Topical Flunarizine Reduces IOP and Protects the Retina against Ischemia-Excitotoxicity

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PURPOSE. To determine whether topical application of flunarizine reduces intraocular pressure (IOP) and acts as a retinal neuroprotectant and to compare the effectiveness of flunarizine with betaxolol and nifedipine at reducing the influx of calcium and sodium.

METHODS. Ischemia was delivered to the rabbit retina by raising the IOP. After 3 days, a flash electroretinogram (ERG) was recorded, and the retina processed for the localization of certain antigens. In the rat, N-methyl-D-aspartate (NMDA) was injected intravitreally, and 8 days later, the retinas were analyzed for the localization of Thy-1 or the relative amounts of mRNAs for antigens located to ganglion cells or photoreceptors. Rats and rabbits received topical flunarizine or vehicle before and after ischemia or NMDA. IOP was measured in rabbits after a single topical application of 2% flunarizine. Studies were conducted on isolated rat retinas, cortical cultures, and brain synaptosomes to compare the effectiveness of flunarizine with nifedipine and betaxolol at reducing the influx of calcium or sodium.

RESULTS. Changes in rabbit retinal choline acetyltransferase and parvalbumin immunoreactivities and the b-wave of the ERG caused by ischemia-reperfusion were blunted by topical treatment with flunarizine. Similarly, NMDA induced reductions in Thy-1 immunoreactivity and mRNA for rat ganglion cell antigens (Thy-1 and neurofilament light form) were counteracted by topical application of flunarizine. Topical application of 2% flunarizine significantly lowered the IOP in rabbits over a period of 5 hours. Flunarizine was more effective than betaxolol and much stronger than nifedipine at attenuating veratridine-induced influx of sodium into synaptosomes. Nifedipine, flunarizine, and betaxolol all reduced the NMDA-induced influx of calcium into the isolated retina or cortical neurons, but betaxolol was the least effective.

CONCLUSIONS. Topically applied flunarizine reduces IOP and attenuates injury to the whole of the retina, including the ganglion cells. The neuroprotective action of flunarizine is to reduce the influx of calcium and sodium into stressed neurons. The potent effect of flunarizine on sodium influx would be particularly protective to axons. (Invest Ophthalmol Vis Sci. 2002;43:1456–1464)

The term neuroprotection describes the process whereby an agent is administered to reach an injured tissue and interacts with specific cellular components to attenuate a death process.1,2 Neuroprotection has particular relevance to the ganglion cells of the retina, which are compromised in a number of pathologic situations—most important, in glaucoma. It is not known what initiates the death of ganglion cells in, for example, glaucoma,3 but probable causes are mechanical and/or ischemic damage to their axons at the level of the lamina cribrosa, induced by either elevated IOP or inadequate blood flow. If this latter idea is tenable, then the use of pharmacologic agents to improve the blood supply to the optic nerve head (i.e., vasoprotection) is also a potential treatment strategy. The ideal antiglaucoma drug for the future may therefore be a substance that not only reduces IOP but also possesses neuroprotective and vasoprotective characteristics.

A common feature of both ischemia to the retina and mechanical damage to the optic nerve in animals is an excessive release of the excitatory neurotransmitter glutamate.5–6 Glutamate and glutamate receptor agonists are toxic to retinal ganglion cells, both in culture7 and when injected intravitreally.8 Ganglion cells are thought to be particularly sensitive to glutamate toxicity because of the presence of N-methyl-D-aspartate (NMDA) receptors on these cells.9 In support of this assertion, NMDA receptor antagonists protect ganglion cells from ischemic and excitotoxic insults8,10 and from optic nerve crush.11,12 A role for glutamate receptor-mediated excitotoxicity in the pathogenesis of both true and experimental glaucoma is supported by the findings that the glutamate levels in the vitreous humor of patients with glaucoma and animals are significantly elevated in comparison with those in control subjects.13 Activation of voltage-dependent sodium and calcium channels (Na+ and Ca2+ channels) are key steps in the release of glutamate and the cascade of detrimental events that occur in ischemia. As a consequence, it is of no surprise to find that substances that diminish the influx of Ca2+ and/or Na+ have been shown to protect neurons in the central nervous system (CNS) from the detrimental effects of ischemia-excitotoxicity.14,15

Interest in the potential neuroprotective action of calcium channel antagonists in retinal ischemia-excitotoxicity models has stemmed from the knowledge that a number of these drugs can lower IOP and improve retinal blood flow. For example, the L-type antagonists verapamil, nifedipine, and diltiazem, applied topically, all reduce IOP in rabbits16 and primates.17 Topical verapamil has also been shown to lower IOP18 and improve microcirculation in the optic nerve head19 in humans. Results to date with calcium channel blockers used in retinal models of ischemia-excitotoxicity are positive. Researchers have reported favorable effects of nifedipine,20 lomerizine,21 nimodipine, and levemopamil.22,23

The ability of Na+ channel antagonists to protect retinal neurons from insults in vivo has thus far been largely ignored. This is surprising, because substances that reduce Na+ influx have been shown to be particularly effective neuroprotectants against ischemia-induced damage to CNS white matter, rather than gray matter.24 Pertinent to the retina, anoxic injury to the isolated rat optic nerve can be ameliorated by exposure to the
potent Na⁺ channel blocker tetrodotoxin. 25 Because the initial insult to ganglion cells in diseases such as glaucoma appears to occur at the level of their axons (white matter), it can be argued that an effective neuroprotectant for this disease may ideally have sodium as well as calcium channel-blocking characteristics. Certain ligands display both calcium and sodium channel-blocking activities, 26 and the rationale for the current research is to provide detailed information on the potential effectiveness of one such drug, flunarizine, in paradigms of ischemia-excitotoxicity.

The diphenylalkylamine flunarizine is classified as a type IV voltage-dependent calcium channel antagonist and a vasodilator. It is regarded as a wide-spectrum Ca²⁺ channel antagonist, but nevertheless displays relative selectivity for the L- and T-type channels. Recent work has shown that topical application of flunarizine reduces IOP in primates 17 and improves blood flow to the optic nerve head in patients with low-tension glaucoma. 27 Flunarizine also exhibits considerable affinity for Na⁺ channels, 28 although whether this property contributes to its effects remains unknown. Flunarizine has been found to attenuate the effect of ischemia to the brain 29–33 and also protects neurons from death after axotomy—nerve crush. 33

In rats, flunarizine reduces ischemic–excitotoxic damage to the retina, 24, 34–36 blunts the effects of argon laser-induced retinal injury, 37 lessens light-induced retinal degeneration, 38 and enhances ganglion cell survival after axotomy. 39 In all prior studies, flunarizine was not applied topically, and rabbits were not the experimental animal. The primary purpose of this work was to provide further support for the idea that topically applied flunarizine is neuroprotective for the ganglion cells of the retina in paradigms of ischemia-excitotoxicity. Initially, we examined whether repeated topical administration of flunarizine reduces retinal cell death after ischemia-reperfusion and NMDA injection. We then investigated whether topical application of flunarizine lowers IOP in rabbits. Experiments were also conducted to compare the effectiveness of flunarizine with that of betaxolol in reducing Ca²⁺ influx (in retinal and cortical neurons) and Na⁺ influx (in brain synaptosomes), so as to obtain a possible measure of their neuroprotective potencies.

Materials and Methods

All animal experiments conformed with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits weighing 3 to 5 kg and Wistar rats were entrained to a light schedule of 12 hours light–12 hours dark and provided with food and water ad libitum. Flunarizine, nifedipine, and verapamil were all bought from Sigma (Poole, UK). MK-801 was purchased from Tocris (Bristol, UK).

Ischemia-Reperfusion

Five litters of rabbits, two comprising six animals and three comprising four animals, were used in the present study. Each litter was divided into two groups, one group received 20 µL 2% flunarizine in 50% polyethylene glycol topically (both eyes) and the other the drug vehicle (both eyes) 1 hour before and 1 hour after ischemia and twice daily during reperfusion. Ischemia was given for 60 minutes to the left eye of each animal from the two groups. This was performed by use of an ophthalmodynamometric cup and raising the IOP to 125 mm Hg to affect the retinal blood flow. Because the degree of blockage of retinal blood flow is dependent on the size of the eye, 7-week-old rabbits were used. After 3 days of reperfusion, a light microscope was used to observe the extent of retinal damage.

NMDA Injection

Two groups of 14 rats (200–250 g) received either 5-µL doses of topical flunarizine (2% in polyethylene glycol) or 5-µL doses of vehicle bilaterally, twice daily for 2 days. On the third day, 5 µL NMDA in sterile water was injected intravitreally into a single eye of each animal (the calculated concentration in the vitreous humor was 200 µM). The other eye was injected with 5 µL sterile water. The rats were further treated topically with flunarizine or vehicle twice daily for 7 days. The rats were then killed and the retinas removed for mRNA analysis (eight rats in each group) or for immunohistochemistry (six rats in each group).

Reverse Transcription–Polymerase Chain Reaction

The levels of Thy-1, rhodopsin, and neurofilament light (NFL; molecular mass 70–80 kDa) from mRNAs present in the retinas were determined with a semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) technique, as described previously. 41 Briefly, total RNA was isolated, and first-strand cDNA synthesis performed on 2 µg DNase-treated RNA. The individual cDNA species were amplified in a reaction containing a cDNA aliquot, PCR buffer, MgCl₂ (4 mM for rhodopsin and Thy-1 primers and 5 mM for NFL). Reactions were initiated by incubating at 94°C for 10 minutes and PCR (94°C, 15 seconds; 52°C, 30 seconds; 72°C, 30 seconds) performed for a suitable number of cycles to ensure that amplification had not occurred (rhodopsin, 25; Thy-1 and NFL, 29), followed by a final extension at 72°C for 5 minutes. Intereperimentation variations were avoided by performing all amplifications in a single run. Oligonucleotides primers used were 5'-GGTTTATCAAGTTTCTTACT-C3' and 5'-GGTTTTTGAGATTAGGACCT3' (sense and antisense for Thy-1), 5'-CATGTTCTCATGTTGATTGACT3' and 5'-ATGTTGGTGTGTTAGTGAGG3' (sense and antisense for rhodopsin), and 5'-ATGTCATTGCTCGTGTGAGATG3' and 5'-GCTTGGCACTGTTCACCG3' (sense and antisense for NFL). PCR reaction products were separated on 1.5% agarose gels with ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels on computer (Labworks, UVP Products, Upland, CA).

Calcium Influx into Cortical Neurons

Cortical neurons were prepared from 16- to 18-day fetal rats, exactly as described previously. 42 Isolated neurons were plated in 12-well multiwell plates at approximately 1 to 2 × 10⁶ cells per well. Cultures were left to grow for 7 days in an atmosphere of 5% CO₂, 95% air at 35.5°C in saturating humidity. Calcium influx studies were performed as outlined previously. 42

Effect of Flunarizine on Calcium Influx into Isolated Rat Retinas

Freshly dissected retinas were preincubated in magnesium-free Krebs-Ringer bicarbonate buffer (158 mM NaCl, 1 mM CaCl₂, 5.6 mM KCl, 1 mM NaH₂PO₄, 11 mM NaHCO₃, 10 mM glucose, and 20 mM HEPES [pH 7.4] and continuously perfused with 95% O₂:5% CO₂ for 25 minutes at 37°C. Test substances were added and, after 10 minutes, calcium influx initiated by the addition of 1 µM ⁴⁵CaCl₂ and 100 µM NMDA in a final volume of 2 mL. After a 15-minute incubation, the reaction was terminated by the addition of 2 mL ice-cold 10 mM EGTA·0.9% NaCl. Retinas were washed twice in 1 mL EGTA·0.9% NaCl and sonicated in 1 mL distilled water. Radioactivity was determined by liquid scintillation spectrometry using 5 mL liquid scintillation cocktail (Insta-Gel Plus; Packard BioScience, Groningen, The Netherlands), and protein concentration was determined with a bicinchoninic protein assay kit (Sigma), with bovine serum albumin as the standard.

Sodium Influx into Rat Cortical Synaptosomes

Sodium influx experiments were conducted as previously described. 44 In brief, aliquots of freshly prepared rat cortical synaptosomes contain
ing approximately 350 to 450 μg protein were preincubated at 37°C for 10 minutes, with or without test agents. After preincubation, 0.5 μCi ²²Na⁺ was added, and the samples were incubated for 10 minutes at 37°C. Uptake was initiated by the addition of 100 μM veratridine and terminated after 30 seconds by washing. Samples were rapidly vacuum filtered and washed and trapped radioactivity measured by liquid scintillation spectrometry. Nonspecific uptake of Na⁺ was determined in the presence of 1 μM tetrodotoxin.

**Intraocular Pressure**

An applanation pneumotometer (Ocular Blood Flow Tonograph; OBF Laboratories, Malmesbury, UK), calibrated for use in rabbits, was used to determine the IOP after topical instillation of 1 drop of 0.4% pilocarpine hydrochloride. All experiments were conducted in a masked manner, and measurements were taken 60 minutes before and at the onset of topical application of 30 μL 2% flurane (in 50% polyethylene glycol) or drug vehicle. After a single application at the midpoint of the light period (t = 0) the IOP was measured at 60-minute intervals for a period of 5 hours and then at two 3-hour intervals during the dark phase.

**Immunohistochemistry**

Similar regions from retinas were excised and fixed in 2% paraformaldehyde and cryopreserved and frozen 10-μm-thick sections produced. Retinal sections were incubated overnight at 4°C with rabbit anti-parvalbumin (1:200; Sigma), rabbit anti-choline acetyltransferase (ChAT; 1:200; Sigma) or mouse anti-Thy-1 monoclonal antibody (clone Ox-7; 1:10) and developed with appropriate secondary antibodies conjugated with fluorescein.

**Statistical Analysis**

All results are expressed as mean ± SEM. Comparisons were made using Student’s paired or unpaired t-tests where appropriate. P < 0.05 was considered significant.

**RESULTS**

**Studies on Rabbit Retina**

Ischemia (raised IOP) for 60 minutes followed by reperfusion for 3 days in vehicle-treated animals showed that the normal b-wave amplitude of the electroretinogram (246 ± 8 μV, n = 14) was reduced by approximately 80% (Fig. 1). However, when animals were treated with five topical applications of flunarizine (n = 10), the reduction in the b-wave (approximately 35%) was significantly less pronounced (Fig. 1). The protective effect of flunarizine was confirmed by immunohistochemistry. The immunoreactivity of parvalbumin and ChAT in retinas subjected to ischemia-reperfusion always showed clear changes when compared with the control tissue. In the case of ChAT, the normal two strata of ChAT immunoreactivity in the inner plexiform layer (Fig. 2A) were reduced in intensity (Fig. 2B). Moreover, the amount of ChAT-positive perikarya was always significantly reduced (Figs. 2A, 2B). The normal immunoreactivity of parvalbumin associated with many amacrine perikarya and a broad band of terminals in the inner plexiform layer (Fig. 2D) was also clearly reduced by ischemia-reperfusion (Fig. 2E). In animals treated topically with flunarizine, it was clear that the changes to the immunoreactivity of ChAT (Fig. 2C) and parvalbumin (Fig. 2F) caused by ischemia-reperfusion were significantly less pronounced than in vehicle-treated rats.

**Studies in Rat Retina**

Thy-1 antigen is primarily associated with retinal ganglion cells, and intraocular injections of NMDA cause a dose- and time-dependent loss of Thy-1 mRNA and protein. NF-L is primarily associated with ganglion cell axons (Fig. 3A), and a reduction in NF-L mRNA level is also synonymous with ganglion cell loss. Determining whether there has been a reduction of Thy-1 and/or NF-L mRNAs caused by NMDA relative to the content of other unaffected retinal mRNA (e.g., rhodopsin) is one way of assaying for ganglion cell survival. Figure 3B shows that relative to the mRNA for rhodopsin, NF-L and Thy-1 mRNAs were reduced by NMDA, and these reductions were partially prevented by topical application of flunarizine, although the effect on NF-L did not quite reach significance. A similar result was observed by "staining" for the localization of Thy-1. The normal thickness of the Thy-1-immunoreactive band in the ganglion and inner plexiform layers (which primarily represents ganglion cell dendrites; Fig. 4A) was much reduced by NMDA treatment (Fig. 4C). Addition of NMDA (100 μM) significantly increased the influx of ⁴⁵Ca²⁺, and this effect was completely blunted by the NMDA antagonist MK-801 (5 μM). Moreover, nifedipine, flunarizine, and betaxolol attenuated the NMDA-induced influx of radioactive calcium to varying degrees. Their effectiveness was similar but not identical in cortical neurons and retina. The solvents used to dissolve nifedipine (0.1% ethanol) and flunarizine (1% DMSO) had no significant effect on the NMDA-stimulated influx of calcium.

**Studies of ⁴⁵Ca²⁺ Influx**

These studies were performed in rat cortical neurons in culture (Fig. 5) and isolated rat retinas (Fig. 6), and the results were qualitatively identical. A steady level of radioactive calcium entered the cortical neurons or retina when they were exposed to a medium containing ⁴⁵Ca²⁺. Addition of NMDA (100 μM) significantly increased the influx of ⁴⁵Ca²⁺, and this effect was completely blunted by the NMDA antagonist MK-801 (5 μM). Moreover, nifedipine, flunarizine, and betaxolol attenuated the NMDA-induced influx of radioactive calcium to varying degrees. Their effectiveness was similar but not identical in cortical neurons and retina. The solvents used to dissolve nifedipine (0.1% ethanol) and flunarizine (1% DMSO) had no significant effect on the NMDA-stimulated influx of calcium.

**Studies on ²²Na⁺ Influx**

Veratridine (100 μM) caused approximately a threefold increase in basal uptake of ²²Na⁺ into rat cortical synaptosomes, which was completely blocked by tetrodotoxin (data not shown).
Flunarizine Therapy for Ischemia-Excitotoxicity

In the present study, the protective effect of topical flunarizine to eyes in the rabbit and rat attenuated both ischemia-reperfusion and NMDA-induced damage, respectively. The protective effect of topical flunarizine in our study appeared to be greater than when the drug was administered intraperitoneally and of a magnitude similar to that achieved with intraperitoneal injection. Direct comparisons are, of course, difficult to make, not only because of the different administration regimens and experimental conditions, but also because of the diverse analytical tools used to assess retinal damage and protection.

Previous studies have shown that flunarizine can counteract ischemia-excitotoxicity-induced insults to the rat retina, when administered intraperitoneally or intravenously. The results of the present study show that topical application of flunarizine to eyes in the rabbit and rat attenuated both ischemia-reperfusion and NMDA-induced damage, respectively. The protective effect of topical flunarizine in our study appeared to be greater than when the drug was administered intraperitoneally and of a magnitude similar to that achieved with intraperitoneal injection. Direct comparisons are, of course, difficult to make, not only because of the different administration regimens and experimental conditions, but also because of the diverse analytical tools used to assess retinal damage and protection.

In previous studies, the Ca²⁺ channel blockers nifedipine and levemopamil have been shown to attenuate the ERG b-wave amplitude loss after an ischemia-like insult to the retina. The present results are therefore consistent with these observations, although how they exert their effects remains uncertain. One obvious possibility is that neurons in the outer retina are adversely affected by ischemia and that Ca²⁺ channel blockers simply act to prevent Ca²⁺ overload. Another possibility is that the cation channel-blocking activity of these substances helps to maintain the normal physiology of the Müller cells.

It is generally accepted that raised IOP is more detrimental to the inner than the outer retina. This is reflected by changes in a number of antigens associated with inner retinal neurons, including Thy-1 (ganglion cells), NF-L (ganglion cell axons), ChAT (small subset of amacrine cells), and parvalbumin (many amacrine cells). It has been shown in the current study that amacrine cells are affected in the rabbit retina by ischemia-reperfusion and that flunarizine partially blunts these effects. It is also known that ischemia-reperfusion or intraocular injections of NMDA cause destruction to rat retinal ganglion cells by similar, but not necessarily identical, mechanisms. Substances that protect against NMDA toxicity to ganglion cells include NMDA antagonists, calcium channel blockers, betaxolol, and α₂-adrenoceptor agonists. The finding that flunarizine also does so, not only confirms the usefulness of calcium channel blockers as neuroprotectants, but also shows that they can be effective when topically applied, at least in the case of flunarizine. Such data support the idea that when topically applied to the human eye, flunarizine may promote survival of some retinal ganglion cells—for example, in glaucoma. Of course, it should be stated that in both glaucoma and ischemia, it is glutamate rather than NMDA that causes damage to ganglion cells. However, because most investigators are in agreement that the predominant form of glutamate excitotoxicity of retinal ganglion cells is mediated by overstimulation of the
NMDA receptor type, results obtained using NMDA are a useful way of mimicking the effect of glutamate. In the normal eye, the retina has efficient mechanisms for uptake of glutamate, which means that very high levels would have to be injected to produce deleterious effects on ganglion cells.

In our investigation, 10 μM flunarizine (an L- and T-type channel blocker) was slightly less effective than the same concentration of nifedipine (an L-type channel blocker) at reducing the NMDA-induced influx of Ca\(^{2+}\) in the rat retina. This suggests that the NMDA-induced influx of Ca\(^{2+}\) involves a significant contribution from L-type but not T-type Ca\(^{2+}\) channels, a conclusion drawn previously.\(^5\)\(^2\) Thus, if the neuroprotective effect of these compounds is dependent solely on their ability to attenuate Ca\(^{2+}\) influx, it can be predicted that nifedipine would be the more effective of the two. Because there is a general consensus that intracellular levels of both Ca\(^{2+}\) and Na\(^{+}\) increase when a neuron is induced to die by ischemia, it would be anticipated that compounds that block the entry of both cations would be more efficacious neuroprotectants. This would be expected to be the case with flunarizine, which was much more effective than nifedipine at blunting Na\(^{+}\) influx (Fig. 7). To be certain of the neuroprotective capabilities of these compounds, a direct comparison would have to be undertaken; however, perhaps in a tissue culture system in which possible confounding factors such as effects of ocular blood flow and/or pharmacokinetic differences could be eliminated. Nevertheless, that voltage-dependent Ca\(^{2+}\) channel blockers do not completely inhibit the NMDA-induced Ca\(^{2+}\) influx in

![Figure 3](image-url)

**FIGURE 3.** (A) Localization of NF-L immunoreactivity in the rat retina, using a monoclonal antineurofilament antibody (against 70-kDa neurofilament protein). NF-L immunoreactivity was primarily localized in ganglion cell axons (large arrows) and to a lesser extent in ganglion cell dendrites (small arrows). Sparse immunoreactivity was also associated with fibers in the outer plexiform layer (arrowbeads). Scale bar, 30 μm. (B) Effect of topical applications of flunarizine on the NMDA-induced reduction of mRNA levels for two ganglion cell antigens, NF-L and Thy-1, relative to the level of mRNA for the photoreceptor marker rhodopsin. (C) Effect of NMDA (20 nmol injected) on mRNA levels in rats treated with vehicle expressed as a percentage relative to the contralateral control eye; (D) results in rats treated with flunarizine+NMDA. Differences in the mRNA level of Thy-1 (flunarizine versus vehicle, n = 8) was significant (Student’s unpaired t-test; *P < 0.05). Differences in the mRNA level for NF-L (flunarizine versus vehicle, n = 8) fell just short of significance (Student’s unpaired t-test; P = 0.057).

![Figure 4](image-url)

**FIGURE 4.** The distribution of Thy-1 immunoreactivity in the rat retina. Thy-1 is a surface antigen and is associated with the whole of the ganglion cell membrane, including dendrites in the normal rat retina. As a consequence, Thy-1 staining appeared as a broad band of immunoreactivity (the bright region between the arrows) beginning at the border of the inner nuclear layer (INL) and extending to the ganglion cell layer (A). Ischemia-reperfusion caused a drastic reduction in the depth of Thy-1 immunoreactivity (B), which was largely blunted by treatment with topical flunarizine (C). Scale bars, 50 μm.
The effect of NMDA was significantly blunted by MK-801 (5 μM), flunarizine (10 or 100 μM), nifedipine (10 μM), and betaxolol (100 μM). The order of effectiveness in blunting the NMDA-induced influx of \(^{45}\text{Ca}^{2+}\) was MK-801 > nifedipine > flunarizine > betaxolol.

**FIGURE 6.** The influence of various substances on the influx of \(^{45}\text{Ca}^{2+}\) in the isolated rat retina induced by NMDA (100 μM). Results are expressed as the mean ± SEM; n = 6. MK-801 (5 μM), flunarizine (10 or 100 μM), nifedipine (10 μM), and betaxolol (100 μM) all significantly blunted the NMDA-induced influx of \(^{45}\text{Ca}^{2+}\). (**P < 0.01, *P < 0.05, by Student’s paired t-test; NMDA+drug versus NMDA). The order of effectiveness was MK-801 > flunarizine > nifedipine > betaxolol. Cultures were exposed, on average, to 2 × 10^5 cpm \(^{45}\text{Ca}^{2+}\) per well, which resulted in the accumulation of approximately 8 × 10^5 cpm per well in control cultures (i.e., 100%).

It appears that the mechanism by which flunarizine acts as a neuroprotectant is very similar to that of the \(\beta\)-adrenoceptor antagonist betaxolol (i.e., reducing Na\(^+\) and Ca\(^{2+}\) influx). The Na\(^+\) channel-blocking activity of these compounds is much more effective than that of nifedipine; this is particularly important in the context of their neuroprotective actions in white matter (ganglion cell axon), as opposed to gray matter (ganglion cell body and dendrites). Anoxic injury to the rat optic nerve can be ameliorated by exposure to Na\(^+\) channel blockers and local substances that prevent the reversal of the sodium-calcium exchanger channel blockers being essentially ineffective. Because it is generally believed that in glaucoma the initial insult to the ganglion cell is to the axon in the optic nerve head, it follows that an effective neuroprotectant has to be particularly directed to preserving the ganglion cell axon. It is therefore suggested that such calcium channel blockers as flunarizine, rather than nifedipine, could help to explain why flunarizine only partially prevented the loss of Thy-1 mRNA caused by NMDA in vivo.

**FIGURE 7.** Comparative effect of 10 μM betaxolol, flunarizine, and nifedipine on \(^{22}\text{Na}^{+}\) influx in rat brain synaptosomes stimulated by veratridine (100 μM). Results are expressed as mean ± SEM; n = 4 (**P < 0.001, *P < 0.05, by Student’s unpaired t-test; drug versus veratridine). Flunarizine and, to a lesser extent, betaxolol attenuated the influx of \(^{22}\text{Na}^{+}\), whereas nifedipine had relatively little effect.

**FIGURE 8.** The effect of topically applied flunarizine (2% in 50% polyethylene glycol) on IOP in normotensive rabbits recorded over 12 hours. Black bar: dark phase of the circadian cycle. Results are expressed as the mean IOP ± SEM; n = 15 (**P < 0.001, *P < 0.01, *P < 0.05, by Student’s paired t-test; flunarizine versus polyethylene glycol). Flunarizine significantly lowered IOP 30 minutes after application in the light phase, and this reduction was maintained for 5 hours.
be more appropriate candidates for use in glaucoma. Moreover, because flunarizine is more effective at reducing the influx of Ca\(^{2+}\) and Na\(^+\) than betaxolol, it follows that theoretically it should be a more potent neuroprotectant.

Future studies on retinal cell cultures will examine this idea in detail in conditions that should make it possible to make a direct comparison of the potencies of the two compounds. As a final point, it should be recognized that the neuroprotective action of flunarizine may occur partly because of increased blood flow to the affected tissues. Flunarizine is known to have vasodilatory properties and to improve blood flow to the optic nerve head in patients with low-tension glaucoma.\(^{27}\)

The observation that topically applied flunarizine attenuates NMDA- and ischemia-induced injuries to the retina raises two questions: How does flunarizine get to the retina, and does the concentration required to be effective relate to the findings observed in the in vitro studies? We suggest that topically applied flunarizine reaches the retina through a combination of systemic and local routes. The reasoning behind this suggestion is that experiments with other topical drugs that are neuroprotective to ganglion cells have shown this to be the case. With regard to the second question, it is likely that the amount of flunarizine required for effective neuroprotection is not necessarily related to the concentration of substance needed to blunt the effect of NMDA in the in vitro studies (Figs. 5, 6). In the in vitro experiments, 100 \(\mu M\) NMDA was necessary to clearly stimulate calcium influx and at least 10 \(\mu M\) flunarizine to attenuate this effect. With more sensitive procedures, less NMDA is likely to be required to obtain a measurable influx of calcium and, as a consequence, less flunarizine to blunt the effect. In addition, in the in vitro studies, NMDA caused a measurable increase in calcium influx within 30 minutes. In contrast, even when NMDA is injected into the vitreous humor so that the concentration is approximately 100 \(\mu M\), ganglion cell death can be clearly measured only after 7 days (Osborne NN, unpublished data, 1998), suggesting that the amount of NMDA actually reaching the ganglion cells is appreciably less than the 100 \(\mu M\) in the vitreous humor. In cell culture experiments, it is known that 100 \(\mu M\) NMDA causes cell death within 2 days, supporting this notion.\(^{27}\) It is therefore postulated that the actual amount of flunarizine that has to reach the retina to attenuate NMDA- or ischemia-induced injuries to the retina is considerably less than that predicted from the in vitro studies. This is partly because the insult to the retina in vivo is likely to be milder (and therefore a measurable effect requires a number of days) than occurs in in vitro studies in which the experiments are completed in a short period (30 minutes).

Various studies have shown that topically applied calcium channel blockers can reduce IOP.\(^{16,17}\) Indeed, flunarizine itself has recently been demonstrated to lower IOP in the monkey eye when applied topically.\(^{17}\) The results of this study support these findings: A single topical application of 2% flunarizine to the rabbit eye caused a significant reduction in IOP that was sustained for 5 hours. The magnitude of the response and the relatively long duration of action are similar to the effects of certain Ca\(^{2+}\) channel blockers in the rabbit and monkey,\(^{17,54}\) implying that the mechanism of action probably involves blockade of Ca\(^{2+}\) channels. However, it should be noted that flunarizine is also an effective antagonist at Na\(^-\) channels, and Na\(^-\) channels are known to be expressed by the ciliary epithelium.\(^{55}\) As a result, a reduction in Na\(^-\) influx may contribute to the decrease of IOP induced by flunarizine, although Na\(^-\) channel-blocking drugs have not, to date, been tested on aqueous dynamics. In addition, flunarizine has effects on the dopaminergic\(^{60}\) and serotonergic\(^{61}\) systems, and each of these neurotransmitters can modulate IOP. To complicate matters further, different Ca\(^{2+}\) channels are associated with different structures in the anterior uvea, and probably the types vary across species. Detailed information regarding the density and types of Ca\(^{2+}\) channels associated with the various structures in the anterior uvea is not available, but it is known that L- and T-type Ca\(^{2+}\) channels are associated with the ciliary processes\(^{62,63}\) and L-type channels alone with the trabecular meshwork.\(^{64}\) Different Ca\(^{2+}\) channel antagonists have differential ion channel selectivities; therefore, flunarizine may not lower IOP by exactly the same mechanism as other Ca\(^{2+}\) channel antagonists, such as verapamil and nifedipine. In support of the argument that Ca\(^{2+}\) channels vary across species are the data obtained using verapamil: Erickson et al.\(^{65}\) reported that flunarizine increased outflow facility in perfused human eyes, whereas Melena et al.\(^{66}\) showed that it decreases the same parameter in rabbits. More comprehensive research is needed to elucidate exactly how Ca\(^{2+}\) channel blockers lower IOP.

In conclusion, we propose that further study is warranted in primates and humans into the potential usefulness of flunarizine in the amelioration of retinal injuries caused by ischemic, hypoxic, or excitotoxic episodes. This is based on studies in rats and rabbits, in which it was shown that when topically applied, this drug reduced IOP and attenuated injury to ganglion cells. Moreover, it protected against general injuries to the retina caused by ischemia-reperfusion. The neuroprotective action of flunarizine does not appear to be weak and is directed to preserving the ganglion cell, including the axon. Such data, together with the findings that flunarizine reduces IOP in monkeys,\(^{17}\) can be safely administered to humans, and even improves blood flow to the optic nerve head in low-tension glaucoma.\(^{27}\) Provide impressive support for the idea that flunarizine may be beneficial in pathologic conditions in which the functioning of ganglion cells is known to be compromised. As a note of caution, however, a wide variety of classes of drug acting through a diverse array of distinct but often complementary mechanisms have been found to offer some degree of neuroprotection to retinal neurons in vitro and in vivo experimental paradigms (for review see Ritch\(^{67}\)). There is still much to be learned about how ganglion cells die in glaucoma\(^{2}\) and other diseases before we can be
confident of selecting the most suitable neuroprotectant for clinical use.

References


