Comparison of the Neuroprotective Effects of Adrenoceptor Drugs in Retinal Cell Culture and Intact Retina

Darryl C. Baptiste,[1,2] *Andrew T. E. Hartwick,*[1,3] *Christine A. B. Jollimore,*[1,2] *William H. Baldridge,*[1,3,4] *Balwantray C. Chauhan,*[1,4,5] *François Tremblay,*[1,4,5] *and Melanie E. M. Kelly*[1,2,4]

PURPOSE. The efficacy of β_1-adrenoceptor (AR)–selective (betaxolol and metoprolol) and nonselective (timolol) antagonists and the α_2-AR agonist UK14,304 as retinal neuroprotectants was compared and contrasted in an in vitro glutamate excitotoxicity model. The ability of UK14,304, brimonidine, and betaxolol to alter glutamate-receptor–induced changes in intracellular calcium ($[Ca^{2+}]_i$) was also determined in isolated retinal neurons and retinal ganglion cells (RGCs) in an intact retina preparation.

METHODS. Neuronal survival was measured in mixed retinal cell cultures treated for 24 hours with media containing 100 μM glutamate, with or without the addition of each of the drugs (1–1000 μM). Effects of glutamate on glia were also investigated in a C6 glioma cell line. Glutamate-induced changes in $[Ca^{2+}]_i$ with and without UK14,304, and its analogue brimonidine were assessed by calcium-imaging techniques in retinal neurons in culture. The effect of betaxolol on $[Ca^{2+}]_i$ was investigated in RGCs in intact rabbit retina.

RESULTS. In cell cultures, 10–1000 μM glutamate resulted in a dose-dependent loss of neurons, but not of glia. The absence of glutamate toxicity in glia was confirmed in C6 glioma cells. Betaxolol, but not timolol or metoprolol, significantly increased survival (from 52% of control in glutamate-only to 78% with 10 μM betaxolol) after excitotoxic insult. UK14,304 also increased survival (from 62% of control in glutamate only to 109% and 101% of control with 10 and 100 μM UK14,304, respectively). This effect was blocked by the specific α_2-antagonist, yohimbine. Both UK14,304 and brimonidine (10–100 μM) reduced glutamate-induced $[Ca^{2+}]_i$ increases in retinal neurons in culture. The actions of the α_2-agonists in reducing glutamate-induced $[Ca^{2+}]_i$ increases were reduced by yohimbine (1 μM). Betaxolol (100 μM) reduced N-methyl-D-aspartate (NMDA)–induced increases of $[Ca^{2+}]_i$ in RGCs in intact retina.

CONCLUSIONS. Betaxolol reduced glutamate excitotoxicity in retinal neurons in vitro through a mechanism independent of β-AR interactions. UK14,304, acting through α_2-ARs, was also neuroprotective in vitro. The neuroprotective actions of betaxolol and the α_2-agonists on retinal neurons may be due, at least in part, to a direct reduction of glutamate receptor-mediated increases of $[Ca^{2+}]_i$. (*Invest Ophthalmol Vis Sci.* 2002;43: 2666–2676)

Retinal ganglion cell (RGC) loss is a feature of a number of diseases that decrease visual function, including primary open-angle glaucoma[1,2] and anterior ischemic optic neuropathy.[3] Although accumulated evidence suggests that RGCs die by apoptosis in open-angle glaucoma and in experimental animal models of optic nerve damage,[4–8] the exact sequelae of damage leading to neuronal loss has not been resolved. Intraocular pressure and a variety of additional factors are probably involved.[9] Interruption of trophic support; oxidative stress; intracellular ion and electrolyte disturbance; increased extracellular glutamate levels, together with excessive stimulation of excitatory amino acid receptors (excitotoxicity); and pathologic increases in intracellular calcium ($[Ca^{2+}]_i$) ion concentration have all been implicated in RGC loss.[10–14]

The presence of apoptosis as a feature of glaucoma indicates that the pathways of cell death that contribute to glaucomatous damage may be similar to those observed in other neurodegenerative diseases of the central nervous system.[15,16] Thus, intervention at various upstream points in the apoptotic cascade, such as glutamate receptors, Ca^{2+} influx, and activation of proapoptotic genes may provide therapeutic targets for drugs that could delay or ameliorate RGC loss in glaucoma and optic nerve neuropathies.[13,14,17] In support of this, glutamate receptor antagonists, Ca^{2+}-channel blockers,[18–20] and caspase protease inhibitors,[21,22] have all been reported to decrease neuronal apoptosis in animal and cell culture models of retinal neuron death.

Adrenoceptor (AR) agonists and antagonists are frequently used in the medical management of glaucoma and decrease IOP by acting at sites in the ciliary process and various outflow pathways to decrease aqueous humor production and increase outflow, respectively.[23–26] An additional neuroprotective action of these agents, either alone or in combination with other drugs would therefore be of considerable benefit in the chronic treatment of glaucoma. Both the β_1-AR blocker, betaxolol, and the α_2-AR agonist, brimonidine, in addition to their ocular hypotensive actions, have been cited as neuroprotectants in various animal models of retinal and optic nerve injury.[27–38] However, because these in vivo studies used systemic or topical drug application, it is not possible to unequivocally rule out the actions of these compounds at target sites remote from retinal neurons (i.e., anterior segment and vascular beds). Therefore, it remains unclear whether the neuroprotection afforded by these drugs is due, at least in part, to a direct action on retinal neurons and, if direct, what mechanisms mediate such neuroprotection.

In this study we compared and contrasted the neuroprotective effects of a variety of β-AR blockers (betaxolol, metoprolol, and timolol) and α_2-AR-agonists (UK14,304 and brimonidine). The objectives of the study were to ascertain whether any of

these agents protect retinal neurons from glutamate excitotoxicity in vitro by increasing neuronal survival, and if so, to determine whether such neuroprotection effects are associated with receptor-mediated mechanisms and/or decreased calcium-influx, as assessed with calcium-imaging in both isolated retinal neurons and in RGCs in an intact retina preparation.

MATERIALS AND METHODS

Materials

All culture reagents were obtained from Gibco BRL (Burlington, Ontario, Canada), unless noted otherwise. All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless noted otherwise. BDNF and CNTF were provided by Regeneron Pharmaceuticals (Tarrytown, NY), betaxolol by Schwarz Pharma (Boulogne-Billancourt, France), and brimonidine tartrate by Allergan (Irvine, CA). Lysing solution (Zapoglobin) was purchased from Coulter Electronics (Hialeah, FL). The Ca^{2+} indicator dyes fura-2 AM, calcium green-1 dextran, and pluronic acid F-127, were purchased from Molecular Probes (Eugene, OR). In all cases L-glutamate was used but is referred to simply as glutamate.

Retinal Cell Cultures

Retinal neurons and glia were isolated from retinas obtained from 6- to 10-day-old Long-Evans rats. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Dalhousie University Committee for the use of Laboratory Animals. Briefly, rat pups were anesthetized with halothane and decapitated. The eyes were enucleated and posterior eyecups were placed in Hanks' balanced salt solution (HBSS) and 1% gentamicin. The neural retinas were gently peeled off with fine forceps and incubated at 37°C in 0.02% trypsin for 5 minutes or for 30 minutes in papain (20 U/mL; Worthington Biochemical, Lakewood, NJ). Fetal bovine serum (10%, FBS) was added to the tube to halt the enzyme reaction. Retinal cells were obtained by trituration in growth medium (Neurobasal medium, 10% FBS, 1 ng/mL ciliary neurotrophic factor [CNTF], 10 ng/mL brain-derived neurotrophic factor [BDNF], N_2 supplement, and 1% gentamicin). The resultant cell suspension was seeded at a density of 250,000 cells/mL on poly-D-lysine–coated coverslips in 24-well culture plates. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air, and half the growth medium was replaced every 2 to 3 days.

The identification of neurons in the mixed neuron-glia cultures was first established by double-label immunocytochemistry with a mouse antibody against neuron-specific β-tubulin (TUJ1; BabCo, Richmond, CA) and contrasted to the number of glial cells identified with a rabbit antibody against glial fibrillary acidic protein (GFAP). Primary antibody labeling was visualized with fluorescent dye conjugated to secondary antibodies (a goat anti-mouse IgG secondary antibody conjugated to Alexa 546 and goat anti-rabbit IgG secondary conjugated to Alexa 488; both from Molecular Probes). Cell density was determined by direct counts of the number of cells in 10 successive 680×460-μm fields (camera field of view for $40\times$ objective) in three separate experiments. A C6 glioma cell line (American Type Culture Collection, Manassas, VA) was used for studies requiring a pure CNS glial culture. These cells were maintained in DMEM containing 10% fetal calf serum.

Cell Survival Assays

Mixed retinal neurons and glial cultures grown for 7 days in 24-well culture dishes were divided into groups and treated for 24 hours with one of the following: (1) treatment medium (TM) consisting of DMEM, 10% FBS, N_2 supplement, 10 ng/mL BDNF, 1 ng/mL CNTF, and gentamicin; (2) TM + glutamate (TMG); or (3) TMG + drug (TMG+D). Cell survival was assayed by a cell lysis method, as previously described.[39] Briefly, the cells were removed from the 24-well plates with trypsin and centrifuged for 3 minutes, and the cell pellet was washed and

resuspended in 300 μL of a 1:10 dilution of lysing solution. Viable cell nuclei were counted with a hemocytometer. Mean cell counts were obtained from three to four wells per treatment group (TM, TMG, or TMG+D) and expressed as a percentage of the number of viable cells in the control TM group. Mean percentage data ± SEM were obtained from three to six separate experiments. The drugs used were betaxolol, metoprolol, and timolol (β-blockers) and UK14,304 and brimonidine (α_2-agonists), and the effect of each was tested over the range of concentrations from 1 to 1000 μM. The specificity of the effect of UK14,304 was further investigated with the α_2-AR antagonist yohimbine at 1 and 10 μM. Solutions of UK14,304 were prepared from stocks dissolved in dimethyl sulfoxide (DMSO). Therefore, in experiments in which UK14,304 was used, equivalent amounts of DMSO were also added to the other treatment solutions (TM, TMG).

Differences between mean data were compared for statistical significance with a two-tailed t-test or one-way analysis of variance (ANOVA) and the Dunnett multiple comparison test (Instat; GraphPad, San Diego, CA).

Calcium Imaging of Retinal Neuron Responses to Glutamate

Isolated rat retinal neurons were maintained in serum-free Neurobasal medium with 1 mM glutamine, 10 μg/mL gentamicin, 2% B27 supplement, 40 ng/mL BDNF, and 10 μM forskolin. The neurons were loaded with the ratiometric calcium-indicator dye fura-2. Cells were incubated in 5 μM fura-2-acetoxymethyl ester (AM) dissolved in modified HBSS (described later) for 20 minutes in the dark at 37°C. The fura-2 AM was first dissolved in DMSO (0.1% final concentration in HBSS) and then solubilized in HBSS containing 0.1% pluronic acid F-127. After loading, the cells were washed in HBSS for at least 15 minutes before imaging.

To assess the effect of glutamate on $[Ca^{2+}]_i$, we used a modified Mg^{2+}-free HBSS (pH 7.4, 20 mM HEPES, 1.5 mM $CaCl_2$). All treatment solutions containing glutamate, UK14,304, and yohimbine were prepared from stocks and dissolved in the modified HBSS. The fura-2–loaded neurons plated on coverslips were maintained in a chamber (ΔTC3; Bioptechs, Butler, PA) that was constantly superfused with HBSS at 34°C to 36°C (SH-27 Inline heater, TC-324B controller; Warner Instruments, Hamden, CT) and bubbled with 100% oxygen. Solutions were delivered to the chamber by a peristaltic pump (Gilson, Middleton, WI) at a rate of 2 mL/min. During treatments, image pairs were collected as often as every 3 seconds, but to limit photodamage, images were collected less frequently (20 seconds) during intervening periods.

The neurons loaded with fura-2 were imaged with a cooled charge-coupled device (CCD) camera (Sensicam; PCO Computer Optic, Kelheim, Germany) fitted to a microscope (Axioskop FS; Carl Zeiss, Oberkochen, Germany) using a water-immersion objective (numeric aperture 0.80; Achroplan $40\times$; Zeiss). Fura-2 fluorescence was produced by excitation from a 75W xenon lamp (Ludl Electronic Products, Hawthorne, NY) and appropriate filters (XF04 set, excitation 340 or 380 nm; emission 510 nm; dichroic >430 nm; Omega Optical, Brattleboro, VT). To reduce photodamage and photobleaching, excitation illumination was filtered with a −0.5 log neutral density filter and 640×512-pixel or 320×256-pixel images acquired. The duration of illumination was limited (400 msec) by an electronic shutter (Uniblitz, Rochester NY). Images at 340 and 380 nm excitation were captured and converted to ratiometric (340/380 nm) images by an imaging system (Imaging Workbench 2.2; Axon Instruments, Foster City, CA) and saved to the hard disk of a computer. The mean ratio over a large area of a neuron, well separated from the edge of the cell, was measured for each captured ratiometric image.

Free $[Ca^{2+}]_i$ was elevated in cultured cells by application of a short pulse (20 seconds) of 10 μM glutamate in HBSS. This protocol was chosen because it resulted in reproducible increases of $[Ca^{2+}]_i$ well within the dynamic range (below saturation) of fura-2 fluorescence. The retinal neurons were treated with UK14,304 (10 – 100 μM) or brimonidine (100 μM) for 5 minutes before and 3 minutes after the glutamate burst. Solutions of UK14,304 were prepared from stocks
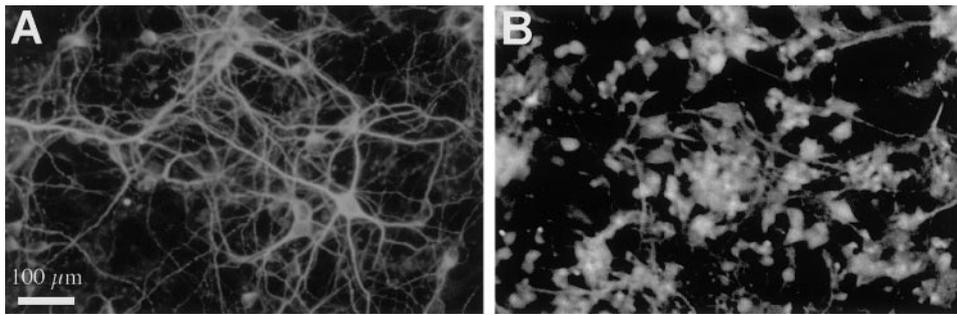
FIGURE 1. Double-label fluorescence photomicrograph of retinal neuron and glia coculture. (**A**) Neurons were labeled with an antibody against neuron-specific β-tubulin and (**B**) glial cells in the same field were labeled with an antibody against GFAP.

dissolved in DMSO. Therefore, in experiments in which UK14,304 was used, equivalent amounts of DMSO were also added to the other treatment solutions. For experiments with yohimbine, the neurons were pretreated first for 5 to 10 minutes with 1 μM yohimbine alone and then with yohimbine and UK14,304 together for the same period as in the experiments with UK14,304 alone. The time between consecutive bursts of glutamate was kept at 10 minutes to allow sufficient time for $[Ca^{2+}]_i$ levels to recover to a baseline level.

The peak fura-2 ratios were normalized to the initial treatment with glutamate and differences between the peak fura-2 ratios (pretreatment, in presence of UK14,304 or brimonidine, and recovery) were tested for statistical significance with the Freidman repeated measures ANOVA followed by a modified Student-Newman-Keuls multiple-comparison test. The differences between the peak ratios for UK14,304 alone versus UK14,304+yohimbine were tested for statistical significance with the Mann-Whitney rank sum test. All analyses were performed on computer (SigmaStat software; SPSS Science, Chicago, IL).

Calcium Imaging in an Intact Rabbit Retina Preparation

Calcium imaging of RGCs in intact retina was performed by a method described previously.[40] Rabbits were used for these experiments, because the isolated retina preparation from this animal is robust and well characterized and is the preparation in which the calcium-imaging technique used was developed. In brief, adult rabbits were killed by barbiturate overdose and both eyes enucleated. The anterior segment and vitreous were removed, and the posterior segment immersed in Ames medium,[41] buffered to pH 7.4 with HEPES, and bubbled continuously with 100% oxygen at room temperature. The retina was dissected from the eyecup, placed on a glass microscope slide, and cut into several pieces with a scalpel blade. The pieces of retina were then mounted on a filter (Millipore, Bedford, MA) with the ganglion cell layer uppermost.

Using a 10-μL syringe fitted with a sharpened tapered 26-gauge needle (Hamilton, Reno, NV) a small amount (<1 μL) of 10% (wt/vol) calcium green-1 conjugated to 10,000 MW dextran (CaGD) and dissolved in purified water was deposited within the substance of the retina. After application of the dye, retinas were incubated in the dark for at least 2 hours at room temperature in Ames medium bubbled continuously with 100% oxygen.

As described previously,[40] dextran-conjugated dye injected into the retina labels a variety of neuronal cell types near the injection site, including amacrine cells displaced to the ganglion cell layer, but it also labels RGC axons that pass through the injection site. In the latter case, the dextran is then retrogradely transported to groups of RGC somata up to 800 μm from the injection site. In the present work, only the RGCs retrogradely labeled by CaGD were studied.

Retinal wholemounts mounted on a filter were transferred to the same imaging sestup as that used in the studies of isolated cells, and the conduct of experiments was similar to that described for the studies of isolated cells. Because CaGD is not a ratiometric dye, fluorescence was imaged using only a single filter set (XF100; Omega Optical) and the data reported as increases of fluorescence intensity divided by the resting intensity ($\Delta F/F$). Free $[Ca^{2+}]_i$ within RGCs was increased by application of 50 μM N-methyl-D-aspartate (NMDA). The effect of

betaxolol (100 μM) on NMDA-induced increases in $[Ca^{2+}]_i$ was assessed by applying betaxolol to the isolated retina both before (8 minutes) and during the treatment with NMDA. Differences in CaGD $\Delta F/F$ during different treatments were tested for statistical significance with a paired t-test of the raw data (Statview SE; Abacus Concepts, Berkeley, CA).

RESULTS

### Immunocytochemical Identification of Neurons and Glia In Vitro

The identification of neurons in the mixed neuron-glia cultures was first established by using double-label immunocytochemistry with antibodies against neuron-specific β-tubulin and GFAP (Fig. 1). The neuron-specific, β-tubulin–labeled cells (neurons) had compact cell bodies and processes longer than twice the size of the somata, whereas GFAP labeling was restricted to cells (glia) with flattened morphology and processes less than twice the size of the somata. Colocalization was never observed. Therefore, in subsequent survival and calcium-imaging studies, cells were classified as neurons if they possessed the appropriate morphology together with processes (neurites) greater than twice the size of the somata. Cells without these long processes were considered to be glia. On average, neurons constituted approximately 35% to 40% of the total population of cultured cells.

Effect of Glutamate on Retinal Neuron Survival

The effects of increasing concentrations of glutamate on neuronal survival were examined in mixed rat retinal neuron-glial cultures. Addition of glutamate at concentrations of 1 to 1000 μM to mixed retinal cell cultures for 24 hours resulted in a significant dose-dependent decrease in the number of viable cells from 57% $\pm$ 2% of the control group at a dose of 1 μM glutamate, to 51% $\pm$ 1%, 37% $\pm$ 1%, and 19% $\pm$ 0.5% of the control group at 10, 100, and 1000 μM glutamate, respectively ($P < 0.05$; Fig. 2A). In subsequent experiments, a dose of 100 μM glutamate was used in cell survival assays, because this was the dose in mixed retinal cell cultures that consistently produced approximately 50% or greater cell loss. The cell loss in mixed cultures exposed to glutamate consisted primarily of neurons (Fig. 2B). After 24 hours' exposure to glutamate, there was a 91% decrease ($P < 0.01$) in the number of neurons, defined by the presence of neurites, but only a 14% decrease ($P > 0.50$) in the number of glial cells, defined by absence of neurites, flattened morphology, and positive staining for GFAP (Fig. 2B). Figure 2C shows the response of a pure glial cell line (C6 glioma) to glutamate treatment. These data demonstrate that 10 and 100 μM glutamate had no significant ($P > 0.40$) effect on glial cell survival.

Effects of Betaxolol, Timolol, and Metoprolol on Retinal Neuron Survival

Figure 3 shows data from experiments examining the survival of retinal cultures in the presence of 100 μM glutamate alone
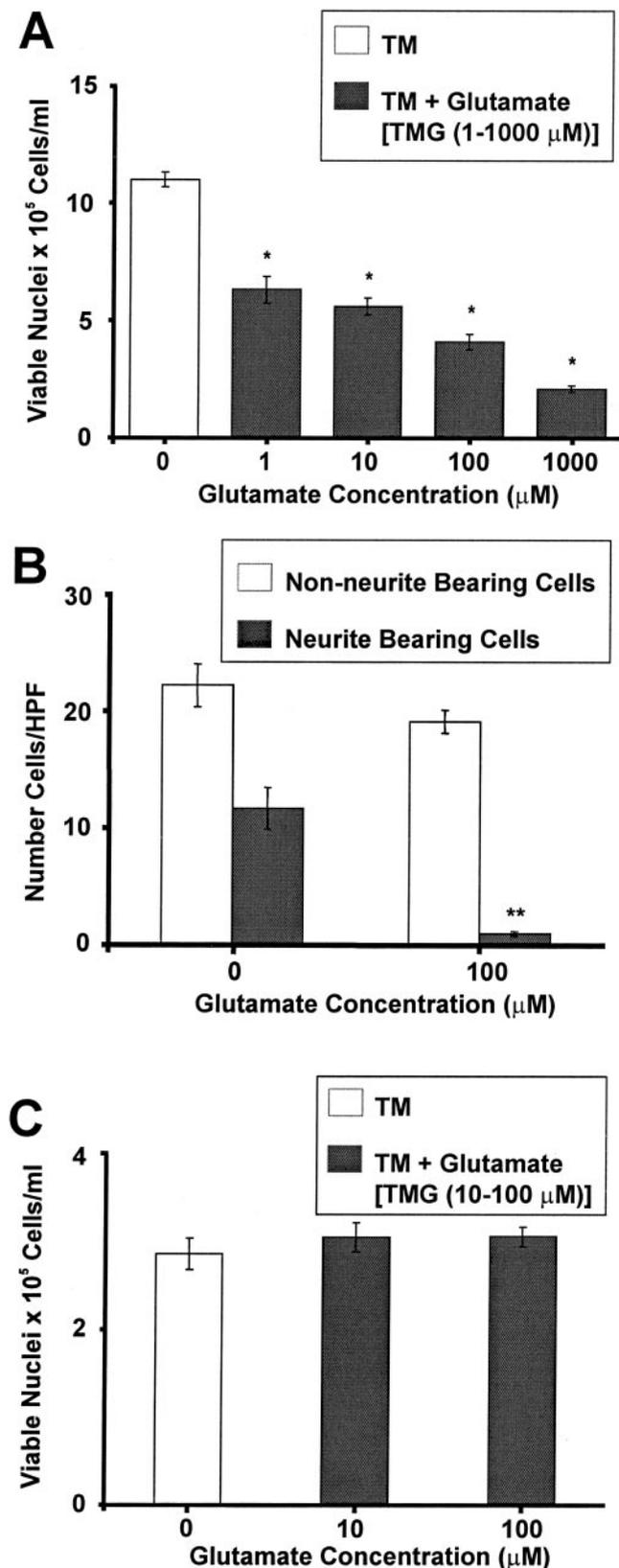
FIGURE 2. Glutamate-induced excitotoxic retinal neuron death. (**A**) Number of viable cells after 24 hours of glutamate treatment, as assessed by counting the density of intact nuclei after cell lysis. Mixed retinal neuron cultures were plated at a density of 250,000 cells/mL and treated with TM plus increasing doses of glutamate (TMG, 1–1000 μM). Control cultures were treated with TM without glutamate (TM, 0). (**B**) Morphologic analysis of the number of neurite-bearing (neu-

rons, processes more than twice the long axis of the somata of origin) and non–neurite-bearing (glia) cells in 10 successive 680×460-μm high-power ($40\times$ objective) fields, before and after 24 hours of glutamate (100 μM) treatment ($n = 3$). (**C**) C6 glioma rat cell line cultures treated for 24 hours with either 10 or 100 μM TM plus glutamate or TM without glutamate. Cell viability was determined from the number of intact nuclei after cell lysis. Data are the mean $\pm$ SEM of results derived from three to four separate experiments. (**A**) *$P < 0.05$ for the number of viable cells in TMG groups compared with the TM-only group; (**B**) **$P < 0.01$ for neurite-bearing cells in TMG (100 μm) compared with the number of neurite-bearing cells in control TM only (0).

or glutamate plus various concentrations (1–1000 μM) of the β_1-selective AR blockers betaxolol and metoprolol or the nonselective β-AR blocker timolol. Betaxolol produced a dose-dependent increase in viable cells up to 100 μM, compared with the glutamate-only treatment group (Fig. 3A). Viable cells (mean $\pm$ SEM) in the presence of 1, 10, 100, and 1000 μM betaxolol were 56% $\pm$ 10%, 78% $\pm$ 2%, 93% $\pm$ 14%, and 86% $\pm$ 30% of control glutamate-free group compared with 53% $\pm$ 4% of control in the presence of glutamate alone. This increase in cell survival was significant at 10 μM betaxolol ($P < 0.01$) with increased variability at higher doses limiting the significance ($P > 0.05$ and $P > 0.30$ at 100 and 1000 μM, respectively) of the effect of betaxolol. In contrast to betaxolol, the nonspecific β-AR blocker timolol failed ($P > 0.50$) to increase cell survival at all doses tested (1–1000 μM) over that observed in the glutamate-only treatment group (Fig. 3B). We also tested another β_1-selective AR blocker, metoprolol, at 1 to 1000 μM (Fig. 3C). As with timolol, the number of cells surviving in the metoprolol-treated groups was not significantly different ($P > 0.50$) from that in the glutamate-only group at all doses tested.
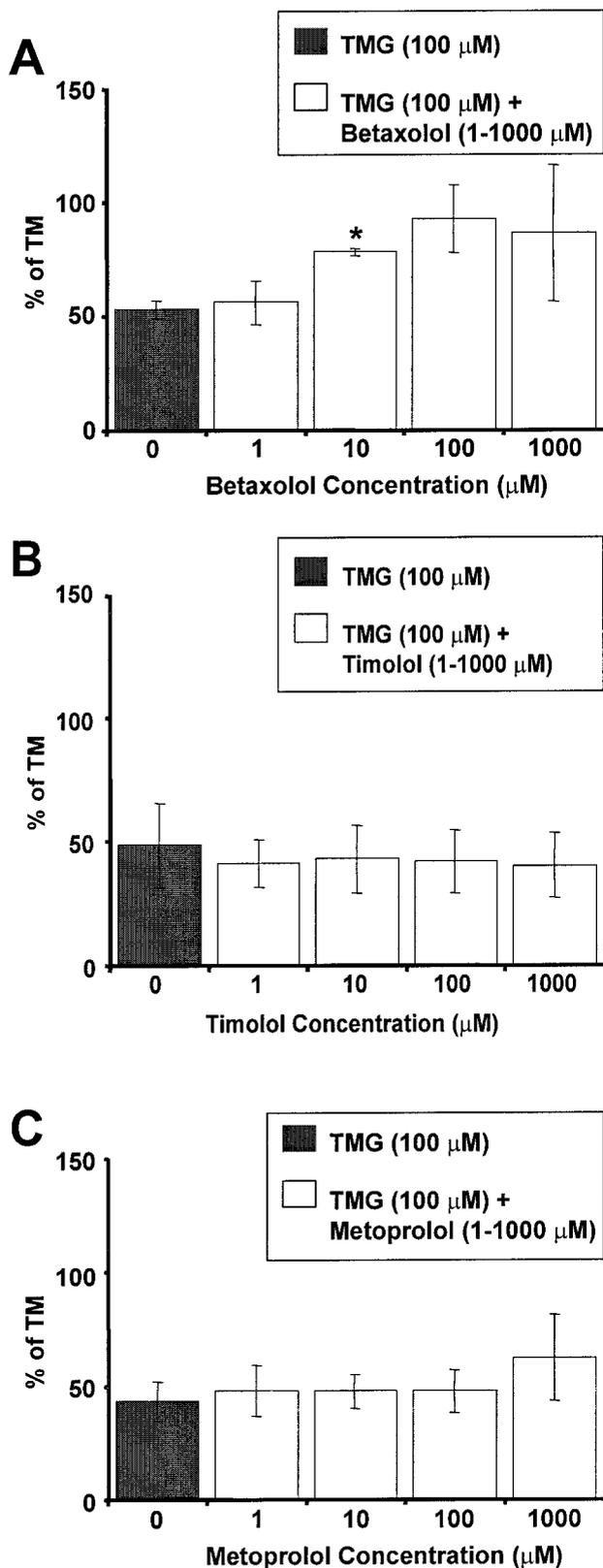
Effect of UK14,304 on Retinal Neuron Survival

In the groups treated with a dose of 10 or 100 μM UK14,304 plus glutamate (Fig. 4A) there was a significant increase ($P < 0.05$) in cell survival (109% $\pm$ 15% and 102% $\pm$ 12% of control, respectively) compared with that in the glutamate-only treatment groups (62% $\pm$ 3%). In the presence of 10 μM UK14,304 and 1 and 10 μM yohimbine (Fig. 4B), the number of surviving cells was not significantly different from that in the glutamate-only treatment group, but was significantly different from the glutamate plus α_2-AR agonist–treated group ($P < 0.05$). This suggests that in contrast to the β-AR blocker betaxolol, the α_2-AR agonist UK14,304 exerts a neuroprotective effect on retinal neurons through interacting with α_2-AR on retinal neurons and/or glia.

Effect of α_2-AR Activation on the Glutamate-Stimulated $[Ca^{2+}]_i$ Increase in Isolated Rat Retinal Neurons

Glutamate (10 μM) increased the fura-2 fluorescence ratio from a resting level of approximately 0.5 (Fig. 5A) to a peak of approximately 1.1 during exposure to 10 μM glutamate (Fig. 5B). One hundred twelve cells were studied and, on average, 10 μM glutamate produced a peak ratio of 1.07 $\pm$ 0.01 (mean $\pm$ SEM), increasing from an average resting ratio of 0.51 $\pm$ 0.01.

In the presence of 10 or 100 μM UK14,304, there was a significant decrease in the peak ratio reached by exposure to 10 μM glutamate. For example, in the cell shown in Figure 6A the presence of 100 μM UK14,304 reduced the glutamate-induced peak fura-2 ratio to 0.997, an 11% decrease from the control response of 1.12. On average, UK14,304 reduced sig-

A

B

C

FIGURE 3. Neuroprotective effects of the β-AR blockers on cultured retinal neurons. Retinal cell cultures were plated at a density of 250,000 cells/mL and divided into treatment groups consisting of TM only, TMG (100 μM), or TMG (100 μM) + various doses (1–1000 μM) of the β-AR blockers (A) betaxolol, (B) timolol, and (C) metoprolol and incubated for 24 hours. Cell survival was then assessed by determining the number of intact nuclei after cell lysis. Data are the mean ± SEM of results of three separate experiments, and expressed as a percentage of the TM control group (100%). *P < 0.05.

A

B
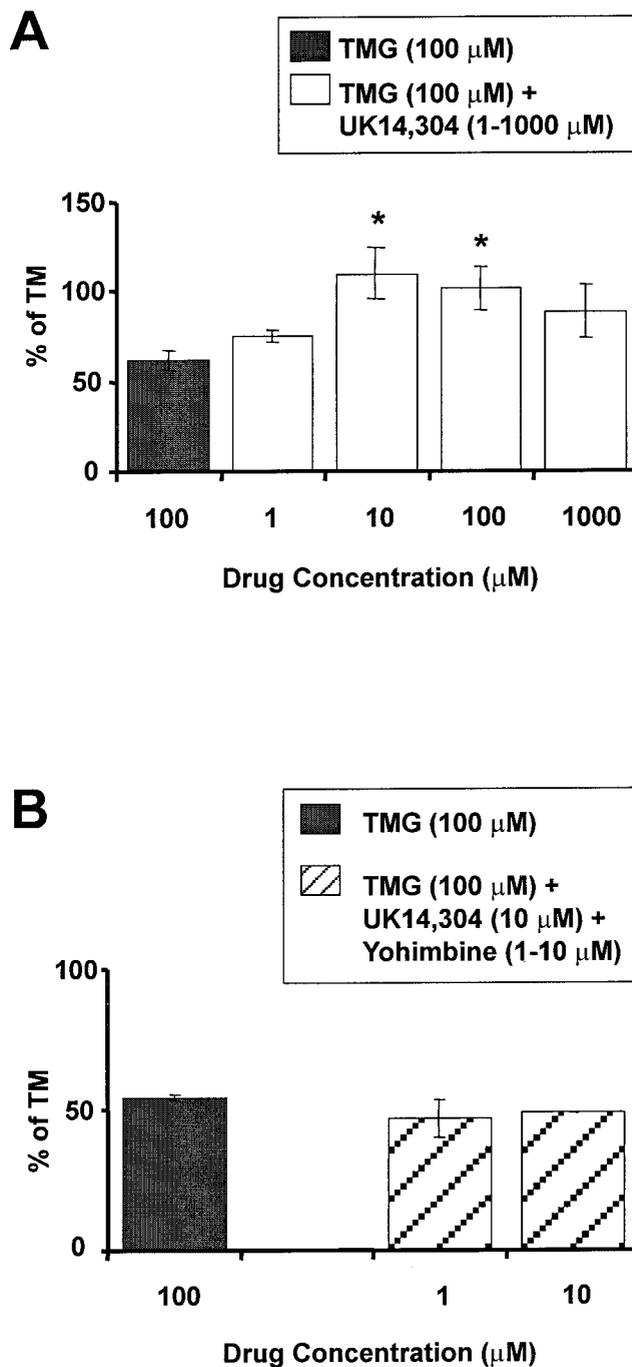
FIGURE 4. Neuroprotective effects of the α_2-AR agonist, UK14,304 on cultured retinal neurons. Retinal cell cultures were plated at a density of 250,000 cells/mL and then divided into experimental groups consisting of (A) TM, TMG (100 μM), or TMG (100 μM) + various doses (1–1000 μM) of UK14,304 and (B) TM, TMG (100 μM), or TMG (100 μM) + 10 μM UK14,304 or 10 μM UK14,304 in combination with 1 or 10 μM yohimbine. All experimental groups were incubated for a further 24 hours before cell survival was assessed from the number of intact nuclei after cell lysis. Data are the mean ± SEM of results in three separate experiments, and expressed as viable cells in each experimental group as a percentage of the TM control group (100%). *P < 0.05.

nificantly (P < 0.01) the effect of 10 μM glutamate on the peak fura-2 ratio with a dose-dependent decrease of 7% ± 1% (n = 21) and 15% ± 1% (n = 31) for 10 and 100 μM UK14,304, respectively (Fig. 6D). In the example shown in Figure 6A, there was essentially complete recovery of the
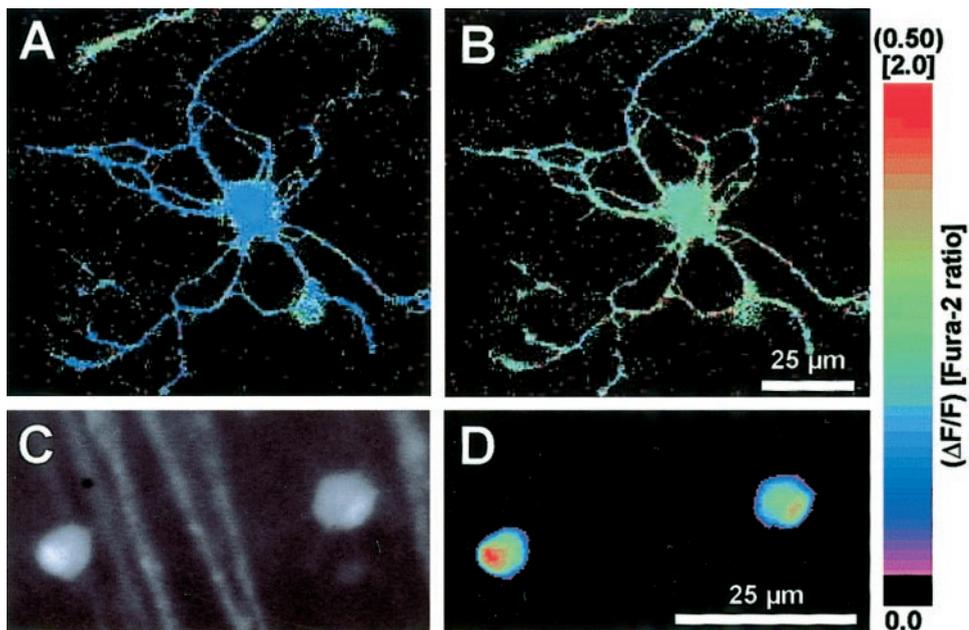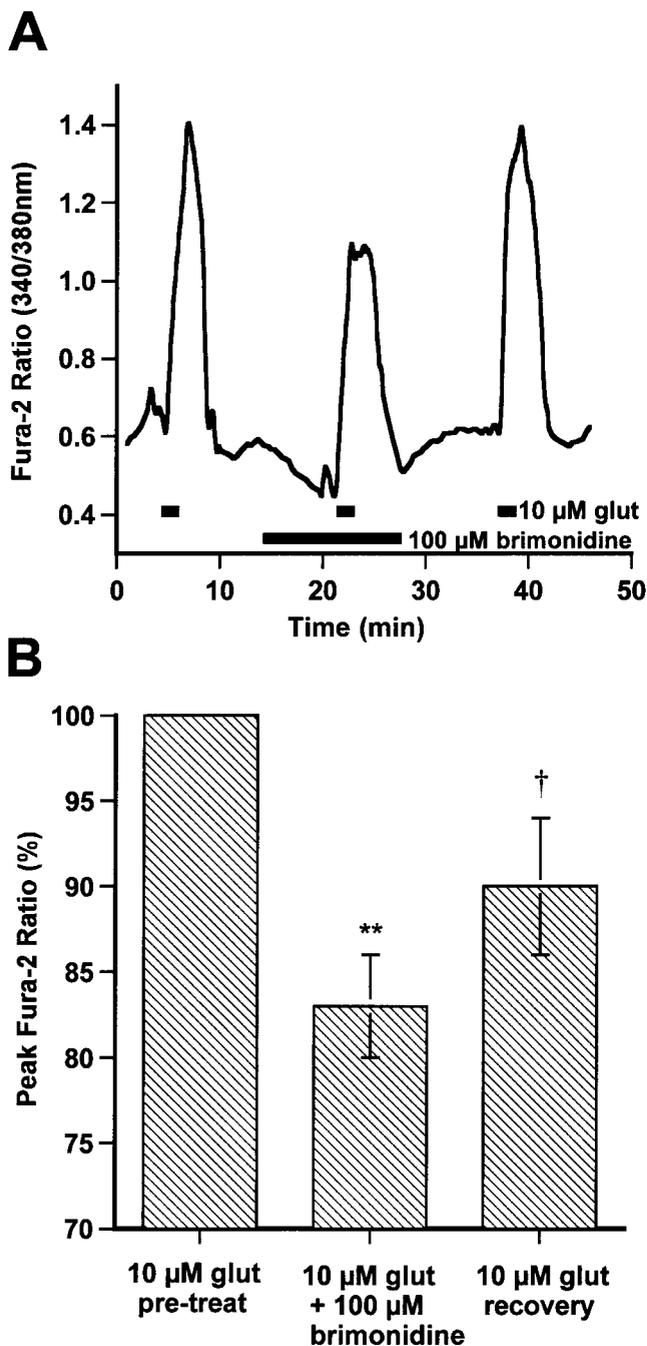
FIGURE 5. (**A**) Pseudocolor representation of the ratio of fura-2 fluorescence in an isolated retinal neuron before and (**B**) at the peak of the response to 10 μM glutamate. (**C**) Fluorescence micrograph showing the loading of two RGCs in the ganglion cell layer of intact rabbit retina with CaGD. (**D**) Pseudocolor representation of the response (ΔF/F) of these cells to 50 μM NMDA.

response to 10 μM glutamate after washout of UK14,304. On average, however, recovery was incomplete (Fig. 6D). Although the response to glutamate after washout of UK14,304 remained significantly different from the response produced before treatment with UK14,304 ($P < 0.01$) the peak ratio was significantly increased compared with that achieved during the treatment ($P < 0.01$). Similar results were achieved when brimonidine, an aqueous-soluble analogue of UK14,304,
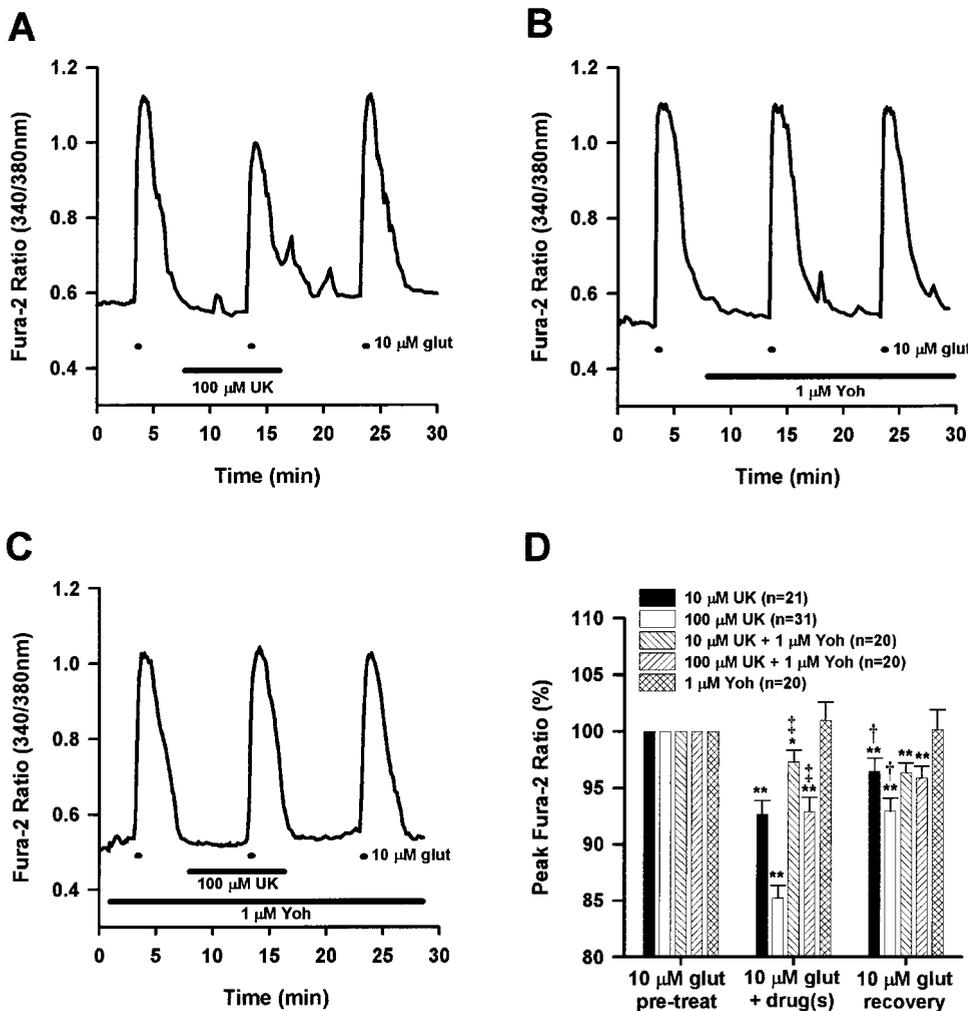
FIGURE 6. Effect of UK14,304 on the increase of $[Ca^{2+}]_i$ in isolated rat retinal neurons. (**A**) Effect of 100 μM UK14,304 on the peak of the fura-2 fluorescence ratio produced by 10 μM glutamate. (**B**) Effect of 1 μM yohimbine alone on the response to 10 μM glutamate. (**C**) Effect of 1 μM yohimbine on glutamate-induced increases of fura-2 fluorescence ratio in the presence of UK14,304. (**D**) Mean data for all cells studied normalized to the first exposure (pretreatment) to 10 μM glutamate. *$P < 0.05$, **$P < 0.01$ compared with glutamate pretreatment; †$P < 0.01$ recovery after pretreatment with UK14,304 + glutamate; ‡$P < 0.01$ effect of yohimbine on UK14,304 + glutamate.
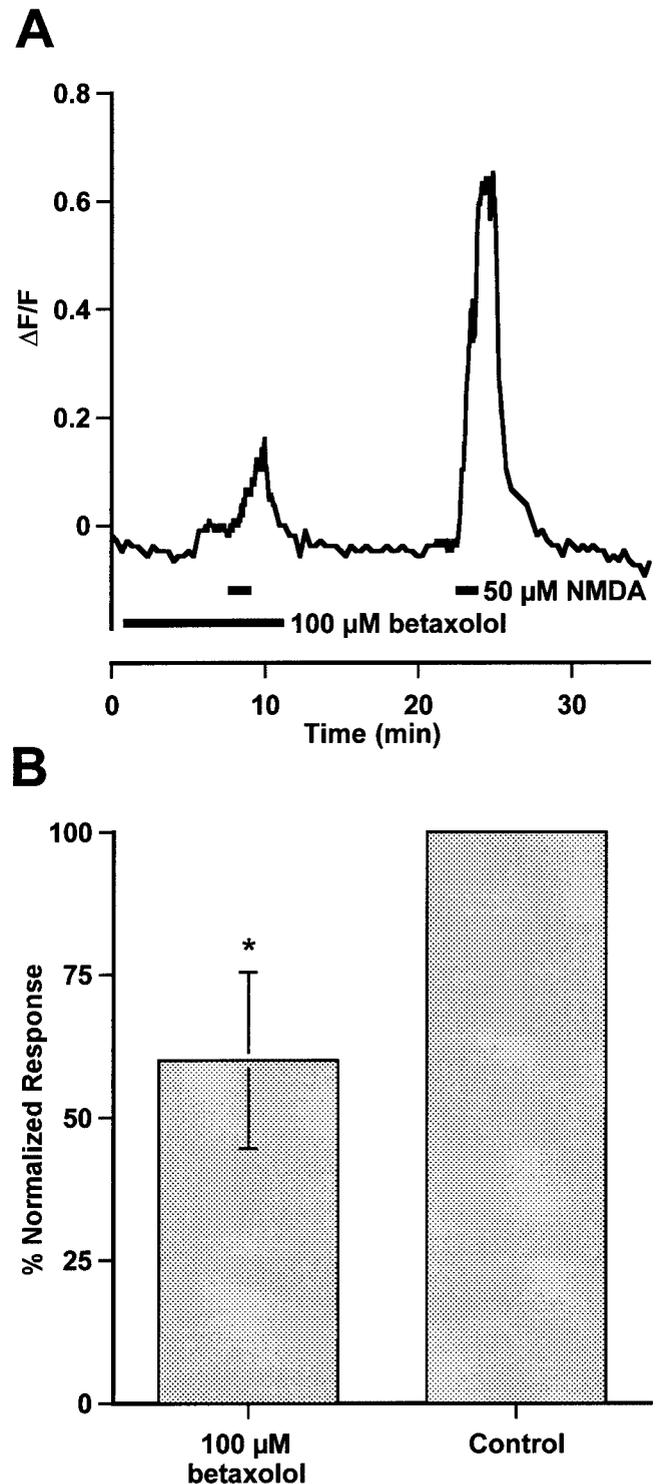
A

B

fura-2 ratio, but on average the decrease was not statistically significant.

To determine whether the effect of UK14,304 on glutamate-induced elevations of $[Ca^{2+}]_i$ were due to the specific action at α_2-ARs, the specific antagonist yohimbine was used. Initial experiments showed that 1 μM yohimbine alone had no effect ($n = 20$; $P > 0.05$) on the glutamate-induced increase of

A

B

FIGURE 7. Brimonidine limits the increase of $[Ca^{2+}]_i$ in isolated rat retinal neurons. (**A**) Effect of 100 μM brimonidine on the peak of the fura-2 fluorescence ratio produced by 10 μM glutamate. (**B**) Mean data for all cells studied ($n = 5$) normalized to the first exposure (pretreatment) to 10 μM glutamate. **$P < 0.01$ compared with glutamate pretreatment; †$P < 0.01$ recovery after pretreatment with brimonidine + glutamate.

was used (Fig. 7). The glutamate response was reduced by 17% $\pm$ 3% ($n = 5$) in the presence of 100 μM brimonidine and showed some recovery (10% $\pm$ 4% less than control response) after washout. The lack of full recovery was not due to a reduction of the response to glutamate, because multiple treatments with glutamate in the absence of UK14,304 or brimonidine produced similar ratio increases (data not shown, but see Fig. 6B). In some cells, such as the cell illustrated in Figure 7A, UK14,304 or brimonidine decreased the resting

FIGURE 8. Effect of betaxolol on the increase of $[Ca^{2+}]_i$ in RGCs in intact rabbit retina. (**A**) Change in fluorescence produced by 50 μM NMDA, in the presence and after washout of 100 μM betaxolol. (**B**) Mean data from 11 cells normalized to the response produced by NMDA. *$P < 0.05$.

$[Ca^{2+}]_i$ (Fig. 6B, 6D). These experiments also demonstrated that the reduced peak ratio during and after UK14,304 treatment was not simply due to a reduction of the response of the cells to glutamate over time or a result of repeated glutamate treatments. Yohimbine (1 μM) limited the effect of UK14,304 on glutamate-induced increases of $[Ca^{2+}]_i$. This is illustrated graphically for a single cell in Figure 6C, where the effect of 100 μM UK14,304 was essentially abolished by yohimbine. On average (Fig. 6D), 1 μM yohimbine reduced but did not completely eliminate the effect of UK14,304. Although glutamate responses in the presence of both yohimbine and UK14,304 did not reach control (pretreatment) levels ($P < 0.05$), the peak ratio obtained was greater than in the presence of UK14,304 alone and this was statistically significant ($n = 20$, Kruskal-Wallis; $P < 0.01$; Fig. 6D).

Effect of Betaxolol on NMDA-Induced Increases of Rabbit RGCs $[Ca^{2+}]_i$ in Intact Retina

RGCs within the ganglion cell layer of the rabbit retina were loaded with CaGD (Fig. 5C) and treated with 50 μM NMDA (Fig. 5D). NMDA was used instead of glutamate, because the uptake of glutamate by neurons and glia in intact retina preparation severely limits the level of extracellular glutamate, even if applied exogenously.[42] Although there was also some loading of axons with CaGD (Fig. 5C), NMDA treatment did not increase fluorescence within axons. Treatment with 100 μM betaxolol reduced the increase of CaGD fluorescence in RGCs produced by 50 μM NMDA (Fig. 8). The protocol used tested the response to 50 μM NMDA first in the presence of 100 μM betaxolol and then after approximately 10 minutes' washout. As described previously,[40] this protocol limits reduction of responses that can occur with multiple increases of $[Ca^{2+}]_i$ in this preparation. An optical recording from a single RGC within the rabbit ganglion cell layer is shown graphically in Figure 8A. The tissue was treated with 100 μM betaxolol for 10 minutes, but at 7 minutes was also exposed to a 2-minute pulse of 50 μM NMDA. In the cell illustrated (Fig. 8A) this resulted in an increase of CaGD fluorescence ($\Delta F/F$) of less than 0.2. After approximately 10 minutes' washout the tissue was treated again with 50 μM NMDA, and the response was much larger, exceeding a $\Delta F/F$ of 0.6. Average results from 11 RGCs are shown in Figure 8B. The responses have been normalized to the control (50 μM NMDA alone) treatment and show that, in the presence of 100 μM betaxolol, there was an approximate 40% decrease ($P < 0.05$) of the response of the cell to 50 μM NMDA.

DISCUSSION

There is now increasing evidence that both the β_1-AR antagonist betaxolol and α_2-AR agonists, such as brimonidine, decrease RGC cell death subsequent to increased intraocular pressure, retinal ischemia, or optic nerve crush.[27–38] However, all these studies were performed in vivo, and drugs were applied topically or systemically, making it impossible to determine whether the action of the drugs was directly neuroprotective or indirect, affecting local blood supply, inflammatory mediators or other nonretinal ocular tissues. Although no studies have assessed the effect of AR drugs on retinal neuron survival in vitro, a few studies have examined the effects of such drugs on ion channels but, for the most part, in neurons isolated from lower vertebrate retinas.[34,43,44]

Our experiments used a mammalian (rat) in vitro cell culture model of retinal excitotoxicity to examine the neuroprotective actions of the β-AR blockers, betaxolol, metoprolol, and timolol and the α_2-AR agonist UK14,304. This in vitro model

allowed the actions of the drugs to be studied directly on retinal neurons and glia in isolation of vascular actions and systemic effects. In our mixed retinal cultures, exposure to exogenous glutamate (10–100 μM) produced an approximate 40% to 50% reduction in the number of cells during a 24-hour period. Immunocytochemical and morphologic identification determined that most of the cell death was neuronal. Glial cells in mixed cultures were relatively resistant to such doses of glutamate, and cells from a purified glial cell line (C6 glioma) were also resistant to glutamate over the same range of concentrations (10–100 μM).

Of the β-blockers tested, only betaxolol increased cell survival after excitotoxic insult in primary rat retinal cultures, whereas the nonspecific β-blocker timolol and another β_1-AR-selective blocker metoprolol did not. These findings seem to demonstrate that the neuroprotective effect of betaxolol on retinal neurons is independent of β-AR interactions. The α_2-agonist UK14,304 was also able to increase retinal cell survival in mixed retinal cultures after excitotoxic stress. This effect was both dose-dependent and receptor-mediated, because it was blocked by the α_2-AR antagonist, yohimbine. The decrease in neuroprotection at higher doses of the α_2-AR agonist may be associated with agonist-mediated receptor desensitization.[45]

An examination of the effect of betaxolol on NMDA-induced increases of $[Ca^{2+}]_i$ in RGCs in isolated intact rabbit retina supports and extends previous results in isolated rat RGCs[43] showing that betaxolol inhibits glutamate-receptor stimulated increases of $[Ca^{2+}]_i$ in RGCs in situ. The use of a different species and a different preparation strengthens the view that betaxolol is capable of limiting excitotoxicity arising from excessive or prolonged Ca^{2+} influx. Glutamate-induced $[Ca^{2+}]_i$ increases in isolated retinal neurons were also decreased by α_2-AR agonists at doses comparable to those found to be neuroprotection in vitro. Therefore, some component of the neuroprotection afforded by betaxolol and α_2-agonists in vitro and in vivo may be due to decreased Ca^{2+} influx. A decrease in Ca^{2+} influx would be expected to increase cell survival after excitotoxic stress by reducing activation of Ca^{2+}-dependent proteases and limiting free radical production and DNA damage.[10,11,15]

It has been demonstrated that certain β-AR blockers have additional properties unrelated to their actions at ARs that include actions at other receptors and ion channels. In the case of betaxolol but not other β-AR blockers such as propranolol, this includes block of voltage-dependent Ca channels in peripheral[46] and retinal vasculature[47,48] and inhibition of neuronal ion channels, Ca^{2+} influx, and neuronal excitability.[34,43,49,50] In our studies, metoprolol, a β_1-AR blocker, failed to increase neuronal survival significantly at a concentration at which betaxolol had significant neuroprotective actions, and timolol, a nonselective β-AR blocker that has been shown not to have Ca channel-blocking action in cultured RGCs,[43] was also ineffective at increasing retinal neuron survival in primary cultures. These data support the hypothesis that in the in vitro retinal cell models used, the neuroprotective actions of betaxolol can be primarily attributed to its direct suppressive actions on neuronal ion channels and Ca^{2+} influx.

In contrast to the results obtained with betaxolol, our data suggest that the neuroprotective effects of UK14,304 on retinal neurons were mediated by a specific action at α_2-ARs, in particular because the neuroprotection was blocked by the α_2-AR antagonist yohimbine. These data are consistent with evidence that the α_2-AR agonists brimonidine and clonidine are neuroprotective in animal models of ischemia, in pressure-induced retinal hypoxia, and after mechanical damage to RGC axons.[28,31,32,51,52] The mechanism(s) for this in vivo neuroprotection also appears to involve α_2-AR activation, because selective antagonists

such as rauwolscine or yohimbine block the neuroprotection produced by agonist treatment.[32,51]

Several lines of evidence have indicated that the mechanisms underlying α_2-AR agonist-induced neuroprotection in the retina may include increased production of growth factors. For example, it has been suggested that photoreceptors are protected from light-induced damage by α_2-AR agonists through the enhancement of basic fibroblast growth factor (bFGF) production.[28] Upregulation of bFGF has been reported in several models of retinal ischemia,[52–54] and it has been suggested that the neuroprotective actions of bFGF may include acting as a free radical scavenger and attenuating ascorbate-iron–induced formation of reactive oxygen species.[52,53] This pathway may be a mechanism for the neuroprotective properties of α_2-AR agonists in the retina and may include nonneuronal targets such as retinal glia. In support of a nonneuronal action are data showing that in vivo administration of the α_2-agonists clonidine and xylazine activates extracellular signal-regulated kinases p42/p44 primarily in Müller cells,[55] an action that may indicate α_2-AR-mediated regulation of the release of neuroactive factors from Müller cells that could influence retinal neuronal survival.

Activation of neuronal α_2-ARs may also act directly to decrease Ca^{2+} influx and excitatory transmitter release.[56] Radioligand binding studies and cloning have demonstrated the presence of several α_2-AR isoforms (α2A, α2C, and α2D) in the inner plexiform layer of the mammalian retina.[56–58] In many types of neurons, α_2-ARs are coupled to voltage-dependent N-type and P/Q type Ca channels through the G_i family of proteins to inhibit Ca^{2+} current, Ca^{2+} influx, and neurotransmitter release.[59–61] RGCs and retinal neurons are known to express different Ca channel subtypes, including a toxin-resistant Ca^{2+} current component and N-type and L-type Ca^{2+} currents.[62,63] Therefore, activation of α_2-ARs may result in inhibition of Ca channels on retinal neurons. Another laboratory has examined binding of the α_2-AR agonist clonidine to voltage-dependent Ca and Na channels as well as the effects of clonidine on radiolabeled Ca^{2+} influx in intact isolated rat retina. Their studies have reported that, unlike betaxolol, clonidine did not directly interact with voltage-dependent L-type Ca channels or Na channels and did not produce a significant decrease in Ca^{2+} influx.[52,64] Our data examining alterations of $[Ca^{2+}]_i$ in isolated retinal neurons indicate that both UK14,304 and brimonidine can reduce the glutamate-induced increases of $[Ca^{2+}]_i$ that may arise through receptor-mediated modulation of voltage-dependent Ca channels and/or release of Ca^{2+}-dependent stores. The inhibitory effect of UK14,304 on glutamate-induced increases of $[Ca^{2+}]_i$ was reduced after pretreatment with the α_2-AR antagonist yohimbine, confirming that activation of α_2-ARs on retinal neurons inhibits increases in glutamate-stimulated increases of $[Ca^{2+}]_i$.

The findings of this study that both the β_1-AR antagonist betaxolol and the α_2-AR agonists UK14,304 and brimonidine can increase survival of isolated retinal neurons in vitro confirm that these drugs can act as neuroprotectants through actions independent of vascular targets. The effective concentrations of drugs used in our studies of isolated cells and retina (1–100 μM) and others using isolated tissue preparations[34,49] are in the clinically relevant range, estimated to be approximately 1 μM in plasma, with greater drug levels accumulating during prolonged administration.[48,65] In vivo experiments in rats and monkeys using topical and systemic application of β-blockers indicate that levels of these drugs with repeated administration accumulate in the micromolar range in the retina and optic nerve head, with even greater levels present in the choroid, sclera, and iris ciliary body.[66,67] Our results comparing different β-AR antagonists support and extend previous data by demonstrating that the neuroprotective properties of

betaxolol in the retina are due, at least in part, to a direct effect on neurons, are independent of specific β-AR receptor interactions and probably arise from the actions of betaxolol on neuronal ion channels and $[Ca^{2+}]_i$.[34,43,49,50,52] In contrast to betaxolol, the survival-promoting actions of the α_2-AR agonist UK14,304 occurred through the activation of α_2-AR receptors. However, our data demonstrating that α_2-AR agonists can modulate $[Ca^{2+}]_i$ after exposure to glutamate suggest that in addition to other reported actions, such as increasing retinal levels of bFGF,[28,52] they may also act directly on retinal neuron α_2-AR receptors to limit increases in $[Ca^{2+}]_i$.

References

1. Quigley HA. Neuronal death in glaucoma. *Prog Retinal Eye Res.* 1999;18:39–57.
2. Nickells RW. Retinal ganglion cell death in glaucoma: the how, the why, and the maybe. *J Glaucoma.* 1996;5:345–356.
3. Anderson DR, Quigley HA. The optic nerve. In: Hart WM, ed. *Adler's Physiology of the Eye: Clinical Application.* 9th ed. St. Louis: Mosby-Year Book; 1992:616–640.
4. Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res.* 1995;61:33–44.
5. Garcia-Valenzuela E, Gorczyca W, Darzynkiewicz Z, Sharma SC. Apoptosis in adult retinal ganglion cells after axotomy. *J Neurobiol.* 1994;25:431–438.
6. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci.* 1995;36:774–786.
7. Nickells RW, Zack DJ. Apoptosis in ocular disease: a molecular overview. *Ophthalmic Genet.* 1996;17:145–165.
8. Kerrigan LA, Zack DJ, Quigley HA, Smith SD, Pease ME. TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch Ophthalmol.* 1997;115:1031–1035.
9. Anderson DR. What happens to the optic disc and retina in glaucoma? *Ophthalmology.* 1983;90:766–770.
10. Kelly MEM, Barnes S. Physiology and pathophysiology of nitric oxide in the retina. *Neuroscientist.* 1997;3:357–360.
11. Nickells RW. Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death. *Surv Ophthalmol.* 1999;43(suppl 1):S151–S161.
12. Sucher NJ, Lei SZ, Lipton SA. Calcium channel antagonists attenuate NMDA receptor-mediated neurotoxicity of retinal ganglion cells in culture. *Brain Res.* 1991;551:297–302.
13. Osborne NN, Chidlow G, Nash MS, Wood JP. The potential of neuroprotection in glaucoma treatment. *Curr Opin Ophthalmol.* 1999;10:82–92.
14. Osborne NN, Ugarte M, Chao M, et al. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol.* 1999;43(suppl 1):S102–S128.
15. Leist M, Nicotera P. Apoptosis, excitotoxicity, and neuropathology. *Exp Cell Res.* 1998;239:183–201.
16. Leist M, Nicotera P. Calcium and neuronal death. *Rev Physiol Biochem Pharmacol.* 1998;132:79–125.
17. Weinreb RN, Levin LA. Is neuroprotection a viable therapy for glaucoma? *Arch Ophthalmol.* 1999;117:1540–1544.
18. Osborne NN, Schwarz M, Pergande G. Protection of rabbit retina from ischemic injury by flupirtine. *Invest Ophthalmol Vis Sci.* 1996;37:274–280.
19. Krieglstein J, Lippert K, Poch G. Apparent independent action of nimodipine and glutamate antagonists to protect cultured neurons against glutamate-induced damage. *Neuropharmacology.* 1996;35:1737–1742.
20. Lipton SA. Calcium channel antagonists in the prevention of neurotoxicity. *Adv Pharmacol.* 1991;22:271–297.
21. Tezel G, Wax MB. Inhibition of caspase activity in retinal cell apoptosis induced by various stimuli in vitro. *Invest Ophthalmol Vis Sci.* 1999;40:2660–2667.

22. Lam TT, Abler AS, Tso MO. Apoptosis and caspases after ischemia-reperfusion injury in rat retina. *Invest Ophthalmol Vis Sci.* 1999; 40:967–975.

23. Diestelhorst M. Medical treatment of glaucoma and the promising perspectives. *Curr Opin Ophthalmol.* 1996;7:18–23.

24. Wilensky JT. Clinical experience with brimonidine tartrate, an α2-agonist. In: Bucci MG, ed. *Glaucoma: Decision Making in Therapy.* New York: Springer-Verlag; 1996:319–322.

25. Epstein DL. Medications used in chronic glaucoma therapy: adrenergic agents: blockers and agonists. In: Epstein DL, Allingham RR, Schulman JS, eds. *Chandler and Grant's Glaucoma.* Baltimore, MD: Williams & Wilkins; 1997:137–151.

26. Buckley MM, Goa KL, Clissold SP. Ocular betaxolol: a review of its pharmacological properties, and therapeutic efficacy in glaucoma and ocular hypertension. *Drugs.* 1990;40:75–90.

27. Morrison JC, Nylander KB, Lauer AK, Cepurna WO, Johnson E. Glaucoma drops control intraocular pressure and protect optic nerves in a rat model of glaucoma. *Invest Ophthalmol Vis Sci.* 1998;39:526–531.

28. Wen R, Cheng T, Li Y, Cao W, Steinberg RH. Alpha 2-adrenergic agonists induce basic fibroblast growth factor expression in photoreceptors in vivo and ameliorate light damage. *J Neurosci.* 1996; 16:5986–5992.

29. Yoles E, Wheeler LA, Schwartz M. Alpha2-adrenoreceptor agonists are neuroprotective in a rat model of optic nerve degeneration. *Invest Ophthalmol Vis Sci.* 1999;40:65–73.

30. Osborne NN, DeSantis L, Bae JH, et al. Topically applied betaxolol attenuates NMDA-induced toxicity to ganglion cells and the effects of ischaemia to the retina. *Exp Eye Res.* 1999;69: 331–342.

31. Ahmed FA, Hegazy K, Chaudhary P, Sharma SC. Neuroprotective effect of alpha(2) agonist (brimonidine) on adult rat retinal ganglion cells after increased intraocular pressure. *Brain Res.* 2001; 913:133–139.

32. Donello JE, Padillo EU, Webster ML, Wheeler LA, Gil DW. alpha(2)-Adrenoceptor agonists inhibit vitreal glutamate and aspartate accumulation and preserve retinal function after transient ischemia. *J Pharmacol Exp Ther.* 2001;296:216–223.

33. Lafuente MP, Villegas-Perez MP, Sobrado-Calvo P, Garcia-Aviles A, Miralles de Imperial J, Vidal-Sanz M. Neuroprotective effects of alpha(2)-selective adrenergic agonists against ischemia-induced retinal ganglion cell death. *Invest Ophthalmol Vis Sci.* 2001;42: 2074–2084.

34. Osborne NN, Cazevieille C, Carvalho AL, Larsen AK, DeSantis L. In vivo and in vitro experiments show that betaxolol is a retinal neuroprotective agent. *Brain Res.* 1997;751:113–123.

35. Wood JP, DeSantis L, Chao HM, Osborne NN. Topically applied betaxolol attenuates ischaemia-induced effects to the rat retina and stimulates BDNF mRNA. *Exp Eye Res.* 2001;72:79–86.

36. Chao HM, Osborne NN. Topically applied clonidine protects the rat retina from ischaemia/reperfusion by stimulating alpha(2)-adrenoceptors and not by an action on imidazoline receptors. *Brain Res.* 2001;904:126–136.

37. WoldeMussie E, Ruiz G, Wijono M, Wheeler LA. Neuroprotection of retinal ganglion cells by brimonidine in rats with laser-induced chronic ocular hypertension. *Invest Ophthalmol Vis Sci.* 2001;42: 2849–2855.

38. Levkovitch-Verbin H, Harris-Cerruti C, Groner Y, Wheeler LA, Schwartz M, Yoles E. RGC death in mice after optic nerve crush injury: oxidative stress and neuroprotection. *Invest Ophthalmol Vis Sci.* 2000;41:4169–4174.

39. Gupta SK, Jollimore CA, McLaren MJ, Inana G, Kelly ME. Mammalian retinal pigment epithelial cells in vitro respond to the neurokines ciliary neurotrophic factor and leukemia inhibitory factor. *Biochem Cell Biol.* 1997;75:119–125.

40. Baldridge WH. Optical recordings of the effects of cholinergic ligands on neurons in the ganglion cell layer of mammalian retina. *J Neurosci.* 1996;16:5060–5072.

41. Ames A III, Nesbett FB. In vitro retina as an experimental model of the central nervous system. *J Neurochem.* 1981;37:867–877.

42. Massey SC, Miller RF. Glutamate receptors on ganglion cells in the rabbit retina: evidence for glutamate as a bipolar cell transmitter. *J Physiol.* 1988;405:635–655.

43. Hirooka K, Kelly ME, Baldridge WH, Barnes S. Suppressive actions of betaxolol on ionic currents in retinal ganglion cells may explain its neuroprotective effects. *Exp Eye Res.* 2000;70: 611–621.

44. Gross RL, Hensley SH, Gao F, Yang XL, Dai SC, Wu SM. Effects of betaxolol on light responses and membrane conductance in retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2000;41:722–728.

45. Invernizzi RW, Parini S, Sacchetti G, et al. Chronic treatment with reboxetine by osmotic pumps facilitates its effect on extracellular noradrenaline and may desensitize alpha(2)-adrenoceptors in the prefrontal cortex. *Br J Pharmacol.* 2001;132: 183–188.

46. Setoguchi M, Ohya Y, Abe I, Fujishima M. Inhibitory action of betaxolol, a beta 1-selective adrenoceptor antagonist, on voltage-dependent calcium channels in guinea-pig artery and vein. *Br J Pharmacol.* 1995;115:198–202.

47. Hoste AM, Andries LJ, Sys SU. The Ca2+ channel blocking action of the β-blockers in bovine retinal microartery. In: Bucci MG, ed. *Glaucoma: Decision Making in Therapy.* New York: Springer-Verlag; 1996:307–312.

48. Hester RK, Chen Z, Becker EJ, McLaughlin M, DeSantis L. The direct vascular relaxing action of betaxolol, carteolol and timolol in porcine long posterior ciliary artery. *Surv Ophthalmol.* 1994; 38(suppl):S125–S134.

49. Gross RL, Hensley SH, Gao F, Wu SM. Retinal ganglion cell dysfunction induced by hypoxia and glutamate: potential neuroprotective effects of beta-blockers. *Surv Ophthalmol.* 1999;43(suppl 1):S162–S170.

50. Chidlow G, Melena J, Osborne NN. Betaxolol, a beta(1)-adrenoceptor antagonist, reduces Na(+) influx into cortical synaptosomes by direct interaction with Na(+) channels: comparison with other beta-adrenoceptor antagonists. *Br J Pharmacol.* 2000; 130:759–766.

51. Wheeler LA, Lai R, Woldemussie E. From the lab to the clinic: activation of an alpha-2 agonist pathway is neuroprotective in models of retinal and optic nerve injury. *Eur J Ophthalmol.* 1999; 9(suppl 1):S17–S21.

52. Chao HM, Chidlow G, Melena J, Wood JP, Osborne NN. An investigation into the potential mechanisms underlying the neuroprotective effect of clonidine in the retina. *Brain Res.* 2000;877:47–57.

53. Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci.* 1994;35:907–915.

54. Zhang C, Takahashi K, Lam TT, Tso MO. Effects of basic fibroblast growth factor in retinal ischemia. *Invest Ophthalmol Vis Sci.* 1994;35:3163–3168.

55. Peng M, Li Y, Luo Z, Liu C, Laties AM, Wen R. Alpha2-adrenergic agonists selectively activate extracellular signal- regulated kinases in Muller cells in vivo. *Invest Ophthalmol Vis Sci.* 1998;39:1721–1726.

56. Bertolino M, Vicini S, Gillis R, Travagli A. Presynaptic alpha2-adrenoceptors inhibit excitatory synaptic transmission in rat brain stem. *Am J Physiol.* 1997;272:G654–G661.

57. Bylund DB, Chacko DM. Characterization of α_2-adrenergic receptor subtypes in human ocular tissue homogenates. *Invest Ophthalmol Vis Sci.* 1999;40:2299–2306.

58. Venkataraman V, Duda T, Galoian K, Sharma RK. Molecular and pharmacological identity of the alpha 2D-adrenergic receptor subtype in bovine retina and its photoreceptors. *Mol Cell Biochem.* 1996;159:129–138.

59. Boehm S, Huck S. Inhibition of N-type calcium channels: the only mechanism by which presynaptic alpha 2-autoreceptors control sympathetic transmitter release. *Eur J Neurosci.* 1996;8:1924–1931.

60. Li YW, Bayliss DA. Activation of alpha 2-adrenoceptors causes inhibition of calcium channels but does not modulate inwardly-rectifying K+ channels in caudal raphe neurons. *Neuroscience.* 1998;82:753–765.

61. Jeong SW, Ikeda SR. Effect of G protein heterotrimer composition on coupling of neurotransmitter receptors to N-type Ca(2+) channel modulation in sympathetic neurons. *Proc Natl Acad Sci USA.* 2000;97:907–912.

62. Rothe T, Grantyn R. Retinal ganglion neurons express a toxin-resistant developmentally regulated novel type of high-voltage-activated calcium channel. *J Neurophysiol.* 1994;72:2542–2546.

63. Karschin A, Lipton SA. Calcium channels in solitary retinal ganglion cells from post-natal rat. *J Physiol.* 1989;418:379–396.

64. Melena J, Stanton D, Osborne NN. Comparative effects of antiglaucoma drugs on voltage-dependent calcium channels. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:522–530.

65. Beresford R, Heel RC. Betaxolol. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in hypertension. *Drugs.* 1986;31:6–28.

66. Dahlin DC, Curtis MA, DeSantis L, Struble CB. Distribution of betaxolol to posterior ocular tissues of the Cynomolgus monkey following a 30-day BID topical ocular regimen of betopic S [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41:S509. Abstract nr 2710.

67. Tan AY, LeVatte TL, Tremblay F, Kelly MEM, Chauhan BC. Timolol concentrations in rat ocular tissues and plasma following topical and intraperitoneal dosing. *J Glaucoma.* 2002;11:134–142.