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α_2-Adrenoceptor Agonists Inhibit Vitreal Glutamate and Aspartate Accumulation and Preserve Retinal Function after Transient Ischemia

JOHN E. DONELLO, EDWIN U. PADILLO, MICHELLE L. WEBSTER, LARRY A. WHEELER, and DANIEL W. GIL

Department of Biological Sciences, Allergan, Inc., Irvine, California

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ABSTRACT

Recent studies have suggested that α_2-adrenergic agonists prevent neuronal cell death in a number of animal models, although the mechanism of α_2-neuroprotection remains unclear. In a retinal ischemia model, the α_2-specific agonist brimonidine (1 mg/kg i.p.) preserves approximately 80% of the electroretinogram (ERG) b-wave. The protective effect of brimonidine is completely blocked by coadministration of the α_2-antagonist rauwolscine. Brimonidine treatment preserves the ERG b-wave if animals are treated 1 or 3 h before ischemia, but has no effect if it is injected during ischemia. The 3-h pretreatment effect is blocked by i.v. injection of rauwolscine 2 h later (1 h before ischemia). A comparison of vitreous humor glutamate levels between untreated and brimonidine-treated eyes shows that 1) after ischemia, glutamate levels rise 2- to 3-fold in the untreated animals, and 2) glutamate levels in the brimonidine-treated animals are comparable to the nonischemic controls. Hence, the mechanism for brimonidine-mediated protection in the retinal ischemia model requires activation of the α_2-adrenergic receptors immediately before and during ischemia. These data suggest that activation of the α_2-adrenergic receptor may reduce ischemic retinal injury by preventing the accumulation of extracellular glutamate and aspartate.

Activation of α_2-adrenoceptors can result in the regulation of multiple signaling pathways and protein targets, some of which are thought to be potentially neuroprotective. α_2-Agonists activate inward rectifying K^+ channels and block voltage-gated Ca^{2+} channels, which hyperpolarizes neurons and inhibits presynaptic neurotransmitter release (Lakhlani et al., 1996). α_2-Adrenoceptors also induce the phosphorylation of mitogen-activated protein kinase and inhibit the cyclic AMP-dependent phosphorylation of cAMP response element-binding protein (Alblas et al., 1993; Fitzgerald et al., 1999). In vivo, systemic administration of the α_2-agonists clonidine and xylazine has been shown to activate the extracellular signal-regulated kinases p42/p44 (Wen et al., 1996).

α_2-Adrenergic agonists significantly improve neurological outcomes in multiple models of brain ischemia. Dexmedetomidine, a selective α_2-agonist, reduced infarct size in a rat forebrain incomplete ischemia model (Hoffman et al., 1991). The drug also reduced central nervous system infarct size in rabbit focal ischemia and gerbil global ischemia (Maier et al., 1993; Koistinaho and Hokfelt, 1997).

Although multiple models have been proposed, the mechanism(s) by which α_2-adrenoceptors prevent neuronal cell death remains unclear. One study has suggested that α_2-agonists prevent ischemia-induced brain damage by inhibiting the accumulation of glutamate (Maier et al., 1993). Consistent with this hypothesis, previous studies have demonstrated that the α_2-agonists dexmedetomidine, mivazerol, and clonidine decrease hypoxia-induced glutamate accumulation in a hippocampal brain slice preparation (Bickler and Hansen, 1996; Talke and Bickler, 1996). A separate study demonstrated that dexmedetomidine partially inhibited the ischemia-induced expression of the immediate early genes c-fos and hsp70 (Wittner et al., 1997). The authors speculated that altered gene regulation may reflect the protective mechanism of dexmedetomidine. Wen et al. (1996) have postulated that α_2-adrenoceptor-mediated transcriptional up-regulation of basic fibroblast growth factor may be sufficient for reduction of light-induced photoreceptor degeneration in rats. The α_2-agonist brimonidine also slows the rate of retinal ganglion cell loss after a partial crush of the rat optic nerve (Yoles et al., 1999). These data suggest that α_2-agonists protect multiple retinal cells from a variety of different insults.

Acute reversible retinal ischemia is an ideal model system for investigating the mechanism by which various compounds reduce ischemia-induced injury. The retina exhibits a well defined structure, is easy to experimentally access, and can be functionally characterized by electroretinography (ERG). These properties enable one to evaluate and quantify the extent of retinal injury electrophysiologically and histologically.

ABBREVIATIONS: ERG, electroretinogram/electroretinography; RGC, retinal ganglion cell; LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; PTI, prior to ischemia.

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Numerous studies suggest that excitotoxicity plays a major role in ischemia-induced retinal cell death; there is a large increase in extracellular glutamate concentrations and glutamate ionotropic antagonists will partially block ischemia-induced damage (Choi and Rothman, 1990). However, the ischemic state is complex and most likely consists of additional insults, including energy depletion and free radicals. The present study demonstrates that pretreatment with α_2-agonists leads to a reduction of ischemic retinal injury and prevents an ischemia-induced rise in extracellular glutamate and aspartate.

Materials and Methods

Retinal Ischemia. Rats were anesthetized with isoflurane and placed onto a warm surface to maintain constant body temperature. Animals' temperature and heart rate were monitored throughout the ischemic period. Pupils were dilated with 1% tropicamide and a drop of Opthaine was instilled in the eye. A 30-gauge cannula, attached to a raised saline reservoir, was placed into the anterior chamber of one eye. The intraocular pressure was raised to over 110 mm Hg, sufficient to induce complete retinal ischemia (Buchi et al., 1991). A hand-held ophthalmoscope was used to visually inspect the retinal blood vessels and verify ischemia. After 50 min, the saline reservoir was lowered and, for 10 min, the intraocular pressure and retinal circulation was allowed to return to normal. The cannula was removed from the cornea and the animals were recovered. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Electrophysiology. Rats were dark adapted for 30 min. A drop of 1% tropicamide was instilled in each eye and the animals were anesthetized with ketamine (65 mg/kg) in combination with xylazine (20 mg/kg). The nonrecorded eye was occluded and subcutaneous needle electrodes were placed in the cheek (reference) and back (ground). The cornea was moistened with Celluvisc, the eyelids gently retracted, a gold ring ERG electrode (i.d. = 3 mm) was placed onto the surface of the cornea and a Grass model 1533 photic stimulator was positioned 10 cm from the eye on the optical axis. ERG responses were elicited by a brief flash of light. Stimuli consisted of brief flashes of 10-μs duration, 0.09 Joule, and delivered at 10-s intervals. The measured values were averaged and the amplitudes of the a- and b-waves were calculated and compared using a Student's *t* test. The ERG response was normalized by dividing the amplitude of the b-wave with the amplitude of the a-wave (Fig. 2). The percentage of protection was calculated by dividing the experimental values by control values and multiplying by 100 {(experimental b-wave + experimental a-wave)/experimental a-wave} − 1/{(control b-wave + control a-wave)/control a-wave} − 1 × 100 to get percentage of protection.

Histology. Animals were euthanized with an overdose of sodium pentobarbital. Both eyes were enucleated and placed in Davidson's fixative overnight. The following day, the eyes were transferred to 10% neutral-buffered formalin. To control the angle of sectioning, the fixed eyes were placed in customized eye block. The block contains a groove with which the ciliary artery was aligned to ensure a consistent plane of blocking. The eyes were then paraffin embedded with the blocked surface flush with the embedding mold. Only sections that included the optic nerve were counterstained with H&E, which ensured that similar sections were comparable between retinas. Images were collected using ImagePro Plus software and then ischemic retinas were visually compared with nonischemic retinas.

Retrograde Labeling of Retinal Ganglion Cells (RGCs). Rats were anesthetized with ketamine (65 mg/kg), xylazine (6.5 mg/kg), and acepromazine (12 mg/kg) and a drop of ophthalmic anesthetic was placed in the right eye. Using a dissecting microscope, a small incision was made in the conjuctiva and, using blunt dissection, the optic nerve was exposed and transected within the dura. Crystals of rhodamine-labeled dextran (3000 mol. wt.; Molecular Probes, Eugene, OR) were placed in the transected site. After 24 h, the animals were euthanized and the eyes were post fixed in 4% paraformaldehyde for 2 h. The retinas were flat mounted onto black nitrocellulose filter paper and RGCs were counted from eight 40× fields that were equal distance from the optic nerve. The fields were averaged for each animal and compared with control eyes using a Student's *t* test.

LC/MS/MS Analysis. Vitreous humor samples collected from the untreated and brimonidine-treated rats were analyzed in a masked manner by high pressure liquid chromatography using an HP 1100 pump and autosampler (Hewlett Packard, Wilmington, DE) equipped with a reversed phase column (LUNA C18, 30 × 2.0 mm, 3-μm particle size) and a linear acetonitrile/10 mM ammonium formate gradient containing 0.5% formic acid at a flow rate of 50 μl/min. The column effluent was introduced into a PE-Sciex API 365 tandem mass spectrometer (PerkinElmer-Sciex, Ontario, Canada) via the Turbo Ionspray interface. Mass spectrometric analysis was performed by multiple reaction monitoring-positive ionization monitoring 148 → 84 *m/z* for glutamate, 132 → 86 *m/z* for leucine, 134 → 74 *m/z* for aspartate, and 154 → 137 *m/z* for dopamine. Calibration standard samples at concentrations of 1.0 to 10,000 ng/ml were prepared by spiking the distilled water with known amounts of analytes. The standards were routinely analyzed with the vitreous humor samples.

Results

Brimonidine Prevents Ischemia-Induced Damage. To determine whether brimonidine can prevent retinal damage in a retinal transient ischemia model, Brown Norway rats were injected i.p. with 1 mg/kg brimonidine 1 h before the transient ischemic insult. Histological examination of the retina 7 days later demonstrated that, compared with saline-treated animals, brimonidine prevents ischemia-induced retinal damage (Fig. 1). Compared with nonischemic retinas (Fig. 1A), the ischemic retinas of the saline-treated animals display significant damage, including the loss of the ordered retinal structure, reduced ganglion cell and inner plexiform layers, and loss of cells in the inner nuclear layer (Fig. 1B). In contrast, ischemic retinas from the brimonidine-treated animals, shown in Fig. 1D, are indistinguishable from the fellow nonischemic retina (Fig. 1C). A masked visual comparison of the nonischemic and brimonidine-treated retinas found that there was no observable loss of the ganglion cell, inner plexiform, or inner nuclear layers. These data demonstrate that the α_2-agonist brimonidine prevents the appearance of overt histological evidence for ischemia-induced structural damage in the retina.

To test whether brimonidine also maintains retinal function after an ischemic insult, retinal function was assessed by ERG. The ERG consists of the a-wave, primarily a measure of photoreceptor cell function, and the b-wave, primarily a measure of bipolar cell function (Kline et al., 1978; Stockton and Slaughter, 1989). A representative ERG response of a saline-treated ischemic eye, shown in Fig. 2B, indicates the ischemic insult selectively abrogates the b-wave response. The a-wave is relatively unchanged, compared with the nonischemic fellow eye (Fig. 2A). Both histological and ERG measures show that injury is limited to the inner retina, consistent with previous studies of this model (Buchi et al., 1991). Brimonidine pretreatment prevents the ischemia-induced loss of the ERG b-wave response (Fig. 2D), which is similar to the nonischemic fellow eye (Fig. 2C). The protection of the

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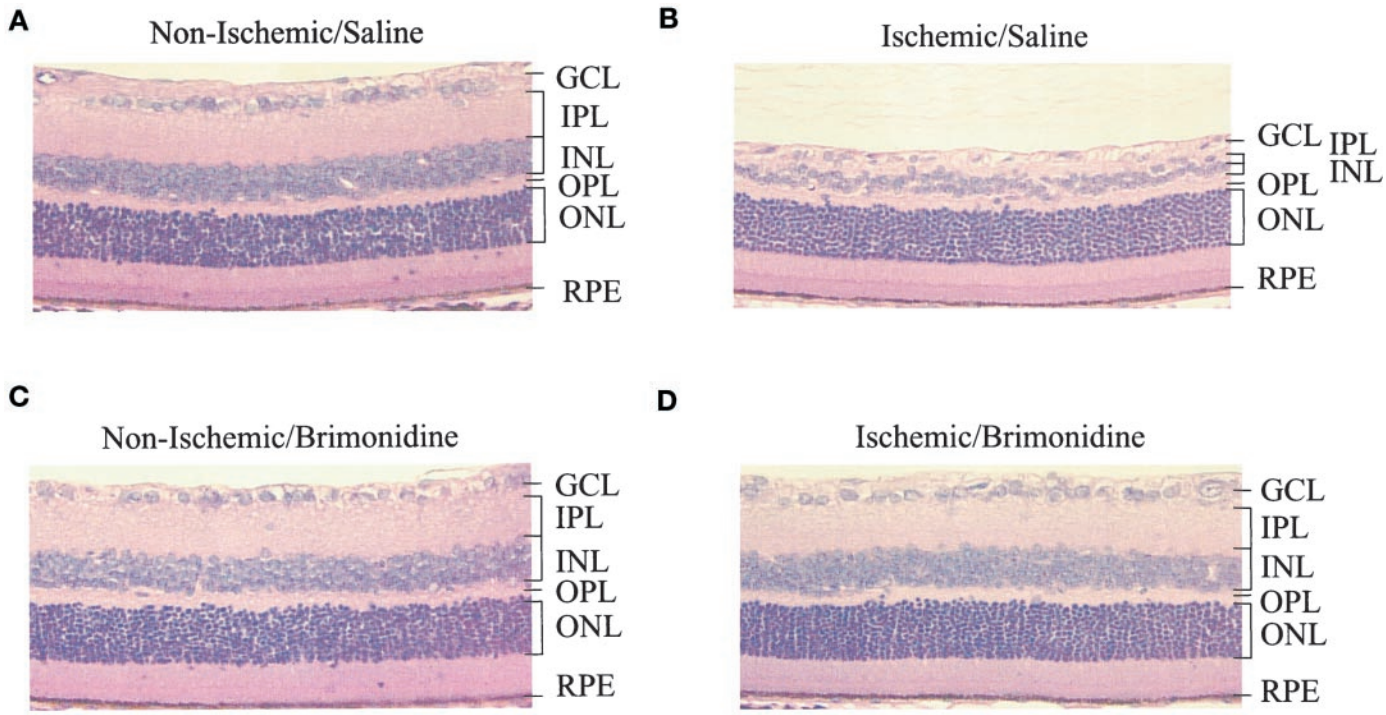

Fig. 1. Brimonidine prevents ischemia-induced damage to the inner retina. Animals were treated with saline or brimonidine (1.0 mg/kg i.p., 1 h before ischemia) and allowed to recover for 7 days. A, nonischemic retina from the saline-treated group. B, ischemic retina from saline-treated group. C, nonischemic retina from the brimonidine-treated group. D, ischemic retina from the brimonidine-treated group. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, pigmented epithelium.

ERG b-wave by brimonidine is dose-responsive (approximately 20% at 0.1 mg/kg and 85% at 0.5 mg/kg) and the α_2-agonist clonidine is also effective at protecting the ERG b-wave (Fig. 2E).

To test whether brimonidine-mediated protection is α_2-adrenoceptor-dependent, animals were cotreated with the α_2-adrenoceptor-specific antagonist rauwolscine (10 mg/kg i.v.) and brimonidine (1 mg/kg i.p.) 1 h before ischemia. Figure 3 demonstrates that brimonidine-mediated protection is blocked by the α_2-antagonist rauwolscine. At high doses rauwolscine may also block 5-hydroxytryptamine receptors, but the 5-hydroxytryptamine antagonist ketanserin did not inhibit the brimonidine-protective effect (data not shown). These data, plus the protective effect of the α_2-agonist clonidine, suggest that the protective effect of brimonidine is mediated by activation of α_2-adrenoceptors.

To assess the ability of brimonidine to preserve retinal ganglion cell viability, ganglion cell retrograde transport of rhodamine-labeled dextran was assessed 7 days after ischemia. The data, shown in Table 1 as number of labeled retinal ganglion cells per field, demonstrate that brimonidine also prevents the loss of retinal ganglion cell retrograde transport induced by ischemia. The protection is dose-responsive and clonidine is as effective as brimonidine. The histology, ERG analysis, and RGC retrograde transport measures demonstrate that activation of α_2-adrenoceptors by α_2-agonists can prevent the loss of multiple retinal cell types and maintain retinal function after an ischemic insult.

Therapeutic Window of Brimonidine in Retinal Ischemia. To determine the therapeutic window for brimonidine treatment, animals were treated with brimonidine at varying times before and during the ischemic insult. Figure 4 demonstrates that animals treated with brimonidine 12 or 6 h before ischemia were not protected from ischemia-induced damage. In addition, brimonidine treatment during ischemia did not rescue the ERG response from ischemic damage. Animals treated 1 h after or 24 h before ischemia were also not protected (data not shown). Brimonidine maintains approximately 80% of the ERG b-wave if animals are treated with brimonidine within 3 h before the start of ischemia. A 30- or 15-min brimonidine treatment before ischemia is sufficient to protect the retina from an ischemic insult. These data demonstrate that the protective mechanism does not require significant time to be activated and that, to preserve retinal function, animals must be treated with brimonidine before the ischemic insult.

To test whether the brimonidine-protective effect requires activation of the α_2-adrenoceptor immediately before and during the onset of ischemia, an agonist pulse-antagonist chase experiment was performed. Animals were treated with brimonidine (1 mg/kg i.p.) 3 h before the ischemic insult. The animals were subsequently treated with rauwolscine (10 mg/kg i.v.) 1 h before ischemia. In this experimental paradigm, brimonidine stimulates the α_2-adrenoceptors for 2 h before rauwolscine administration. Once rauwolscine is administered, brimonidine is blocked from further stimulating the α_2-adrenoceptors. The ERG data, shown in Fig. 4B, demonstrate that rauwolscine blocks the brimonidine-protective effect even if α_2-adrenoceptors have already been stimulated for 2 h. The protective mechanism of brimonidine requires α_2-adrenoceptor stimulation immediately before and during ischemia. The time course, antagonist, and pulse-chase data suggest that brimonidine protects retinal function from ischemic damage by an immediate and acute mechanism that is downstream of α_2-adrenoceptor activation.

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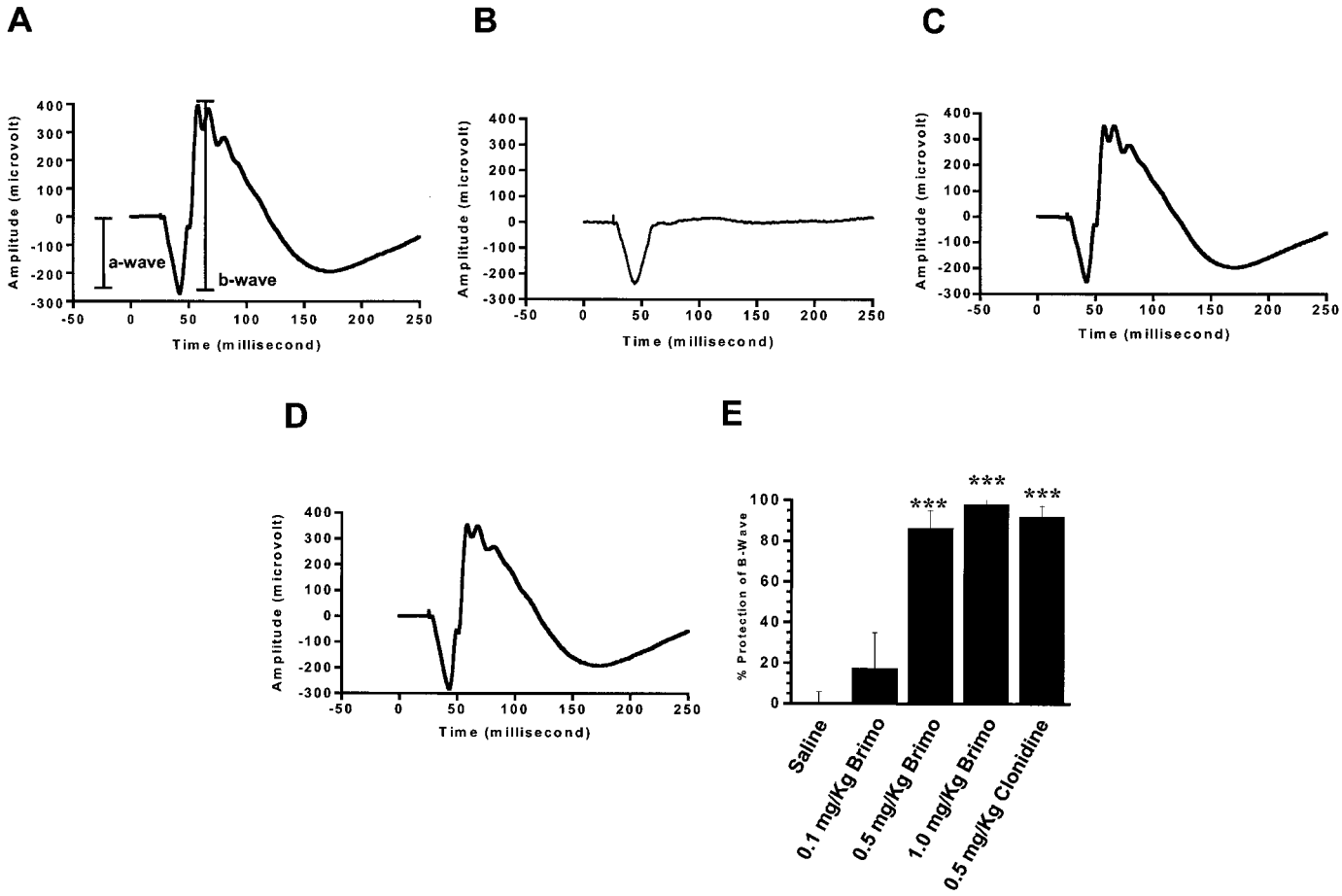

Fig. 2. α_2-Agonists prevent the loss of the ERG b-wave in a dose-responsive manner. Animals were treated with saline or the indicated α_2-agonist 1 h before ischemia. The ERG measurement was taken 7 days after ischemia and represents an average of 10 responses from a single animal. Representative ERG traces of A, nonischemic eye from the saline-treated group; B, ischemic fellow eye from the saline-treated group; C, nonischemic eye from the brimonidine-treated group (1 mg/kg i.p.); D, ischemic eye from the brimonidine-treated group (1 mg/kg i.p.); and E, dose response of ERG b-wave protection by 0.1, 0.5, and 1.0 mg/kg brimondine and 0.5 mg/kg clonidine. All values are the average ± S.E.M. of results from six animals. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Inhibition of Ischemia-Induced Glutamate and Aspartate Accumulation by α_2-Adrenergic Agonists. Excitotoxicity, driven by high extracellular concentrations of glutamate, is thought to play a significant role in ischemia-induced neuronal cell death. Multiple studies have shown that after ischemia, there is a large rise in the extracellular concentration of glutamate. In the retinal ischemia model, an increase in extracellular retinal glutamate would result in an increase of glutamate in the vitreous humor. To determine whether extracellular glutamate concentrations rise after retinal ischemia, vitreous humor was isolated from saline- and brimonidine-treated animals at varying times during and after ischemia. The samples were analyzed by LC/MS/MS for the concentrations of glutamate, aspartate, dopamine, and leucine. Figure 5A demonstrates that brimonidine inhibits an ischemia-induced increase in the vitreal glutamate concentrations. In the nonischemic group, the average vitreal glutamate concentration was approximately 30 μM. Glutamate levels rise during ischemia and continue to rise after ischemia, reaching 60 μM at 30 min after ischemia. In the brimonidine group, the vitreal glutamate concentration is significantly lower than the saline group after ischemia. These data suggest that brimonidine inhibits the vitreal accumulation of glutamate during and after an ischemic insult.

Figure 5B demonstrates that pretreatment with brimonidine also inhibits the ischemia-induced vitreal accumulation of aspartate. In nonischemic eyes, the vitreal aspartate concentration is approximately 4 μM. The vitreal concentrations of aspartate in the saline-treated ischemic eyes increase during and after ischemia, reaching a peak of more than 9 μM at 45 min after ischemia. However, aspartate does not accumulate in the brimonidine-treated ischemic group. Interestingly, the time courses of ischemia-induced aspartate and glutamate accumulation are remarkably similar and brimonidine inhibits accumulation of both.

To determine the specificity of brimonidine-mediated inhibition of glutamate and aspartate accumulation, the vitreal concentrations of the catecholamine dopamine and the amino acid leucine were analyzed. Figure 5C indicates that vitreal dopamine accumulates rapidly immediately after the onset of the ischemic insult. Thirty minutes of ischemia induces a large increase in vitreal dopamine concentrations in the saline- and brimonidine-treated groups, to approximately 12 and 8 μM, respectively, from a nonischemic control dopamine concentration of 1 μM. Brimonidine partially inhibits the rise in vitreal dopamine concentrations because the dopamine concentrations are significantly different between the saline- and brimonidine-treated groups at the 0-, 10-, and 20-min

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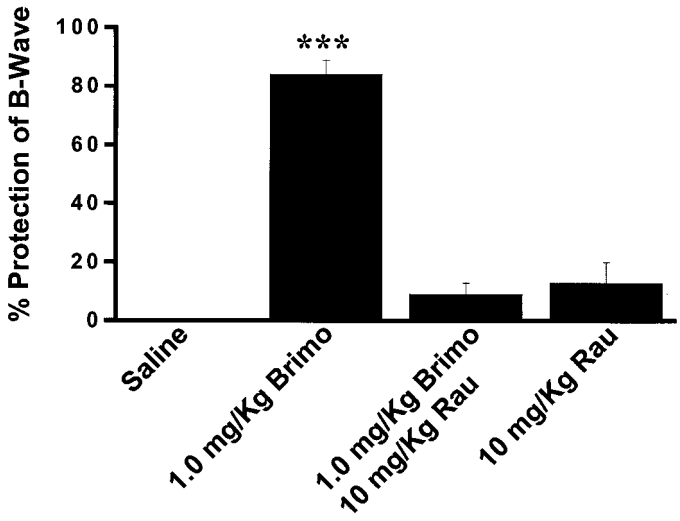

Fig. 3. Protective effect of brimonidine is α_2-adrenoceptor-mediated. Animals were treated 1 h before ischemia with saline (n = 4), 1.0 mg/kg brimonidine i.p. (n = 6), 1.0 mg/kg brimonidine i.p. + 10.0 mg/kg rauwolscine i.v. (n = 11), or 10.0 mg/kg rauwolscine i.v. (n = 5) and allowed to recover for 7 days. All values are the average ± S.E.M. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 1

α_2-Agonists prevent ischemia-induced loss of RGC retrograde transport

Animals were injected i.p. 1 h prior to ischemia (dose as indicated). Animals recovered for 8 days following ischemia. Retinal ganglion cells were retrograde labeled and counted (n = 5). Values indicate the average number of ganglion cells per microscope field ± S.E.M. determined in eight fields from five animals.

Treatment	Mean RGCs/Field	±S.E.M.
Saline/ischemic	135.2	±8.4
0.1 mg/kg brimonidine	129.0	±10.2
0.5 mg/kg brimonidine	197.3**	±11.0
1.0 mg/kg brimonidine	200.0**	±6.3
0.5 mg/kg clonidine	215.0***	±6.0
Nonischemic/saline	252.3***	±6.8

Statistical significance, compared to saline: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

time points after ischemia. Vitreal dopamine concentrations in both groups decrease rapidly, and return to baseline levels 1 h after ischemia. The vitreal dopamine accumulation time course is markedly different from the accumulation time course of aspartate and glutamate. Vitreal leucine concentrations do not change during or after ischemia in either the saline- or brimonidine-treated groups. There is no consistently significant difference from the nonischemic eye (Fig. 5D). The lack of accumulation of leucine and markedly different time course of dopamine accumulation suggest that brimonidine inhibits the vitreal accumulation of aspartate and glutamate during and after an ischemic insult by a specific mechanism.

Figure 6 illustrates that the inhibition of ischemia-induced glutamate accumulation by brimonidine is dose-responsive and α_2-adrenoceptor-dependent. Vitreous humor was isolated 45 min after the end of ischemia. The accumulation of vitreal glutamate was inhibited approximately 30% at 0.1 mg/kg and 95% at 0.5 mg/kg. Topical dosing of brimonidine (0.2 or 0.5%) also inhibited glutamate accumulation in a dose-responsive manner. Clonidine (0.5 mg/kg) also inhibited glutamate accumulation (data not shown). The inhibition of vitreal glutamate accumulation by brimonidine was significantly blocked by coadministration of the α_2-antagonist rauwolscine. The inhibition

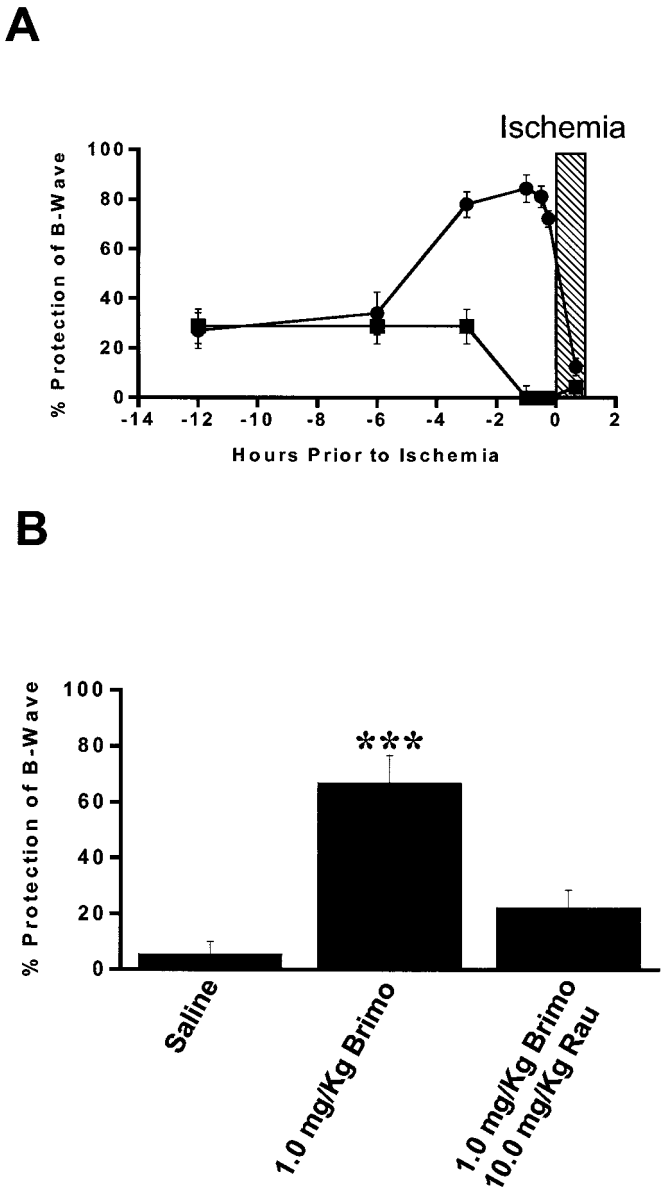

Fig. 4. Brimonidine protects the ERG-b wave by acutely activating α_2-adrenoceptors. A, therapeutic window of brimonidine. Brimonidine-treated animals (●) were dosed 1.0 mg/kg 15 min prior to ischemia (PTI) i.v. (n = 6); 30 min PTI i.p. (n = 5); 60 min PTI i.p. (n = 12); 3 h PTI i.p. (n = 12), 6 h PTI i.p. (n = 15); 12 h PTI i.p. (n = 13); and 40 min into ischemia (n = 8). Saline-treated animals (■) were dosed for 3, 6, and 12 h (n = 8), or for 1 h, 30 min, and 15 min (n = 6). The gray hatched bar represents the 50-min ischemic period. B, brimonidine must activate the α_2-adrenoceptors immediately before ischemia. Animals were treated as indicated and allowed to recover for 7 days: saline (n = 4), brimonidine 3 h PTI i.p. (n = 5), brimonidine 3 h PTI i.p. + rauwolscine 1 h PTI i.v. (n = 12). All values are the average ± S.E.M. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of aspartate accumulation was also dose-responsive and blocked by rauwolscine cotreatment (data not shown).

Discussion

Previous studies have suggested that α_2-adrenergic agonists will decrease ischemia-induced damage in the brain (Maier et al., 1993; Berkman et al., 1998). In this study, we demonstrate that the α_2-agonist brimonidine will protect retinal structure and function from a transient ischemic stress.

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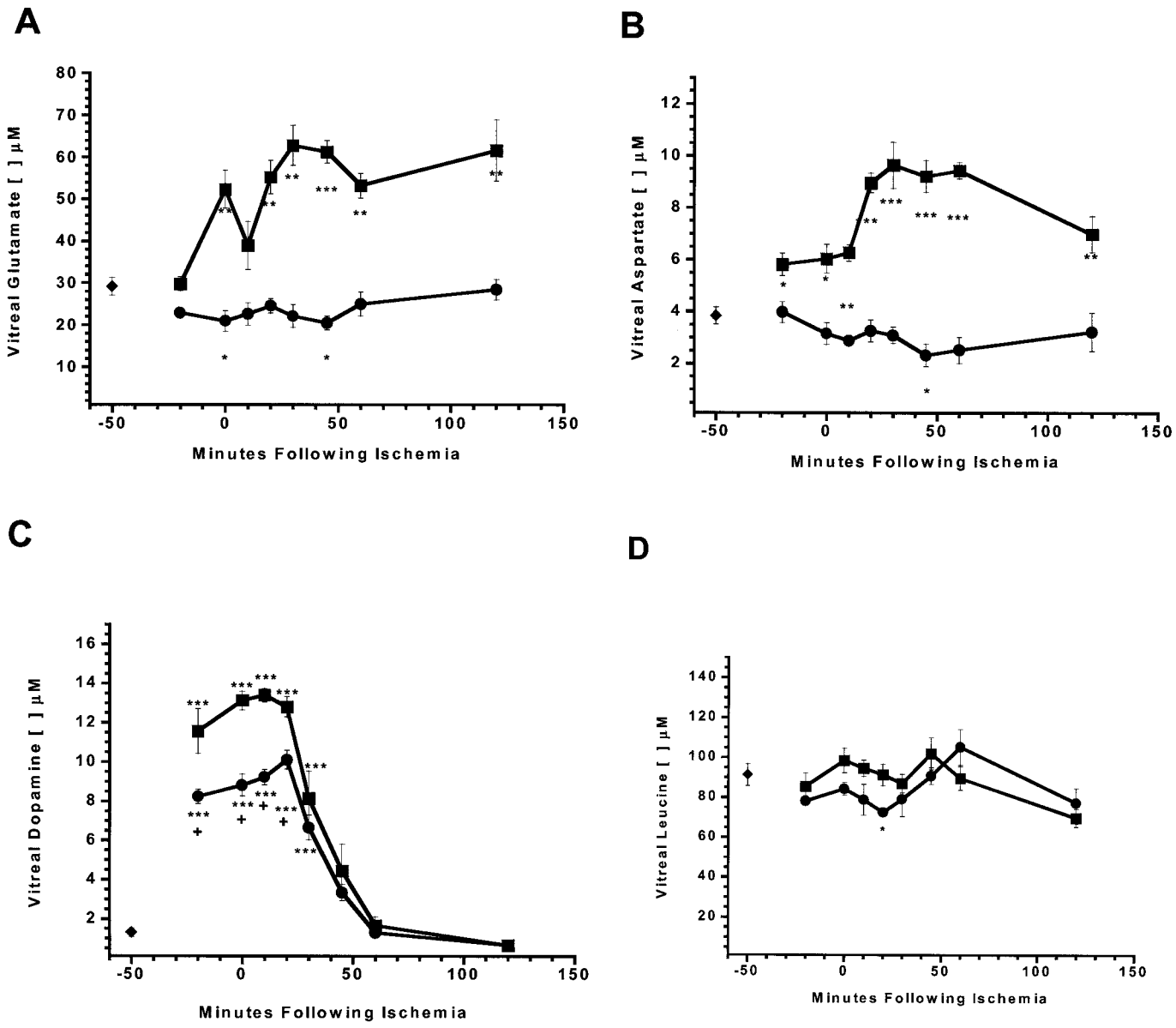

Fig. 5. Brimonidine inhibits the accumulation of intravitreal glutamate and aspartate. Nonischemic eye (♦); brimonidine-treated, ischemic eye (●); saline-treated ischemic eye (■). A, intravitreal glutamate concentration at varying times during and after ischemia. B, intravitreal aspartate concentrations at varying times during and after ischemia. C, intravitreal dopamine concentrations at varying times during and after ischemia. D, intravitreal leucine concentrations at varying times during and after ischemia. Animals were treated with saline or 1.0 mg/kg brimonidine 1 h before ischemia, i.p. At the specified time during or after ischemia, the animals were sacrificed and vitreous humor was isolated. Each point represents a minimum of five vitreal samples. The same samples were analyzed for glutamate, aspartate, dopamine, and leucine. All values are the average ± S.E.M. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with nonischemic eye. $^+P < 0.05$ compared with saline-treated eye for panel C.

Figure 1 illustrates histologically that brimonidine pretreatment prevents the loss of ganglion cell, inner plexiform, and inner nuclear structural layers. The ERG b-wave analyses, an electrophysiological measure of bipolar cell function, demonstrate that brimonidine also protects retinal function (Kline et al., 1978; Stockton and Slaughter, 1989). Retrograde labeling of RGCs (Table 1) demonstrates that brimonidine will also prevent an ischemia-induced loss of RGCs. These data show that brimonidine treatment prevents the ischemia-induced injury to multiple cell types in the inner retina. Dose-response studies and experiments with the α_2-agonist clonidine and the α_2-antagonist rauwolscine demonstrate that brimonidine-mediated protection is α_2-adrenoceptor-dependent.

A number of neuroprotective molecular mechanisms have been proposed for the α_2-adrenergic receptors, including the inhibition of glutamate release (Talke and Bickler, 1996), transcriptional up-regulation of basic fibroblast growth factor (Wen et al., 1996), or activation of an antiapoptotic signaling cascade (p44/p42 extracellular signal-regulated kinases) (Peng et al., 1998). To elucidate the neuroprotective mechanism of brimonidine in the retinal ischemia model, the therapeutic window of brimonidine was defined. The beneficial therapeutic window of brimonidine is small and tightly

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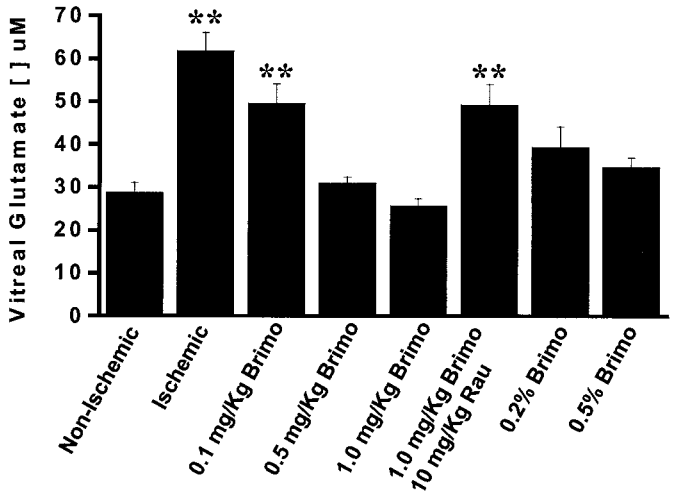

Fig. 6. The inhibition of glutamate accumulation by brimonidine is dose-responsive and α_2-adrenoceptor-dependent. Animals were treated 1 h before ischemia with saline; 0.1, 0.5, or 1.0 mg/kg brimonidine i.p.; 1.0 mg/kg brimonidine i.p. + 10.0 mg/kg rauwolscine i.v.; and 0.2 or 0.5% topical brimonidine. Animals were sacrificed 45 min after the end of ischemia. The animals were sacrificed and vitreous humor was isolated. Each point represents a minimum of five vitreal samples. Statistical significance: $^{**}P < 0.01$ compared with nonischemic eye.

correlates with the approximately 3-h systemic half-life of brimonidine (data not shown). For instance, a single dose 6 or 12 h before ischemia, which would result in very low levels of plasma brimonidine at the time of ischemia, was not protective. However, a single brimonidine dose 15 min before ischemia is efficacious. These data demonstrate that, to prevent the ischemia-induced damage, brimonidine must be present immediately before or during ischemia. The agonist pulse-antagonist chase data (Fig. 4B) also suggest that, to be protective, the α_2-adrenoceptors must be stimulated immediately before and during ischemia. The time course and pulse-chase data are inconsistent with a protective mechanism that requires the activation of gene transcription or translation.

Glutamatergic excitotoxicity, driven by high extracellular concentrations of glutamate, is thought to play a significant role in ischemia-induced neuronal cell death (Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). Figure 5, A and B, demonstrates that, after ischemia, there was a significant accumulation of aspartate and glutamate within the vitreous humor of the saline-treated animals. Previous reports of rat and rabbit retinal ischemia models have reported similar increases of glutamate (Louzada-Junior et al., 1992). Pretreatment with brimonidine completely prevented the ischemia-induced vitreal accumulation of glutamate and aspartate. The inhibition of glutamate accumulation by brimonidine was dose-responsive and blocked by rauwolscine, suggesting that inhibition of glutamate accumulation is also α_2-adrenoceptor-dependent. The time course of dopamine accumulation was markedly different from glutamate and aspartate, and brimonidine only partially inhibited the accumulation of dopamine. Previous reports have shown that α_2-adrenoceptor agonists can decrease the presynaptic release of dopamine. The partial inhibition of dopamine accumulation can be explained if only a subset of the retinal dopaminergic cells express α_2-adrenoceptors. The brimonidine-mediated inhibition of glutamate and aspartate accumulation is specific because vitreal leucine concentrations were not altered by ischemia and the ischemia-induced dopamine accumulation was not greatly inhibited by brimonidine. Therefore, the brimonidine-mediated inhibition of glutamate and aspartate is not likely to be a secondary effect of preventing cells from dying and subsequently disgorging their intracellular contents. Taken together, these data argue that activation of α_2-adrenoceptors will prevent an ischemia-induced accumulation of glutamate and aspartate by a specific mechanism.

Several pieces of evidence suggest that the inhibition of glutamate accumulation is the main mechanism by which brimonidine prevents ischemia-induced damage. First, the dose-response and antagonist profiles are remarkably similar for the protection of the ERG b-wave, the protection of retinal ganglion cells, and the inhibition of glutamate accumulation. Second, the data demonstrate that glutamate and aspartate begin to accumulate by the end of the ischemic period. These data are consistent with the therapeutic window of brimonidine; if brimonidine is not present in the retina immediately before ischemia, and therefore before glutamate and aspartate accumulation, brimonidine cannot prevent the ischemia-induced damage.

If mechanisms other than the accumulation of glutamate and aspartate are involved in neuroprotection by brimonidine during transient ischemia, brimonidine should protect in the presence of acute elevations of glutamate. Previous studies have demonstrated that the inner retina is sensitive to glutamate and the glutamate analog kainate. An intravitreal injection of kainate produces ischemia-like damage, including loss of the inner retina and ERG b-wave. A high extracellular concentration of kainate kills neurons by an excitotoxic mechanism, including Ca^{2+} overload and death. Brimonidine does not prevent the loss of the b-wave caused by an intravitreal injection of kainate (J. E. Donello and E. U. Padillo, unpublished data). These data support the hypothesis that brimonidine protects the retina by inhibiting glutamate and aspartate accumulation and inhibiting subsequent excitotoxicity. However, α_2-agonists may protect neurons by mechanisms other than the inhibition of glutamate accumulation in slower retinal degeneration models, such as the partial optic nerve crush.

The molecular mechanism by which α_2-agonists inhibit ischemia-induced accumulation of aspartate and glutamate is not clear. Previous studies have demonstrated that activation of α_2-adrenoceptors will result in the activation of inward rectifying G-protein-coupled K^+ channels and block voltage-gated Ca^{2+} channels (Aghajanian and Vander-Maelen, 1982; Williams et al., 1985). Hence, activated α_2-adrenoceptors will hyperpolarize neurons and inhibit the presynaptic release of glutamate, aspartate, and norepinephrine (Kamisaki et al., 1992) Brimonidine might inhibit glutamate accumulation by decreasing the amount of presynaptic glutamate released during ischemia.

Alternatively, brimonidine might prevent glutamate accumulation by maintaining the glutamate-buffering activity of Muller cells. Muller cells are retinal glial cells and are critical for maintaining low levels of extracellular glutamate, via the electrogenic Na^+-dependent glutamate/aspartate transporter GLAST (Harada et al., 1998; Pow et al., 2000). Glutamate/aspartate transporters require a negative membrane potential to transport extracellular glutamate and aspartate into the cell. It has been proposed that, during ischemia, the electrogenic glutamate/aspartate transporter will reverse and begin to pump intracellular glutamate into the extracel-

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lular space (Rossi et al., 2000). The similarity between the glutamate and aspartate time course and accumulation levels suggest that activation of α_2-adrenoceptors may prevent glutamate and aspartate accumulation by maintaining activity of the glutamate/aspartate transporters. Future studies of isolated Muller cells will be able to address whether α_2-adrenoceptors might regulate the glutamate/aspartate transporters.

Glaucoma is a leading cause of blindness worldwide. As a disease, glaucoma remains relatively uncharacterized and the causative factors are not understood. Glaucomatous patients are phenotypically characterized by a gradual loss of visual field, due to the degeneration of the optic nerve, and often display abnormally high intraocular pressure. Although the causative agent of visual field loss is controversial, one hypothesis states that stresses such as abnormally high intraocular pressure may directly result in an ongoing insult to which retinal ganglion cells are especially sensitive. Dreyer et al. (1996) have demonstrated that intravitreal glutamate concentrations are elevated 2-fold in glaucoma patients undergoing cataract surgery. Animal models of glaucoma also exhibit elevated levels of intravitreal glutamate (Dreyer et al., 1996; Brooks et al., 1997). It has been subsequently shown that a chronic exposure of retinal ganglion cells to a low dose of glutamate is toxic to RGCs (Vorwerk et al., 1996). The "excitotoxicity glaucoma hypothesis" would suggest that preventing excess intravitreal glutamate levels or blocking the glutamate ionotropic receptors would prevent glaucomatous vision loss (Sucher et al., 1997). Indeed, there has been much effort in identifying new classes of compounds that can protect retinal cells from excitotoxic injury.

Brimonidine is a therapeutic agent currently used to lower high intraocular pressure in glaucomatous patients (Burke and Schwartz, 1996). This study demonstrates that brimonidine exhibits additional properties, beyond its pressure-lowering activity, that are potentially beneficial for glaucoma patients.

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Send reprint requests to: John E. Donello, Ph.D., Department of Biological Sciences, Allergan, Inc., 2525 Dupont Dr., Irvine, CA 92612. E-mail: Donello_john@allergan.com

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