α2 Adrenergic Modulation of NMDA Receptor Function as a Major Mechanism of RGC Protection in Experimental Glaucoma and Retinal Excitotoxicity

Cun-Jian Dong, Yuanxing Guo, Peter Agey, Larry Wheeler, and William A. Hare

Purpose. α2 Agonists, such as brimonidine, have been shown to protect retinal ganglion cells (RGCs) in animal models of glaucoma and acute retinal ischemia. In this study, the authors investigated the neural mechanism that may underlie α2 neuroprotection of RGCs.

Methods. The authors used in situ RGCs in the isolated rat retina to investigate possible interactions between α2 and N-methyl-D-aspartate (NMDA) receptors and rat glaucoma or rabbit retinal NMDA excitotoxicity models to verify in vitro findings under in vivo conditions.

Results. Application of NMDA elicited a robust intracellular Ca^{2+} signal and inward current in individual in situ RGCs voltage clamped at −70 mV. NMDA-elicited responses were blocked by D-AP5 (D-2-amino-5-phosphonopentanoic acid), a selective NMDA receptor antagonist. Brimonidine pretreatment also significantly reduced NMDA-elicited whole-cell currents and cytosolic Ca^{2+} signals in RGCs. This suppressive action of brimonidine was blocked by α2 antagonists, cAMP analogs, an adenylate cyclase activator, and a cAMP-specific phosphodiesterase (PDE4) inhibitor, indicating that this brimonidine effect is mediated by the α2 receptor through a reduction of intracellular cAMP production. Brimonidine or NMDA receptor blockers protected RGCs in rat glaucoma and rabbit retinal NMDA excitotoxicity models. The brimonidine neuroprotective effect was abolished by an α2 antagonist or a PDE4 inhibitor in both in vivo models.

Conclusions. The results demonstrate α2 modulation of NMDA receptor function as an important mechanism for neuroprotection. These results suggest a new therapeutic approach based on neuromodulation, instead of direct inhibition, of the NMDA receptor for the treatment of glaucoma and other CNS disorders associated with NMDA receptor overactivation. (*Invest Ophthalmol Vis Sci.* 2008;49:4515–4522) DOI:10.1167/iovs.08-2078

The α2 adrenergic receptor participates in the regulation of a broad range of physiological functions. It modulates the release of key hormones, such as insulin and adrenaline,[1,2] and neurotransmitters, such as serotonin and glutamate.[3,4] The α2 receptor also mediates a variety of therapeutic effects, including neuroprotective, analgesic, antiepileptic, and anesthetic-sparing effects. These effects have been largely attributed to its classic presynaptic inhibition of signaling molecule release by inhibiting Ca^{2+} channels, activating K^+ channels, or reducing active release sites.[5–9]

The NMDA type of ionotropic glutamate receptor plays an important role in health and disease.[10,11] Its overactivation is thought to be a key contributing factor in the pathophysiology of many CNS disorders, such as Alzheimer disease,[12] Huntington disease,[13] pain,[14,15] and experimental glaucoma.[16–18] The NMDA receptor has a relatively high permeability to Ca^{2+} ions. Its excessive activation under disease conditions can cause intracellular Ca^{2+} overload that leads to neuronal cell death (excitotoxicity).[19,20]

In rodent and monkey glaucoma models, α2 agonists such as brimonidine (also known as UK14304)[21,22] and NMDA blockers such as memantine[16–18] protect RGCs against injury associated with high intraocular pressure (IOP). While the neuroprotective effect of memantine is achieved by preventing excessive activity of the NMDA receptor, the mechanism that underlies the protective effect of brimonidine is unclear. Presynaptic modulation of glutamate release by brimonidine is likely a contributing factor,[23] as suggested for acute retinal ischemia.[24] RGCs express α2[25] and NMDA[26,27] receptors. NMDA receptor function is known to be modulated by a variety of endogenous molecules.[28–31] In addition, α2 agonists have been shown to modulate glutamate and NMDA-elicited responses in dissociated neurons[32–34] and to reduce glutamate induced injury in cultured retinal neurons.[33] These results from dissociated neurons raise the possibility of direct α2 modulation of NMDA receptor activity as a mechanism for neuroprotection. However, it is not known how α2 agonists protect cultured retinal neurons and whether this mechanism contributes to the observed neuroprotective effect of α2 agonists in in vivo models, particularly in models of chronic disease.

In this work, we investigated possible interactions between α2 and NMDA receptors as a major mechanism for neuroprotection in a rat glaucoma model and a rabbit retinal NMDA excitotoxicity model. We used in situ RGCs in the isolated rat retina to investigate α2 regulation of NMDA receptor signaling and rat glaucoma or rabbit retinal NMDA excitotoxicity models to verify our in vitro findings under in vivo conditions. We found that, in these two in vivo models, brimonidine protects RGCs through the inhibition of NMDA receptor function. In both models, this appears to be the major mechanism that underlies the neuroprotective effect of brimonidine. Our results suggest a new therapeutic approach based on neuromodulation, instead of direct inhibition, of the NMDA receptor for the treatment of glaucoma and other CNS disorders associated with NMDA receptor overactivation.

Materials and Methods

Isolated Retina and In Situ Retinal Ganglion Cells

We used in situ retinal ganglion cells (RGCs) in the isolated retina (Figs. 1A, 1B), an ex vivo model, to study possible α2 modulation of NMDA

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Submitted for publication March 26, 2008; revised May 30, 2008; accepted August 20, 2008.

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receptor function. Ex vivo models maintain a natural extracellular environment with normal cell–cell interactions and are useful tools for the study of neural processing and drug action under conditions similar to those in vivo. The present study was conducted in accordance with guidelines outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by an institutional animal care and use committee. Male Brown Norway rats (275–300 g) were deeply anesthetized by intramuscular injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). After enucleation of both eyes, rats were humanely killed immediately by intracardiac injection of Eutha-6 (120 mg/kg). Retinas were carefully isolated, and a small piece (approximately 3 × 6 mm) was placed photoreceptor-side down in a recording chamber. A glass pipette filled with normal Ringer solution was used to expose the somas of in situ RGCs by mechanically removing a small portion of the inner limiting membrane and cleaning the surface of the cell membrane for whole-cell patch clamp (Fig. 1A).

Whole-Cell Patch Clamp, Ca^{2+} Imaging, and Drug Application to In Situ RGCs

The isolated retina was perfused continuously with normal Ringer by whole chamber and local perfusion systems to accelerate delivery and removal of the test agents. To better monitor NMDA receptor activity, we recorded simultaneously the NMDA-induced whole cell current and the cytosolic Ca^{2+} signal. Normal Ringer contained 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM KH_2PO_4, 10 mM D-glucose, 26 mM $NaHCO_3$, 0.005 mM strychnine, and 0.02 mM SR95530. Conventional methods for whole-cell patch clamp were used. RGCs were voltage clamped at −70 mV. After whole-cell patch clamp was established, normal Ringer was replaced with 0 Mg^{2+} Ringer, which was made by removing $MgSO_4$ from normal Ringer and adding 0.02 mM ascorbic acid to it. The normal intracellular (patch pipette) solution contained 125 mM $CsCH_3SO_3$, 1 mM $MgCl_2$, 15 mM TEA·Cl, 10 mM HEPES, 4 mM ATP-Mg, 0.5 mM GTP-Na_3, 12 mM phosphocreatine, 5 mM QX-314, and 0.1 mM fluo-4. NMDA (100 μM NMDA + 5 μM glycine) was applied for 8 seconds through a multichannel local perfusion system. In experiments using brimonidine or atipamezole, these two agents were added to the whole-chamber and local perfusion systems. Ca^{2+} images were obtained with a spinning disc confocal system (Nipkow; Solamere Technology Group, Salt Lake City, UT) mounted on a fixed-stage upright microscope (BX51WI; Olympus, Tokyo, Japan).

Rat Glaucoma Model and Systemic Dosing of Test Agents

Male Sprague-Dawley rats (weight range, 350–400 g) were used. IOP was elevated by laser photocoagulation of episcleral and limbal veins.[21] To avoid IOP spike and achieve persistent IOP elevation, two laser treatments, 1 week apart, were performed. IOP was measured with a tonometer (TonoLab; Colonial Medical Supply, Franconia, NH). Brimonidine and other agents were systemically and continuously delivered using osmotic pumps (Alzet; Durect, Cupertino, CA). RGCs were labeled in a retrograde fashion with dextran tetramethylrhodamine (Molecular Probes, Eugene, OR) according to the procedure described elsewhere[21] and were counted in 24 fields (see Fig. 5A) using imaging software (Image-Pro Plus; Media Cybernetics, Bethesda, MD) and a fixed-stage upright microscope (BX51WI; Olympus) equipped with an automated microscope stage (H101A; Prior Scientific Inc., Rockland, MA).

Rabbit Retinal Excitotoxicity Model

In vivo excitotoxic insult to RGCs was induced by a single intravitreal injection of 3.6 μmol NMDA (in 50 μL PBS). The effects of brimonidine (3.6 nmol), atipamezole (24 nmol), MK-801 (0.12 μmol), memantine (0.36 μmol), and rolipram (12 and 120 nmol) on NMDA-induced RGC injury were evaluated by coinjection with NMDA. In addition, these agents were also injected 1 hour before and 24 hours after NMDA injection. In the NMDA alone group, 50 μL saline was also injected 1 hour before and 24 hours after NMDA injection to control for any injection-induced effects on cell survival. Two weeks after intraocular NMDA application, the rabbits were humanely killed and the retinas were isolated (see Fig. 6 legend). RGCs were fluorescence labeled by intravitreal injection of a fluorescent nuclear dye 4′-6-diamidino-2-phenylindole (DAPI; 1 mM, 50 μL) 24 hours before euthanatization, and RGC injury was evaluated using a fixed-stage upright microscope (BX51WI; Olympus) equipped with an automated microscope stage (H101A; Prior Scientific Inc), a high-sensitivity CCD camera (Orcaer; Hamamatsu Photonics, Hamamatsu, Japan), and imaging software (Image-Pro Plus; Media Cybernetics). Neurons in the ganglion cell layer were counted within an 8-mm diameter sample of the central retina. To minimize sampling error, the optic nerve head was used as a marker to ensure that all retinal samples from different eyes were obtained from the same region and that neurons were counted within 25 fields comprising a 5 × 5 array (Fig. 6A).

Statistical Analysis

Group data are expressed as mean ± SEM. Statistical comparisons were made using a two-population Student *t*-test (Origin; OriginLab, Northampton, MA). $P < 0.05$ was chosen to indicate a statistically significant difference.

Results

NMDA-Elicited Responses in In Situ RGCs

In the mammalian retina, the NMDA receptor is expressed mostly in RGCs.[26,27] A brief application of NMDA (with glycine) elicited a robust inward current and Ca^{2+} signal that was blocked by D-AP5, a specific NMDA receptor antagonist (Figs. 1C, 1E). For simplicity, we use the terms *NMDA elicited* and *NMDA responses* instead of the terms *NMDA/glycine-elicited* or *NMDA/glycine responses*. NMDA responses were sensitive to Mg^{2+} block and required glycine as a coagonist (data not shown). Figure 1D shows Ca^{2+} images taken at the time indicated by the vertical line in Figure 1C under the three test conditions. The yellow squares in Figure 1D represent the area where the Ca^{2+} signals in Figure 1C were measured. Figure 1E shows normalized group data from four different RGCs. Thus, these NMDA-elicited responses in RGCs were indeed mediated by NMDA receptors.

α2 Modulation of NMDA-Elicited Responses in In Situ RGCs

Next, we tested whether α2 agonists could modulate NMDA responses. Figure 2 shows that activation of α2 receptors by brimonidine, a selective α2 agonist, leads to a suppression of NMDA receptor function. Pretreatment with brimonidine caused a significant reduction ($P < 0.01$) of the NMDA-induced current and the Ca^{2+} signal. NMDA responses were fully recovered after washout of brimonidine (Fig. 2). Suppressive effects of brimonidine were mimicked by other selective α2 agonists, such as medetomidine (data not shown), and were completely blocked by pretreatment with atipamezole, a highly selective α2 receptor antagonist, indicating that the suppressive effect of brimonidine requires α2 receptor activation. The brimonidine effect was also blocked by intracellular application of GDP-β-S, a G-protein inhibitor, indicating that this effect requires G-protein activation (Fig. 2C).

α2 Modulation of NMDA Receptor Function via a cAMP Second-Messenger Pathway

The α2 receptor is coupled to G_i/G_o. α2 Receptors can signal through a number of effector mechanisms, including modulating the activity of adenylate cyclase (AC), Ca^{2+} and K^+ channels, and Na^+/H^+ exchangers.[5,6] The inhibition of AC is

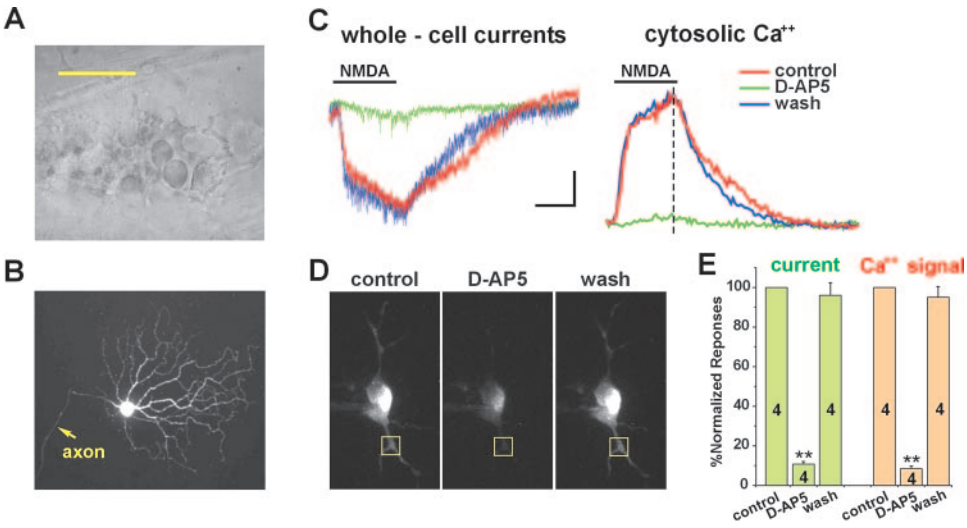

FIGURE 1. NMDA-elicited whole-cell currents and cytosolic Ca^{2+} signals in in situ RGCs in the isolated rat retina. (**A**) Exposed somas of in situ RGCs. Calibration bar, 50 μm. (**B**) Confocal image of a fluorescent dye (Alex 488)–filled in situ RGC. (**C**) Representative NMDA-induced currents and Ca^{2+} signals (recorded in 0 Mg^{2+} Ringers) in control, in the presence of 50 μM D-AP5, and after washing out D-AP5. Vertical calibration bar, 120 pA; horizontal calibration bar, 5 seconds. (**D**) Ca^{2+} images taken at the time indicated by the *vertical line* in (**C**) under the same three conditions. *Yellow squares*: areas in which the Ca^{2+} signals in (**C**) were measured. (**E**) Normalized group data from four different RGCs. $^{**}P < 0.01$ between control responses and responses recorded in the presence of D-AP5. For all Ca^{2+} signals (including those in Figs. 2–4), arbitrary units were used during data acquisition. The *y*-axis measures the arbitrary fluorescent intensity. Peak fluorescence intensity of the control trace is represented as 100%. Fluorescence intensity measured in the presence of D-AP5 is normalized against the control value.

through $G_{i\alpha}/G_{o\alpha}$ (a cAMP-dependent signaling pathway), whereas the modulation of Ca^{2+} and K^+ channels or the number of active release sites is believed to be through $G_{i\beta\gamma}/G_{o\beta\gamma}$-mediated direct action (cAMP-independent pathways).[5,6,8,35]

To determine whether α2 modulation of NMDA receptor function requires a cAMP second-messenger pathway, we tested agents known to affect AC activity or intracellular cAMP concentration. To limit the activity of these agents to the recorded RGCs, the agents were delivered intracellularly through the patch electrode. We selected membrane-permeable agents so that drug effects could be confirmed after the patch electrode was removed. Intracellular application of Sp-cAMPS, a hydrolysis-resistant cAMP analog, abolished the suppressive brimonidine effect on the NMDA-elicited whole cell current and the cytosolic Ca^{2+} signal (Figs. 3A, 3C). After simultaneously recording the effect of brimonidine on the NMDA-elicited whole-cell current and the cytosolic Ca^{2+} signal (in the presence of intracellular Sp-cAMPS), the patch electrode was successfully removed from five RGCs without causing significant damage to these cells (in many cases, the somas were also removed with the electrodes or the RGCs were severely damaged). A second set of Ca^{2+} images was obtained

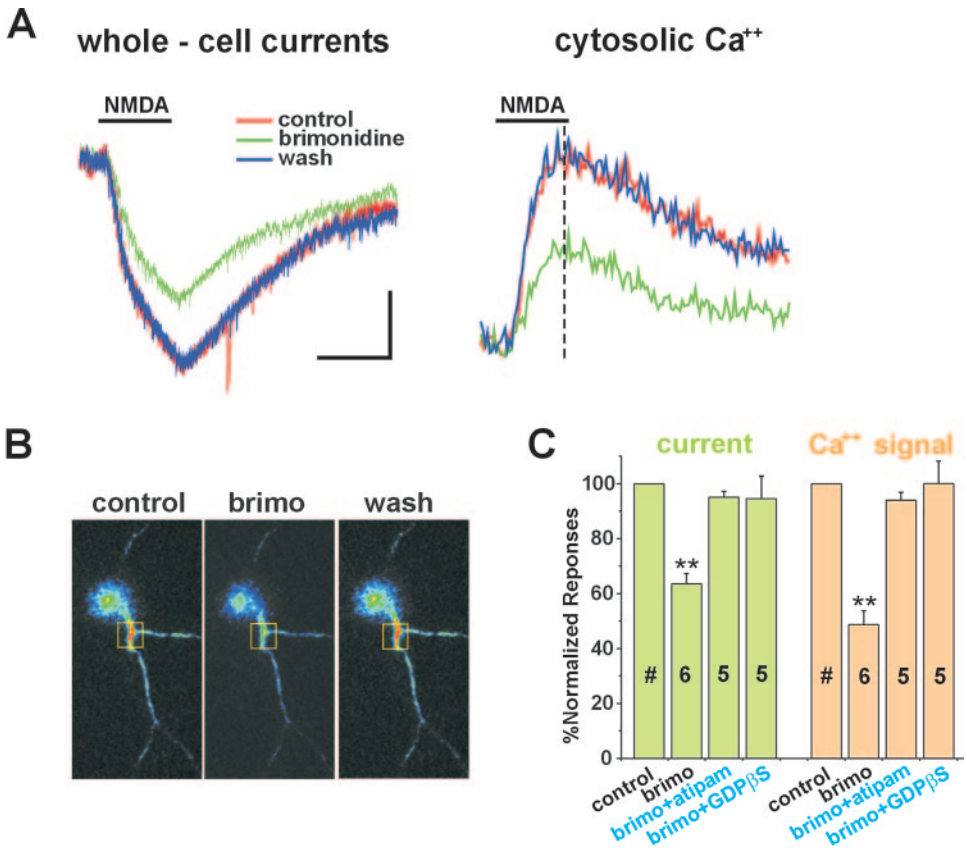

FIGURE 2. Brimonidine, a selective α2 agonist, modulates NMDA-elicited whole-cell currents and cytosolic Ca^{2+} signals in in situ RGCs. (**A**) NMDA-induced currents and Ca^{2+} signals from a representative RGC in control, in the presence of 3 μM brimonidine, and after washing out brimonidine. Vertical calibration bar, 150 pA; horizontal calibration bar, 8 seconds. (**B**) Ca^{2+} images from the same RGC in (**A**) that were taken at the time indicated by the *vertical line* in (**A**) under the same three conditions. *Yellow squares*: areas in which the Ca^{2+} signals in **A** were measured. (**C**) Normalized group data that show the suppressive brimonidine (brimo) effect on NMDA responses was blocked by a selective α2 receptor antagonist (atipamezole, atipam, 15 μM; $n = 5$) or a G-protein inhibitor (GDP-β-S; 1 mM in the intracellular solution; $n = 5$). $^{**}P < 0.01$ between control responses and responses recorded in the presence of brimonidine or brimonidine plus other test agents. The number in each bar indicates how many RGCs were tested in the group. The bars that have a # sign represent controls for all test groups in the plot. Their number is the same as that of the corresponding drug-treated groups.

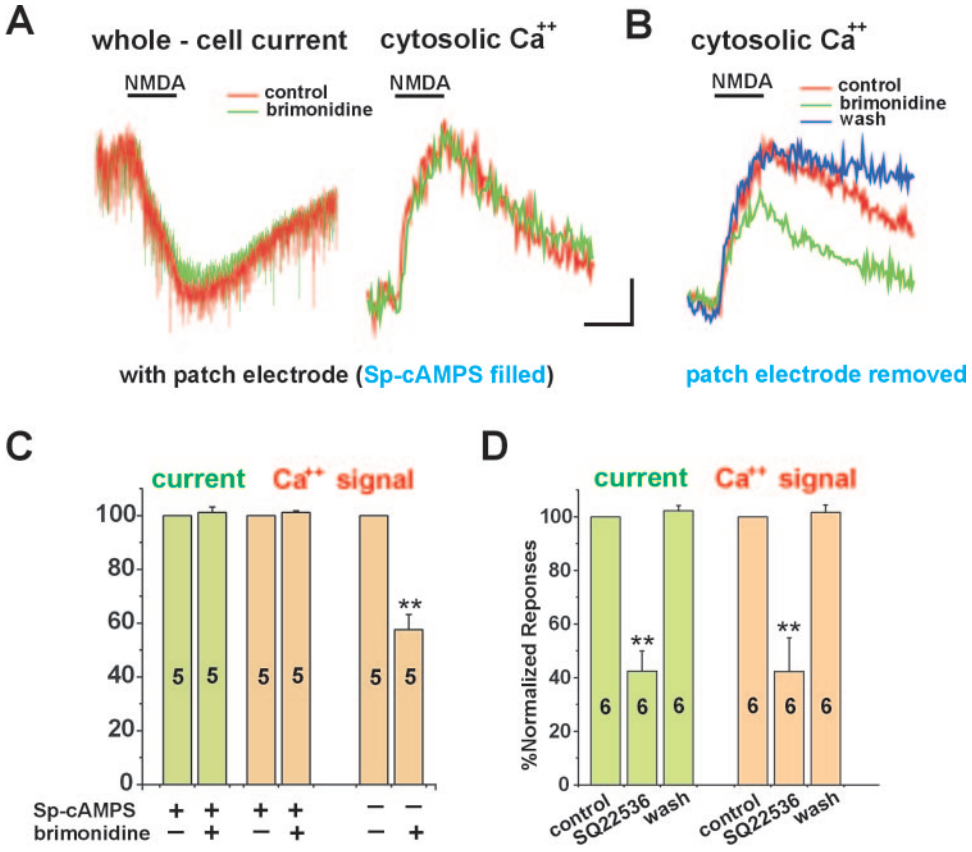

FIGURE 3. α2 Receptor–mediated modulation of NMDA receptor function is abolished by a cAMP analog. (**A**) Electrophysiological and optical recordings from a representative in situ RGC showing that an intracellularly applied cAMP analog (Sp-cAMPS, 200 μM) abolished the brimonidine effect on NMDA responses. Vertical calibration bar, 100 pA; horizontal calibration bar, 8 seconds. (**B**) Typical brimonidine effect on Ca^{2+} signals appeared 8 minutes after removal of the Sp-cAMPS–filled patch electrode from the same RGC shown in (**A**). (**C**) Statistical results from five RGCs in the presence and absence of intracellular Sp-cAMPS. (**D**) Statistical results from six RGCs showing that application of SQ22536 (200 μm), a selective AC inhibitor, suppressed dramatically the NMDA-induced current and Ca^{2+} signal. $^{**}P < 0.01$ between control responses and the responses recorded in the presence of brimonidine or SQ22536. The number in each bar indicates how many RGCs were tested in the group.

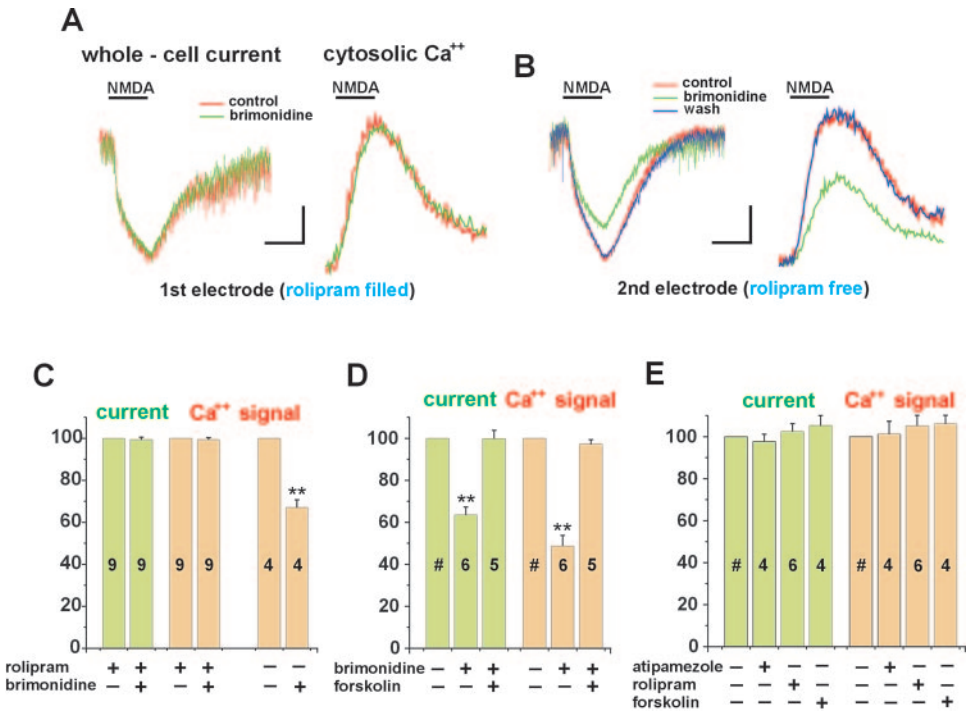

FIGURE 4. α2 Receptor–mediated modulation of NMDA receptor function is abolished by rolipram and forskolin. (**A**) Electrophysiological and optical recordings from a representative in situ RGC showing that intracellularly applied rolipram (3 μM) abolished the effect of brimonidine on NMDA responses. (**B**) Typical brimonidine effect on whole-cell currents and Ca^{2+} signals appeared 8 minutes after removal of the rolipram-filled patch electrode and rerecording from the same RGC shown in (**A**) with a rolipram-free patch electrode. (**C**) Statistical results from nine RGCs. In four of those RGCs, a second set of Ca^{2+} images was obtained after successful removal of the rolipram-filled patch electrode and after waiting 5 to 8 minutes to allow rolipram to diffuse out of the cells and be washed away by local and background perfusions. (**D**) Statistical results from five RGCs showing that intracellular application of adenylate cyclase stimulator forskolin (10 μM) blocked the effect of brimonidine on NMDA responses. (**E**) Statistical results from three groups of RGCs in which effects of atipamezole (15 μM), forskolin(10 μM), or rolipram (3 μM) alone on NMDA-elicited responses were tested. $^{**}P < 0.01$ between control responses and responses recorded in the presence of brimonidine or brimonidine plus forskolin. The number in each bar indicates how many RGCs were tested in the group. The bars that have a # sign represent controls for all test groups in the plot. Their number is the same as that of the corresponding drug-treated groups. Vertical and horizontal calibration bars: (**A**) 150 pA; (**B**) 170 pA; (**A**, **B**) 8 seconds, respectively.

TABLE 1. Tool Compounds and Their Mechanisms of Action

Name	Mechanism of Action	Reference
D-AP5	Competitive NMDA receptor antagonist	Davis and Watkins[36]
Atipamezole	α_2 Adrenergic antagonist	Virtanen[37]
Brimonidine	α_2 Adrenergic agonist	MacDonald et al.[38]
Forskolin	Cell-permeable adenylyl cyclase activator	De Souza and Dohadwalla[39]
GDP-β-S	Cell-impermeable competitive inhibitor of G-protein	Kucera and Rittenhouse[40]
MK-801	Noncompetitive NMDA receptor antagonist	Thompson et al.[41]
NMDA	Agonist of NMDA receptor	Collingridge and Lester[42]
Rolipram	Inhibitor of phosphodiesterase type 4	Teixeira et al.[43]
Sp-cAMPS	Cell-permeable cAMP analogue	O'Brian et al.[44]
SQ 22536	Cell-permeable adenylyl cyclase inhibitor	Fabbri et al.[45]

from the same RGCs after waiting 8 minutes to allow Sp-cAMPS to diffuse out of the cells and to be washed away by local and background perfusion. After washout, the typical suppressive effect of brimonidine on NMDA-elicited cytosolic Ca^{2+} signal was observed (Figs. 3B, 3C; traces in Figs. 3A and 3B are from the same RGC). Thus, maintaining intracellular cAMP concentration with an exogenous cAMP analog abolished the suppressive brimonidine effect, indicating that the effect was associated with lowering intracellular cAMP concentration. This notion was further supported by the effect of SQ22536, a selective membrane-permeable inhibitor of AC. Local perfusion of SQ22536 dramatically and reversibly suppressed the NMDA-elicited current and the cytosolic Ca^{2+} signal (Fig. 3D).

The effect of rolipram, a selective inhibitor of PDE4, on NMDA-elicited responses provides further confirmation that brimonidine acts by lowering cAMP. As shown in Figures 4A to 4C, intracellular application of rolipram through the patch electrode also completely abolished the effect of brimonidine ($n = 9$). In four RGCs, after the brimonidine effect on whole-cell current and Ca^{2+} signal was evaluated (Fig. 4A), the rolipram-filled patch electrode was successfully removed and a second set of Ca^{2+} images was obtained from the same RGCs after rolipram had diffused out of the cells and washed away. After the rolipram washout, a suppressive effect of brimonidine on the Ca^{2+} signal was observed (Fig. 4C, right pair of columns). In one of these four RGCs, whole-cell recording was successfully made with a second (rolipram-free) electrode in the same cell (Fig. 4B; traces in Figs. 4B and 4A are from the same RGC). In this cell, the brimonidine effect on whole-cell current and Ca^{2+} signal could be demonstrated clearly after rolipram washout.

In another group of five RGCs, the AC activator forskolin was added intracellularly through the patch electrode This treatment also abolished the brimonidine effect on both types of NMDA responses (Fig. 4D). Figure 4E shows the effect of forskolin, atipamezole, and rolipram alone on NMDA responses. By themselves, these agents did not have a significant effect on the NMDA-elicited current or Ca^{2+} signal. Taken together, the results shown in Figures 2 to 4 demonstrate strongly that the activation of α2 receptors by brimonidine suppresses NMDA receptor function by decreasing intracellular cAMP production. This is consistent with a $G_{\alpha i}$-mediated inhibition of AC because direct inhibition of AC by the selective inhibitor (Fig. 3D) produced a similar suppressive effect. Preserving intracellular cAMP concentration by adding an exogenous cAMP analog, by blocking the degradation of endogenous cAMP, or by directly stimulating AC, can block the brimonidine effect. Table 1 lists the names of tool compounds used in this study, their sites of action, and references.

Elimination of Brimonidine Protection of RGCs by Rolipram in a Rat Glaucoma Model

Because NMDA receptor overactivation is believed to be an important contributing factor in the pathophysiology of many CNS disorders, our results (Figs. 2–4) suggest that a direct modulation of NMDA receptor function by α2 agonists could mediate neuroprotective effects in models of CNS disorder or injury. We tested this hypothesis in two in vivo models: a rat glaucoma model and a rabbit retinal NMDA excitotoxicity model. In the rat glaucoma model (chronic ocular hypertension [COHT]), high IOP produced by laser photocoagulation of episcleral and limbal veins leads to RGC loss.[21] We show that chronic systemic administration of brimonidine or memantine is neuroprotective in this glaucoma model (Figs. 5B, 5C) without lowering IOP (Fig. 5E). These results are in good agreement with previous observations.[16,21,22] Next, we demonstrated that brimonidine protection was indeed mediated by α2 receptors because the coadministration of brimonidine with the selective α2 antagonist atipamezole abolished brimonidine protection (Figs. 5C; see also Fig. 2C). Based on the results shown in Figures 3 and 4, we hypothesized that the neuroprotective effect of brimonidine may be achieved at least in part by a suppression of NMDA receptor function through the inhibition of intracellular cAMP production. If this is true, preserving intracellular cAMP concentration by inhibiting its degrading enzyme PDE 4 (Fig. 4) should attenuate or abolish the neuroprotective effect of brimonidine. This was indeed the case. Coadministration of brimonidine with the PDE 4 inhibitor rolipram abolished the neuroprotective effect of brimonidine in this rat glaucoma model (Figs. 5B, 5C). Thus, these in vivo data are consistent with our hypothesis that direct modulation of NMDA receptor function by α2 agonists mediates their neuroprotective effect in this experimental glaucoma model.

Elimination of Brimonidine Protection of RGCs by Rolipram in a Rabbit Retinal Excitotoxicity Model

To more directly establish α2 modulation of NMDA receptor function as an important mechanism for neuroprotection, we developed an in vivo rabbit retinal NMDA excitotoxicity model. This in vivo rabbit model has two major advantages: the cause of RGC injury (NMDA receptor overactivation) is well defined, and difficulties associated with in vivo compound testing (i.e., in vivo drug delivery, systemic side effects, bioavailability of test agents) are either overcome or minimized. Test agents were injected directly into the vitreal cavity (bypassing the blood-retina barrier), where they have direct access to RGCs. In addition, any systemic side effects of these test agents are minimized with intravitreal drug delivery.

The rabbit eye and vitreal space are much larger that those of the rat, which makes intravitreal drug application substan-

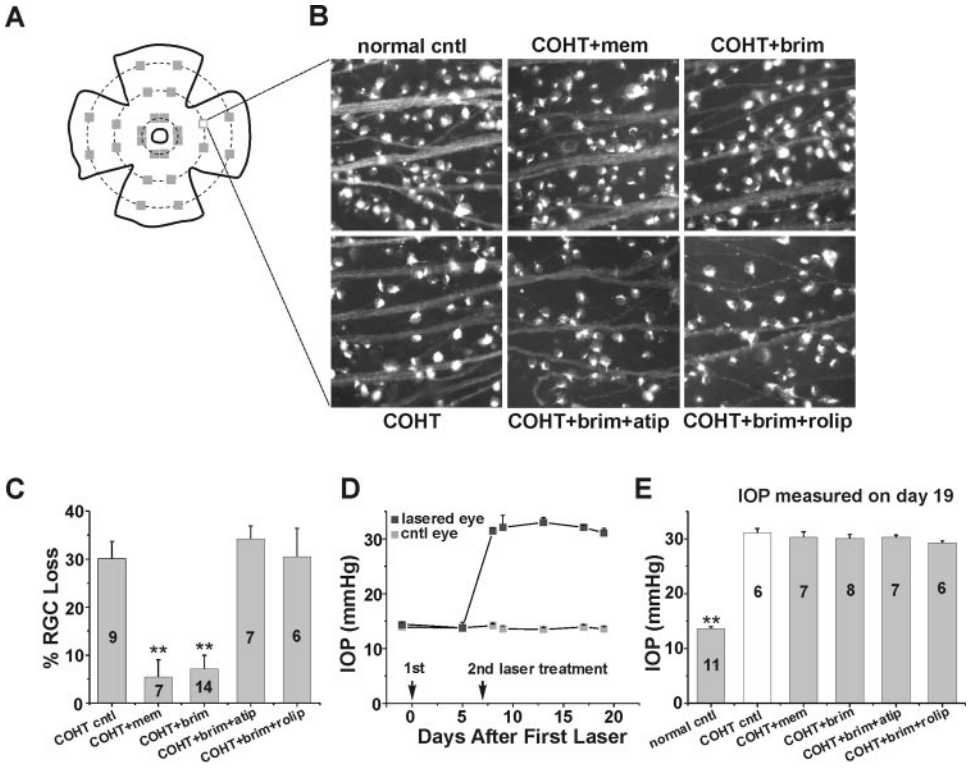

FIGURE 5. Brimonidine protection of RGCs in a rat glaucoma model was abolished by rolipram. (**A**) Diagram illustrating the 24 fields in the rat retina in which the number of RGCs was counted. (**B**) Representative images from the intermediate fields that show the effects of laser treatment alone (COHT), 2-week treatment (using osmotic pumps) with memantine (3 mg/kg/d; COHT + mem), brimonidine (72 μg/kg/d; COHT + brim), brimonidine + atipamezole (72 μg/kg/d and 0.36 mg/kg/d respectively, COHT + brim + atip), and brimonidine + rolipram (72 μg/kg/d and 0.24 mg/kg/d, respectively, COHT + brim + rolip). (**C**) Group statistical data showing the percentage of RGC loss under various experimental conditions. Dosing with brimonidine or memantine significantly reduced RGC loss. Brimonidine protection of RGCs was abolished by cotreatment with atipamezole or rolipram. (**D**) IOP values of unlasered (*left*, cntl) and lasered (*right*) eyes of the same animals ($n = 12$). (**E**) Group statistical data showing that the effects of brimonidine and other compounds on RGC survival are independent of any effect on IOP. $^{**}P < 0.01$ between COHT control group and normal control or other drug-treated COHT groups.

tially easier. A single intravitreal injection of 3.6 μmol NMDA resulted in loss of approximately 40% of the neurons in the ganglion cell layer 2 weeks after injection (Figs. 6B–6D). Based on analysis of the DAPI staining pattern, the lost neurons were predominantly or exclusively RGCs. This cell loss was either blocked or significantly reduced by pretreatment with MK-801 or memantine (two NMDA channel blockers), respectively, confirming that neuronal cell loss was caused by NMDA receptor overactivation (Fig. 6D). Pretreatment with brimonidine also significantly reduced NMDA-induced RGC loss (Figs. 6B–6D). The neuroprotective effect of brimonidine was blocked by pretreatment with atipamezole (Figs. 6B–6D). This verifies that the neuroprotective effect of brimonidine is mediated by the α2 receptor, similar to the results from the glaucoma model shown in Figure 5. Our results with in situ RGCs (Figs. 3, 4) show that α2 modulation of NMDA receptor function was mediated through the inhibition of intracellular cAMP production. Blocking intracellular cAMP degradation with the PDE4 inhibitor rolipram abolished the suppressive brimonidine effect on NMDA-elicited responses (Fig. 4) and the neuroprotective effect on RGCs in experimental glaucoma (Fig. 5). Figures 6B to 6D show that the effect of brimonidine to reduce NMDA-induced RGC injury was also blocked by intravitreal injection of rolipram (see Fig. 6 legend for details). Taken together, these results provide evidence that brimonidine protects RGCs in rat glaucoma and rabbit retinal excitotoxicity models by modulating NMDA receptor function.

DISCUSSION

The results from our ex vivo and in vivo models provide compelling evidence for α2 modulation of NMDA receptor function as a major cellular mechanism that mediates the neuroprotective effects of α2 agonists in experimental glaucoma and NMDA-induced RGC excitotoxicity. Unlike its presynaptic effect (modulation of Ca^{2+}, K^+ channels, or number of active release sites) mediated largely by direct actions of $G_{i\beta\gamma}$/$G_{o\beta\gamma}$,[5,6,8,35] this postsynaptic α2 modulation of NMDA receptor function occurs through the suppression of intracellular cAMP production, consistent with a $G_{i\alpha}$/$G_{o\alpha}$-coupled mechanism.

Our ex vivo and in vivo results also provide evidence in the RGCs (a type of CNS projection neuron) of important intracellular events that regulate NMDA receptor function. First, at rest, intracellular cAMP is likely at or near a saturating level in terms of its permissive effect on NMDA receptor function. This high cAMP level appears to be maintained mainly by high AC activity because direct inhibition of AC with a selective inhibitor (SQ22536) has a dramatic suppressive effect on the NMDA receptor-mediated current and Ca^{2+} signal (Fig. 3D). In addition, the PDE4 inhibitor rolipram and the AC activator forskolin can block brimonidine modulation of NMDA receptor function (Figs. 4A–4D), but neither by itself can significantly enhance NMDA receptor activity in RGCs (Fig. 4E). Second, intracellular cAMP appears to undergo rapid turnover. Once cAMP production is reduced by α2 receptor-mediated inhibition of AC, blocking PDE4 with rolipram abolishes effectively the suppressive brimonidine effect on NMDA receptor function (Fig. 4), suggesting that the intracellular cAMP level drops rapidly because of PDE4-mediated degradation after AC is inhibited. Thus, AC and PDE4 are two key enzymes that dynamically and precisely set the level of the intracellular cAMP in RGCs. This in turn sets the level of NMDA receptor activity that determines the severity of excitotoxic RGC injury under disease conditions. Our in vivo findings (Figs. 5, 6) support this notion.

Our results also suggest that, in experimental glaucoma and retinal excitotoxicity models, α2 modulation of NMDA receptor function appears to be the principal mechanism that mediates the neuroprotective effect of exogenous α2 agonists because preserving NMDA receptor function by preventing the degradation of intracellular cAMP with rolipram (Fig. 4) abolishes completely the neuroprotective effect of brimonidine (Figs. 5, 6). These results also provide further support for the notion that overactivation of

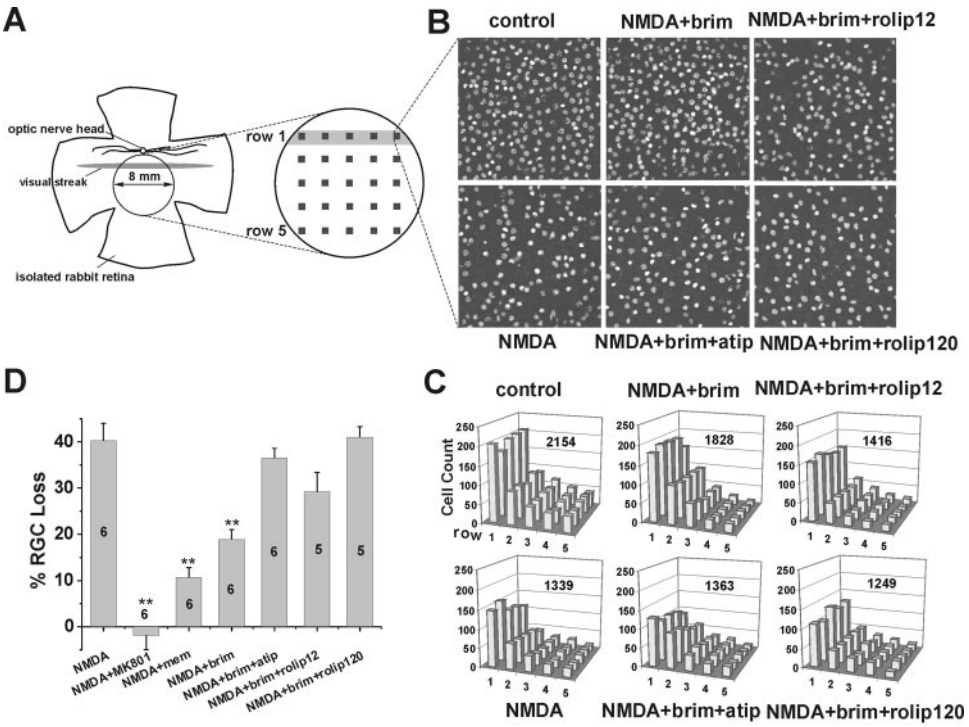

FIGURE 6. Brimonidine protection of RGCs in a rabbit retinal NMDA excitotoxicity model was abolished by rolipram. (**A**) Diagram illustrating the 25 fields in the rabbit retina in which the number of neurons in the ganglion cell layer was counted. Each field measures 220 μm × 220 μm. (**B**) Representative images from row 1 (visual streak area) that show the effects of NMDA treatment alone (single intravitreal injection of 50 μL saline containing 3.6 μmol NMDA) and cotreatment with brimonidine and other compounds on neuronal survival at 2 weeks after NMDA injection. Each fluorescent dot in an image is the DAPI-labeled nucleus of a neuron in the ganglion cell layer. Brimonidine (brim, 3.6 nmol), atipamezole (atip, 24 nmol), and rolipram (rolip, 2 doses at 12 or 120 nmol) were dissolved in 50 μL saline and were coinjected with NMDA. In addition, they were injected (at the same dose) 1 hour before and 24 hours after NMDA injection. In the NMDA-alone group, 50 μL saline was also injected 1 hour before and 24 hours after NMDA injection to control for any injection-induced changes on cell survival. (**C**) Three-dimensional plots of cell counts at all 25 fields of representative retinas from the same six groups shown in (**B**). Numbers inside each plot represent total cell counts for all 25 fields of that particular retina. (**D**) Group statistical data showing the percentage of RGC loss under various experimental conditions. MK-801 (0.12 μmol) completely blocked NMDA-induced RGC loss. Brimonidine and memantine application also significantly reduced RGC loss. Brimonidine protection of RGCs was blocked by preapplication and coapplication of atipamezole. Brimonidine protection was also blocked by rolipram at both doses. $^{**}P < 0.01$ between NMDA control and other groups.

NMDA receptors makes a significant contribution to RGC injury in experimental glaucoma models[16–18] (Fig. 5).

In addition to a neuroprotective action, α2 agonists have other CNS effects, including analgesic, antiepileptic, and anesthetic-sparing actions, that have been attributed largely to a presynaptic inhibition of signaling molecule release.[5,6,9] Interestingly, NMDA antagonists/blockers also have analgesic, antiepileptic, and anesthetic effects.[14,46,47] Our results suggest that these other effects of α2 agonists may be mediated at least in part by postsynaptic modulation of NMDA receptor function.

The NMDA receptor has long been recognized as a therapeutic target for CNS disorders.[14,46,47] However, a major obstacle for using NMDA antagonists/blockers as therapeutic agents is their potential for unacceptable side effects.[47,48] The introduction of memantine, a use-dependent and well-tolerated NMDA channel blocker, has significantly reduced the side effects and improved safety.[49] Our results suggest a novel approach based on neuromodulation, instead of direct inhibition, of the NMDA receptor, which may also provide safe and effective therapy for those CNS disorders associated with excessive activation of the NMDA receptor.

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