brain Res* 2006;1115:217–222. [PubMed: 16938282]
- Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;414:813–820. [PubMed: 11742414]
- Chang Y, Weiss DS. Channel opening locks agonist onto the GABA_C receptor. *Nat Neurosci* 1999;2:219–225. [PubMed: 10195213]

- Croci C, Brandstatter JH, Enz R. ZIP3, a new splice variant of the PKC- ζ -interacting protein family, binds to GABA_C receptors, PKC- ζ , and Kv β 2. *J Biol Chem* 2003;278:6128–6135. [PubMed: 12431995]
- Davies NP, Morland AB. The Hermann-Hering grid illusion demonstrates disruption of lateral inhibition processing in diabetes mellitus. *Br J Ophthalmol* 2002;86:203–208. [PubMed: 11815348]
- Davis MD, Fisher MR, Gangnon RE, Barton F, Aiello LM, Chew EY, Ferris FL 3rd, Knatterud GL. Risk factors for high-risk proliferative diabetic retinopathy and severe visual loss: Early Treatment Diabetic Retinopathy Study Report #18. *Invest Ophthalmol Vis Sci* 1998;39:233–252. [PubMed: 9477980]
- Duarte JM, Nogueira C, Mackie K, Oliveira CR, Cunha RA, Kofalvi A. Increase of cannabinoid CB(1) receptor density in the hippocampus of streptozotocin-induced diabetic rats. *Exp Neurol* 2007;204:479–484. [PubMed: 17222407]
- EDIC. Epidemiology of Diabetes Interventions and Complications (EDIC). Design, implementation, and preliminary results of a long-term follow-up of the Diabetes Control and Complications Trial cohort. *Diabetes Care* 1999;22:99–111. [PubMed: 10333910]
- Enz R, Cutting GR. GABA_C receptor ρ subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. *Eur J Neurosci* 1999;11:41–50. [PubMed: 9987010]
- Enz R, Brandstatter JH, Wassle H, Bormann J. Immunocytochemical localization of the GABA_C receptor ρ subunits in the mammalian retina. *J Neurosci* 1996;16:4479–4490. [PubMed: 8699258]
- Enz R, Brandstatter JH, Hartveit E, Wassle H, Bormann J. Expression of GABA receptor ρ 1 and ρ 2 subunits in the retina and brain of the rat. *Eur J Neurosci* 1995;7:1495–1501. [PubMed: 7551175]
- Euler T, Wassle H. Different contributions of GABA_A and GABA_C receptors to rod and cone bipolar cells in a rat retinal slice preparation. *J Neurophysiol* 1998;79:1384–1395. [PubMed: 9497419]
- Feigenspan A, Wassle H, Bormann J. Pharmacology of GABA receptor Cl⁻ channels in rat retinal bipolar cells. *Nature* 1993;361:159–162. [PubMed: 7678450]
- Filippova N, Dudley R, Weiss DS. Evidence for phosphorylation-dependent internalization of recombinant human ρ 1 GABA_C receptors. *J Physiol (Lond)* 1999;518:385–399. [PubMed: 10381587]
- Fletcher EL, Koulen P, Wassle H. GABA_A and GABA_C receptors on mammalian rod bipolar cells. *J Comp Neurol* 1998;396:351–365. [PubMed: 9624589]
- Hanley JG, Koulen P, Bedford F, Gordon-Weeks PR, Moss SJ. The protein MAP-1B links GABA_C receptors to the cytoskeleton at retinal synapses. *Nature* 1999;397:66–69. [PubMed: 9892354]
- Honda M, Inoue M, Okada Y, Yamamoto M. Alteration of the GABAergic neuronal system of the retina and superior colliculus in streptozotocin-induced diabetic rat. *Kobe J Med Sci* 1998;44:1–8. [PubMed: 9846053]
- Ishikawa A, Ishiguro S, Tamai M. Changes in GABA metabolism in streptozotocin-induced diabetic rat retinas. *Curr Eye Res* 1996a;15:63–71. [PubMed: 8631205]
- Ishikawa A, Ishiguro S, Tamai M. Accumulation of gamma-aminobutyric acid in diabetic rat retinal Muller cells evidenced by electron microscopic immunocytochemistry. *Curr Eye Res* 1996b;15:958–964. [PubMed: 8921217]
- Kamal A, Biessels GJ, Gispen WH, Ramakers GM. Synaptic transmission changes in the pyramidal cells of the hippocampus in streptozotocin-induced diabetes mellitus in rats. *Brain Res* 2006;1073-1074:276–280. [PubMed: 16455062]
- Kowluru RA, Engerman RL, Case GL, Kern TS. Retinal glutamate in diabetes and effect of antioxidants. *Neurochem Int* 2001;38:385–390. [PubMed: 11222918]
- Lechuga-Sancho AM, Arroba AI, Frago LM, Garcia-Caceres C, de Celix AD, Argente J, Chowen JA. Reduction in the number of astrocytes and their projections is associated with increased synaptic protein density in the hypothalamus of poorly controlled diabetic rats. *Endocrinology* 2006;147:5314–5324. [PubMed: 16873533]
- Li Q, Puro DG. Diabetes-induced dysfunction of the glutamate transporter in retinal Muller cells. *Invest Ophthalmol Vis Sci* 2002;43:3109–3116. [PubMed: 12202536]
- Lukasiewicz PD, Eggers ED, Sagdullaev BT, McCall MA. GABA_C receptor-mediated inhibition in the retina. *Vision Res* 2004;44:3289–3296. [PubMed: 15535996]

- Milligan CJ, Buckley NJ, Garret M, Deuchars J, Deuchars SA. Evidence for inhibition mediated by coassembly of GABA_A and GABA_C receptor subunits in native central neurons. *J Neurosci* 2004;24:9241–9250.
- Morris BJ. Control of receptor sensitivity at the mRNA level. *Mol Neurobiol* 1993;7:189–205. [PubMed: 7514000]
- Ng YK, Zeng XX, Ling EA. Expression of glutamate receptors and calcium-binding proteins in the retina of streptozotocin-induced diabetic rats. *Brain Res* 2004;1018:66–72. [PubMed: 15262206]
- Ogurusu T, Shingai R. Cloning of a putative gamma-aminobutyric acid (GABA) receptor subunit rho 3 cDNA. *Biochim Biophys Acta* 1996;1305:15–18. [PubMed: 8605242]
- Ogurusu T, Eguchi G, Shingai R. Localization of gamma-aminobutyric acid (GABA) receptor rho 3 subunit in rat retina. *Neuroreport* 1997;8:925–927. [PubMed: 9141066]
- Palmer MJ. Functional segregation of synaptic GABAA and GABAC receptors in goldfish bipolar cell terminals. *J Physiol* 2006;577:45–53. [PubMed: 17008372]
- Pan Y, Qian H. Interactions between ρ and γ_2 subunits of the GABA receptor. *J Neurochem* 2005;94:482–490. [PubMed: 15998298]
- Pan Y, Ripps H, Qian H. Random assembly of GABA ρ_1 and ρ_2 subunits in the formation of heteromeric GABA_C receptors. *Cell Mol Neurobiol* 2006;26:289–305. [PubMed: 16767514]
- Qian H, Dowling JE. GABA_A and GABA_C receptors on hybrid bass retinal bipolar cells. *J Neurophysiol* 1995;74:1920–1928. [PubMed: 8592185]
- Qian H, Ripps H. Response kinetics and pharmacological properties of heteromeric receptors formed by coassembly of GABA ρ - and γ_2 -subunits. *Proc R Soc Lond B Biol Sci* 1999;266:2419–2425.
- Qian, H.; Ripps, H. The GABA_C receptors of retinal neurons. In: Kolb, H.; Ripps, H.; Wu, S., editors. *Progress in Brain Research*. Elsevier Science B.V.; 2001. p. 295–308.
- Qian H, Pan Y. Co-assembly of GABA ρ subunits with the GABA_A receptor γ_2 subunit cloned from white perch retina. *Brain Res Mol Brain Res* 2002;103:62–70. [PubMed: 12106692]
- Qian H, Dowling JE, Ripps H. Molecular and pharmacological properties of GABA- ρ subunits from white perch retina. *J Neurobiol* 1998;37:305–320. [PubMed: 9805275]
- Qian H, Pan Y, Zhu Y, Khalili P. Picrotoxin accelerates relaxation of GABA_C receptors. *Mol Pharmacol* 2005;67:470–479. [PubMed: 15509715]
- Ramsey DJ, Ripps H, Qian H. An electrophysiological study of retinal function in the diabetic female rat. *Invest Ophthalmol Vis Sci* 2006;47:5116–5124. [PubMed: 17065533]
- Santiago AR, Pereira TS, Garrido MJ, Cristovao AJ, Santos PF, Ambrosio AF. High glucose and diabetes increase the release of [3H]-D-aspartate in retinal cell cultures and in rat retinas. *Neurochem Int* 2006a;48:453–458. [PubMed: 16513217]
- Santiago AR, Rosa SC, Santos PF, Cristovao AJ, Barber AJ, Ambrosio AF. Elevated glucose changes the expression of ionotropic glutamate receptor subunits and impairs calcium homeostasis in retinal neural cells. *Invest Ophthalmol Vis Sci* 2006b;47:4130–4137. [PubMed: 16936133]
- Song XQ, Meng F, Ramsey DJ, Ripps H, Qian H. The GABA ρ_1 subunit interacts with a cellular retinoic acid binding protein in mammalian retina. *Neuroscience* 2005;136:467–475. [PubMed: 16198491]
- Takeo-Goto S, Doi M, Ma N, Goto R, Semba R, Uji Y. Immunohistochemical localization of amino acids in the diabetic retina of Goto-Kakizaki rats. *Ophthalmic Res* 2002;34:139–145. [PubMed: 12097796]
- Tzekov R, Arden GB. The electroretinogram in diabetic retinopathy. *Surv Ophthalmol* 1999;44:53–60. [PubMed: 10466588]
- Vilchis C, Salceda R. Effect of diabetes on levels and uptake of putative amino acid neurotransmitters in rat retina and retinal pigment epithelium. *Neurochem Res* 1996;21:1167–1171. [PubMed: 8923476]
- Wachtmeister L. Oscillatory potentials in the retina: what do they reveal. *Prog Retin Eye Res* 1998;17:485–521. [PubMed: 9777648]
- Wachtmeister L. Some aspects of the oscillatory response of the retina. *Prog Brain Res* 2001;131:465–474. [PubMed: 11420963]
- Ward MM, Jobling AI, Kalloniatis M, Fletcher EL. Glutamate uptake in retinal glial cells during diabetes. *Diabetologia* 2005;48:351–360. [PubMed: 15688208]

- Zhang D, Pan ZH, Awobuluyi M, Lipton SA. Structure and function of GABA_C receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol Sci* 2001;22:121–132. [PubMed: 11239575]
- Zhang D, Pan ZH, Zhang X, Brideau AD, Lipton SA. Cloning of a γ -aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. *Proc Natl Acad Sci U S A* 1995;92:11756–11760. [PubMed: 8524843]
- Zhu Y, Ripps H, Qian H. A single amino acid in the second transmembrane domain of GABA ρ receptors regulates channel conductance. *Neurosci Lett* 2007;418:205–209. [PubMed: 17398006]

Figure 1.

(A) A phase contrast image showing the typical morphology of a rod bipolar cell isolated from the rat retina (scale bar = 10 μm). (B) The membrane current responses elicited by GABA (3 μM) from a control and diabetic bipolar cell. Both control and diabetic bipolar cells have a major response component, mediated by GABA_C receptors, that is insensitive to the co-application of 100 μM bicuculline with GABA. (C) Bar graph illustrating the significant differences in the peak amplitudes of the responses recorded from normal (n = 55) and diabetic (n = 47) rod bipolar cells; shown is for the total GABA-activated current and for GABA_C receptor mediated responses, as well as the GABA_A mediated response estimated by their difference (**, p<0.01; ***, p< 0.001).

Figure 2.

Dose-response relation of the GABA_C receptor-mediated responses from rod bipolar cells. Examples of current responses elicited from a normal (**A**) and a diabetic bipolar cell (**B**) by application of 3, 10, and 30 μ M GABA in combination with 100 μ M bicuculline to block activation of the GABA_A receptor. (**C**) Averaged dose-response data of 7 cells from diabetic animals, and 8 bipolar cells from age-matched control retinas. Data were normalized to their respective maxima, which were 415 pA for control and 506 pA for diabetic cells. The curves were fit by the Hill equation with EC₅₀ values of 1.72 μ M and 4.95 μ M, and Hill coefficients of 2.2 and 1.41 for diabetic and control cells, respectively.

Figure 3.

The membrane current responses mediated by GABA_C receptors from a control (A) and a diabetic (B) bipolar cell. Note the slower current decay of the diabetic cell at drug offset. The normalized inactivation time course for the two responses is shown in (C). Both traces can be fit with a single exponential function (thin lines). (D) Bar graphs illustrating the average time constants (τ) show that diabetic bipolar cells (n=39) had significantly slower relaxation kinetics than control cells (n=58) (*, $p < 0.05$).

Figure 4.

Single channel properties of the GABA_C receptors on retinal bipolar cells. Example of GABA-elicited (3 μ M) currents responses from a control (**A**) and diabetic (**B**) bipolar cells. A 2-Hz digital filter was used to separate the noise associated with each response (plotted above each trace). (**C**) The relationship between the variance and the mean of the GABA-elicited current fluctuations from control and diabetic bipolar cells. The larger single channel conductance for the control bipolar cell is indicated by the steeper slope, and averaged values (**, $p < 0.01$) are shown in the bar graphs (**D**); ($n = 49$ for control and $n = 34$ for diabetic cells).

Figure 5.

GABA responses elicited from receptors formed by ρ subunits expressed in HEK cells. The whole cell currents were elicited by application of 10 μ M GABA to cells expressing the rat $\rho 1$ subunit alone (A), and from cells containing a mixture of rat $\rho 1$ and $\rho 2$ subunits (B). The smaller currents evoked by brief application of GABA are shown in (C) and (D), and the digitally-filtered noise associated with each trace is illustrated above the recordings. The mean vs variance plot of each response segment is shown in (E). Bar graphs in F show the time constants of GABA inactivation, and the single channel conductances for homomeric $\rho 1$ (n=6) and heteromeric $\rho 1\rho 2$ receptors (n=10; **, p<0.01).

Figure 6.

Expression of GABA p1 and p2 subunits mRNA in the retina. (A) Bar graphs illustrate the difference in Ct value (ΔCt) between the GABA p1 subunit and the house keeping gene L7, measured with real-time RT-PCR. Lower ΔCt values for diabetic sample indicate slight higher p1 subunit expression than for normal retinas. (B) Bar graph shows the differences in Ct values between the p2 subunit and the housekeeping gene L7. (C) The ratio of p1/p2 subunits measured from normal and diabetic retinas. There is a significant difference (**, $p < 0.01$) in the values obtained from normal and diabetic retinas.