Ocular Hypotensive Action of Topical Flunarizine in the Rabbit: Role of σ₁ Recognition Sites

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ABSTRACT
In a previous study we ascertained the presence of σ₁ and σ₂ recognition sites in the rabbit iris-ciliary body, an ocular structure involved in aqueous humor production and drainage. We characterized the σ₁ sites using the preferential ligand (+)-pentazocine, which caused a significant reduction of intraocular pressure (IOP). In the present study, flunarizine, a calcium channel blocker with a complex pharmacological profile, bound to σ₁ sites expressed in the iris-ciliary body with moderate affinity (Kᵢ = 68 nM). Unilateral topical flunarizine (0.01–0.1%) caused a dose-related reduction of IOP in ocular normotensive rabbits and in the α-chymotrypsin model of ocular hypertension, without altering the IOP of the contralateral eye. This activity was blocked by the σ₁ site antagonist NE-100 (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine HCl) which, by itself, had no effect on IOP. Detection of flunarizine in rabbit iris-ciliary body homogenates, after topical instillation, showed that it adequately penetrates the rabbit eye. To investigate mechanisms that may contribute to ocular hypotension induced by σ₁ agonists, we carried out in vitro studies on the isolated rabbit iris-ciliary body. Flunarizine (IC₅₀ = 5.96 nM) and (+)-pentazocine (IC₅₀ = 3.81 nM) inhibited [³H]norepinephrine release. Moreover, flunarizine (IC₅₀ = 6.34 nM) and (+)-pentazocine (IC₅₀ = 27.26 nM) also antagonized isoproterenol-induced cAMP accumulation. The action of flunarizine and (+)-pentazocine was sensitive to NE-100 antagonism; however, this latter compound partially prevented their effect on [³H]norepinephrine and cAMP accumulation. These findings indicate that flunarizine and (+)-pentazocine interact with ocular σ₁ sites and may prove effective in the control of ocular hypertension.

Sigma (σ) recognition sites are a unique class of binding sites, heterogeneously distributed in the nervous system and in peripheral organs, that presumably serve as receptors for some unidentified endogenous ligand (Walker et al., 1990; Quiron et al., 1992). The σ recognition sites bind an array of structural classes of compounds including haloperidol, 1,3-di-O-tolylguanidine (DTG), and (+)-benzomorphans, such as (+)-pentazocine and (+)-N-allylnormetazocine (Su and Junien, 1994). On the basis of biochemical and radioligand binding data, σ recognition sites have been classified into at least two types, σ₁ and σ₂ (Quiron et al., 1992). The σ₁ recognition sites display preferential affinity and stereoselectivity for (+)-benzomorphans (DeHaven-Hudkins et al., 1992). A σ₂ binding protein has been cloned (Hanner et al., 1996), and its sequence shows significant similarities with sterol C₈-C₇ isomerases from fungi.

The functional role of σ recognition sites and the cellular mechanisms responsible for the effects produced by σ-site ligands have not been clearly determined, although these compounds may act as neuromodulators. Previous reports have associated σ-site ligands with calcium homeostasis (Brent et al., 1996; Hayashi et al., 2000; Hayashi and Su, 2001). σ-site ligands can influence [³H]dopamine and [³H]norepinephrine (NE) release from rat brain slices, acting, at least partially, presynaptically (Gonzalez-Alvear and Werling, 1995; Gonzalez and Werling, 1997).

Several σ-site ligands influence electrically evoked contractions in the guinea pig longitudinal muscle/myenteric plexus preparation (Campbell et al., 1989). These latter findings add evidence to the theory that σ recognition sites participate in the regulation of autonomic functions and that σ-site ligands may interfere with neurotransmitter release, modulating their action on innervated tissue (Su and Junien, 1994).

Several of these studies (Brent et al., 1996; Gonzalez and Werling, 1997; Hayashi et al., 2000) proposed that σ₁ site-preferential (+)-benzomorphans behave as agonists. In contrast, NE-100 ([N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine HCl]) stimulation 1 through 4; [Ca²⁺], intracellular Ca²⁺ levels.

ABBREVIATIONS: DTG, 1,3-di-O-tolylguanidine; NE, norepinephrine; NE-100, (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine HCl; DuP 734, 1-(cyclopentylmethyl)-4-(2′-4′-fluorophenyl)-2′-oxoethyl)piperidine HBr; IOP, intraocular pressure; PD, pupil diameter; S₁–S₄, stimulations 1 through 4; [Ca²⁺], intracellular Ca²⁺ levels.
(Tanaka et al., 1995) and DuP 734 (1-(cyclopropylmethyl)-4-(2’-(4’-fluorophenyl)-2’-oxoethyl) piperidine HBr) (Gonzalez-Alvear and Werling, 1995), which have no effects by themselves but reverse the effects of \( \sigma_1 \) site agonists, are defined as antagonists.

In the eye, \( \sigma \) recognition sites have been reported in bovine retinal membranes (Senda et al., 1997). In a previous study (Bucolo et al., 1999), we found distinguishable populations of \( \sigma_1 \) and \( \sigma_2 \) recognition sites in the rabbit iris-ciliary body, a structure that contains both parasympathetic and sympathetic innervation and contributes to the regulation of intraocular pressure (IOP) and pupil diameter (PD) (Nomura and Smelser, 1974). Topical (–)-pentazocine caused a dose-related reduction of IOP in ocular normotensive rabbits and in the \( \alpha \)-chymotrypsin model of ocular hypertension. This reduction was blocked by NE-100 which, by itself, had no effect on IOP. Recently, Ola et al. (2001) have confirmed, by in situ hybridization and immunohistochemistry, the occurrence of \( \sigma_1 \) sites in the mouse iris-ciliary body.

Several studies have reported that topical and systemic calcium channel blockers reduce IOP in experimental animals and in humans (Netland et al., 1993; Siegner et al., 2000). This action has been observed with selective L-type calcium channel blockers such as dihydropyridines and verapamil (Segarra et al., 1993) or diltiazem (Melena et al., 1998) and of drugs such as flunarizine, a nonsel ective calcium blocker (Cellini et al., 1997; Osborne et al., 2002). The mechanisms underlying these effects have been explored very little.

Flunarizine, a difluorinated piperazine derivative, is a mixed L- and T-type calcium channel blocker (Holmes et al., 1984) and sodium channel antagonist (Pauwels et al., 1991). The complexity of its pharmacological profile is further borne out by findings suggesting that it inhibits the dopamine (DA) uptake process and binds to DA receptors, mainly of the D2 type, with an effect similar to the action of the dopaminergic antagonist haloperidol (Belforte et al., 2001). Flunarizine, as well as other DA receptor blockers, interacts with \( \sigma \) recognition sites (DeHaven-Hudkins and Fleissner, 1992), inhibits (–)-[\(^3\)H]pentazocine binding to \( \sigma_1 \) recognition sites (Basile et al., 1992), and has a mixed agonist-antagonist action on opioid receptors (Weizman et al., 1999).

The present study was designed to investigate whether flunarizine interacted with \( \sigma_1 \) recognition sites in the rabbit iris-ciliary body by receptor binding, and to elucidate its effect on IOP in ocular normotensive albino rabbits and in the \( \alpha \)-chymotrypsin model of ocular hypertension. We extended our previous in vivo findings of the ocular effects of \( \sigma_1 \) ligands by investigating the action of (–)-pentazocine and flunarizine on electrically stimulated release of [\(^3\)H]NE from postganglionic sympathetic neurons and on isoproterenol-induced cAMP accumulation in isolated iris-ciliary body of albino rabbits.

**Materials and Methods**

**Animals.** Male New Zealand White albino rabbits (Charles River Italia, Calco, Italy) weighing 1.8 to 2.2 kg, with no signs of ocular inflammation or gross abnormality, were used. Animal procedures followed the guidelines of the Animal Care and Use Committee of the University of Bologna and conformed to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research.

**Drugs and Chemicals.** (+)-Pentazocine, haloperidol, DTG, flunarizine, and isoproterenol were from Sigma/RBI (Milan, Italy). Cinna- 

**Binding Assays.** Membranes from the rabbit iris-ciliary body were prepared according to a procedure previously described (Bucolo et al., 1999). Rabb its were killed by i.v. injection of 0.3 ml/kg Tanax T-61 (Tanax; Aventis, Strasbourg, France), and the eyes were enucleated. The iris-ciliary body was rapidly removed, weighed, and homogenized in ice-cold 10 mM Tris-sucrose buffer (0.32 M sucrose in 10 mM Tris-HCl, pH 7.4; 10 ml/g wet tissue weight) using a Potter-Elvejehm homogenizer. The homogenate was centrifuged at 1000g for 10 min at 4°C, and the supernatant was saved. The pellet was suspended in 2 ml/g Tris-sucrose buffer and centrifuged at 1000g for 10 min at 4°C. The supernatants were combined and centrifuged (15 min, 30,000g, 4°C). The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, in a volume of 3 ml/g, and incubated for 30 min at 25°C. After recentrifugation as above, the pellet was resuspended in 10 mM Tris-HCl, pH 7.4, in a final volume of 1.5 ml/g wet tissue, and aliquots were stored at 80°C until use. The protein concentration of the suspension was determined (Bucolo et al., 1999), and it corresponded to 65 ± 3 µg/mg of wet tissue (\( n = 24 \)).

For \( \sigma_1 \) competition binding assays, membranes from rabbit iris- 

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**PD and IOP Measurements.** Conscious rabbits were placed in restraint boxes to which they had been habituated, with unrestricted head and eye movements. PD (in millimeters) was measured with a Castroviejo caliper under constant light. Then, 10.0 µl of 0.4% oxybuprocaine hydrochloride (Novesina; Novartis, Milan, Italy) was ap-
plied to the cornea to minimize any discomfort to the animal, and IOP (millimeters of mercury) was measured using a Tono-Pen XL tonometer (Mentor Corp., Norwell, MA), calibrated according to the manufacturer's instructions and against a Goldmann applanation tonometer in different groups of normotensive and hypertensive rabbits (Mermoud et al., 1995); the local anesthetic had no effect on PD and IOP. Before IOP measurement, the anterior segment of each eye was macroscopically observed to check for discomfort or signs of inflammation, adopting the procedure previously described (Bucolo et al., 1999). For each IOP determination, three readings were taken on each eye, alternating the left and right eyes, and the mean was calculated. Two baseline readings were taken 30 min before and at \( t = 0 \) (this was taken as baseline), and then 0.5, 1, 1.5, 2, 3, and 4 h after the instillation of eyedrops into the conjunctival sac. A stock solution of flunarizine (10\(^{-2}\) M) was prepared in absolute ethanol and further diluted in phosphate-buffered saline (pH 7.4; vehicle), and 50.0 \( \mu \)l/eye was instilled. NE-100 was dissolved in phosphate-buffered saline. PD values are expressed as mean \( \pm \) S.E.M. in millimeters; IOP values are expressed as mean \( \pm \) S.E.M. in millimeters of mercury and as the difference from baseline.

\[ \text{a-Chymotrypsin-Induced Ocular Hypertension in Rabbit.} \]

Ocular hypertension was induced in the left eye by injection of \( \alpha \)-chymotrypsin into the posterior chamber, as described elsewhere (Sears and Sears, 1974). Briefly, a single dose of \( \alpha \)-chymotrypsin (50 Unité d’Activation Enzymatique, Pharmacoépée Française; dissolved in 200 \( \mu \)l of sterile saline) was administered using a 30-gauge needle into the posterior ocular chamber in rabbits anesthetized by an i.m. injection of 35 mg/kg ketamine (Ketalar; Parke-Davis, Milan, Italy) and 5 mg/kg xylazine HCl (Rompun 2%; Bayer AG, Leverkusen, Germany). The tip of the needle was swept across so as to distribute the enzyme coming into contact with the cornea, and the external surface of the lens was washed with 10 ml of sterile saline. Ten minutes before \( \alpha \)-chymotrypsin injection and after 4, 12, and 24 h, 20 \( \mu \)l of 0.4% oxybuprocaine hydrochloride was instilled to minimize discomfort to the rabbit.

For 7 days after \( \alpha \)-chymotrypsin injection, two chloramphenicol eyedrops (Vitamfenicolo; Allergan, Milan, Italy) were administered twice a day. In case of severe ocular inflammation (which occurred in about 5% of animals), the rabbits were not included in the study. IOP was checked after 4 weeks, and only rabbits with pressure of 26 mm Hg or more (i.e., approximately 12 mm Hg above the IOP in the contralateral, untreated eye) and no sign of ocular inflammation were used.

\[ \text{[3H]NE Overflow in Rabbit Iris-Ciliary Body.} \]

Rabbit iris-ciliary body preparations were perfused with solutions containing increasing concentrations of (+)-pentazocine or flunarizine to investigate \([3H]NE\) overflow in vitro in response to electrical field stimulation. This preparation is useful for determining the prejunctional activity of a variety of agonists in iris-ciliary body preparations (Jumblatt and North, 1986; Ogidjben et al., 1994; Russell and Potter, 2001).

The rabbits were killed by i.v. injection of Tanax T-61 and the eyes were enucleated. The eye was dissected to isolate the iris-ciliary body preparations were perfused with solutions containing indomethacin 2.2 mg/100 ml; pH 7.4). Iris-ciliary bodies were incubated for 30 min at 37°C in a humidified incubator in an oxygenated (95% O\(_2\), 5% C\(_2\)O\(_2\)) environment. Tissues were then treated with 3-isobutyl-1-methylxanthine (1 mM, a nonselective phosphodiesterase inhibitor) for an additional 10 min under the conditions described above. Isoproterenol (1.0 \( \mu \)M) and (+)-pentazocine or flunarizine (10.0 nM-1.0 \( \mu \)M) were added sequentially to the tissue for 10 min intervals in the presence or absence of NE-100 (1.0 \( \mu \)M). All experiments were done in triplicate and were repeated at least six times.

After completion of the treatment procedures, tissues were quickly collected, immediately frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). Tissue was extracted for [3H]NE assay, which was performed within 2 weeks. [3H]NE was measured using a commercial kit (Amersham). Protein concentration was determined by a commercial kit (Pierce, Rockford, IL).

\[ \text{Flunarizine in the Iris-Ciliary Body.} \]

Rabbits received a topical instillation (5 \( \mu \)l/eye) of flunarizine (0.05% w/v), and the levels in the iris-ciliary body were measured at 15, 30, 60, 120, and 240 min by gas chromatography. The rabbits were killed by i.v. injection of Tanax T-61 (in the marginal vein of the ear). The iris-ciliary body was removed, cut into two pieces, and incubated for 1 h at 37°C in an oxygenated (95% O\(_2\), 5% C\(_2\)O\(_2\)) bicarbonate-rich, HEPES-buffered solution (115 mM NaCl, 5 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 0.9 mM Na\(_2\)HPO\(_4\), 25 mM NaHCO\(_3\), 10 mM glucose, 10 mM HEPES, 1 mM sodium ascorbate, indomethacin 2.2 mg/100 ml, pH 7.4). Iris-ciliary bodies were incubated for 30 min at 37°C in a humidified incubator in an oxygenated (95% O\(_2\), 5% C\(_2\)O\(_2\)) environment. Tissues were then treated with 3-isobutyl-1-methylxanthine (1 mM, a nonselective phosphodiesterase inhibitor) for an additional 10 min under the conditions described above. Isoproterenol (1.0 \( \mu \)M) and (+)-pentazocine or flunarizine (10.0 nM-1.0 \( \mu \)M) were added sequentially to the tissue for 10 min intervals in the presence or absence of NE-100 (1.0 \( \mu \)M). All experiments were done in triplicate and were repeated at least six times.

The mixture was shaken for 4 min and then centrifuged (15 min, 5000 g, 4°C). The organic layer was removed and extraction was repeated with another 300 \( \mu \)l of ethyl acetate. The organic layers were combined and back-extracted with 300 \( \mu \)l of 1 N HCl. After shaking and centrifugation (15 min, 5000 g, 4°C), the organic layer was aspirated and discarded. The aqueous layer was then mixed with 200 \( \mu \)l of 10 N NaOH and extracted twice with ethyl acetate.
After centrifugation (15 min, 5000g, 4°C), the organic layers were reunited and evaporated to dryness under nitrogen.

The residue was reconstituted in 100 μl of methanol, and 3 μl were injected into a gas chromatography apparatus (Hewlett Packard model 5890 II; Hewlett Packard, Milan, Italy) equipped with a nitrogen-phosphorus detector. To detect flunarizine in iris-ciliary body extracts, a 25 m × 0.2 mm i.d. cross-linked methylsilicone gum (0.33-μm film) HP-1 fused-silica capillary column (Hewlett Packard) was used. The carrier gas was ultra-high-purity helium adjusted to a flow rate of 0.9 ml/min. The injector and detector temperatures were maintained at 180°C and 300°C, respectively. The oven temperature was held at 100°C for 10 min, then programmed at 35°C/min up to 290°C and held for 20 min.

Standard solutions were prepared by adding flunarizine [10 μl of 0.2 to 3 μg/ml solutions (corresponding to 2–300 ng), in duplicate, were added to each homogenate] and cinnarizine (internal standard) to iris-ciliary body homogenates from untreated rabbits, and processing was done as described. Results were quantified by plotting the flunarizine to internal standard peak-area ratios against the flunarizine concentration (a typical chromatogram is shown in Fig. 1). Recovery of flunarizine was at least 75% and was linear over the concentration range of 3 to 300 ng (r = 0.94). The limit of detection was 0.15 ng. Under the conditions described, chromatograms of blank iris-ciliary body samples did not present any interfering peak (data not shown). Calibration graphs in the ranges investigated were linear for all tissues and plasma, with regression coefficients over 0.998. Borwin software (JMBS Developpements, Grenoble, France) was used for data acquisition and integration. Data are expressed as micrograms of flunarizine per gram of wet tissue.

**Statistical Analysis.** Data are expressed as mean ± S.E.M. Statistical comparisons were made by analysis of variance for repeated measures and post hoc Dunnett’s multiple comparison test, with differences of P < 0.05 being considered significant (GraphPad Software, San Diego, CA). To obtain IC₅₀ values (the concentrations of test compound resulting in 50% inhibition of the vehicle response), nonlinear regression analysis of data was carried out using GraphPad software.

## Results

**Flunarizine Binds to σ₁ Recognition Sites in Rabbit Iris-Ciliary Body Membranes.** In competition binding studies, flunarizine showed moderate affinity for σ₁ recognition sites labeled with (+)-[3H]pentazocine and very low affinity for σ₂ recognition sites in iris-ciliary body homogenates (Table 1). This compound had n₁₁ values not significantly different from unity, indicating the absence of positive or negative cooperative binding at each site (Table 1). In a previous study (Bucolo et al., 1999), using the same experimental conditions, (+)-pentazocine had higher affinity for σ₁ recognition sites expressed in the rabbit iris-ciliary body (Table 1).

**IOP and PD.** The unilateral instillation of 0.01%, 0.05%, and 0.1% solutions (w/v) of flunarizine lowered the IOP of normotensive rabbits in a dose-related manner (Fig. 2A), with a maximum fall of 2 mm Hg 60 min after instillation of the 0.1% solution. By 4 h, IOP had returned to baseline (Fig. 2A). The elevated IOP of the a-chymotrypsinized rabbit eye was significantly lowered, in a dose-related manner, by flunarizine (Fig. 2B), with maximum reduction (by 12 mm Hg) at 60 min with the 0.1% solution; the effect lasted from 1.5 to 3 h, depending on the dose (Fig. 2B). The σ₁ receptor antagonist NE-100 (50 μl of 0.1% solution instilled 10 min before 0.1% flunarizine) blocked the effect of this compound in normotensive and hypertensive rabbit eyes; the vehicle alone had no effect on IOP (Fig. 2, and the IOP of the contralateral eye was not affected by topical treatments (data not shown). Topical NE-100 does not alter IOP in the normotensive and hypertensive rabbit eye (Bucolo et al., 1999).

Flunarizine did not significantly affect PD in ocular normotensive and hypertensive rabbit eyes (Bucolo et al., 1999).

<table>
<thead>
<tr>
<th>Compound</th>
<th>(+)-[3H]Pentazocine (σ₁-Site)</th>
<th>[3H]DTG (σ₂-Site)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵣ (nM)</td>
<td>nᵣ</td>
</tr>
<tr>
<td>(+)-Pentazocine (3)</td>
<td>2.1</td>
<td>942</td>
</tr>
<tr>
<td>Flunarizine (4)</td>
<td>68</td>
<td>3542</td>
</tr>
<tr>
<td></td>
<td>K₁ (nM)</td>
<td>n₁₁</td>
</tr>
<tr>
<td>(+)-Pentazocine (3)</td>
<td>90</td>
<td>–0.90</td>
</tr>
<tr>
<td>Flunarizine (4)</td>
<td>–0.84</td>
<td>–0.78</td>
</tr>
</tbody>
</table>

**Table 1**

Affinities (Kᵣ) and Hill coefficients (nᵣ) of σ-site ligands for (+)-[3H]pentazocine and [3H]DTG binding sites in rabbit iris-ciliary body

Results are from computer analysis of competition curves obtained by adding various concentrations of a competing ligand and a fixed concentration (3.0 nM) of (+)-[3H]pentazocine or of [3H]DTG in the presence of 200 nM (+)-pentazocine. Data related to (+)-pentazocine were taken from Bucolo et al. (1999). Number of experiments performed in duplicate is shown in parentheses.
motensive (Table 2) and hypertensive rabbits up to 4 h after treatment (data not shown).

Iris-Ciliary Body Distribution. The levels of flunarizine in the iris-ciliary body after a single instillation (50 μl) of 0.05% solution are reported in Fig. 3. The compound was detectable after 15 min, reaching a peak at 30 min, with a Cmax of 3.05 μg/g of wet tissue. It was still measurable up to 240 min.

Fig. 2. Effect of topical flunarizine on ipsilateral IOP and antagonism of flunarizine-induced hypotension by NE-100. Compounds and vehicle were instilled (50 μl) in the left eye. NE-100 was administered 10 min before flunarizine (flun.). IOP responses were evaluated in ocular normotensive rabbits (A) and in rabbits pretreated with α-chymotrypsin at least 4 weeks earlier (B). Each value is the mean ± S.E.M. of seven or eight animals, and results are expressed as the difference in mm Hg from the pretreatment value. The average basal IOP was 12.1 mm Hg in normotensive and 30.5 mm Hg in hypertensive eyes. *p < 0.05; **p < 0.01 versus the corresponding vehicle-treated group (Dunnett’s test after analysis of variance).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PD before Treatment</th>
<th>0.5 h</th>
<th>1 h</th>
<th>1.5 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.80 ± 0.20</td>
<td>6.85 ± 0.20</td>
<td>6.80 ± 0.20</td>
<td>6.80 ± 0.23</td>
<td>6.85 ± 0.20</td>
<td>6.80 ± 0.25</td>
<td>6.85 ± 0.30</td>
</tr>
<tr>
<td>Flunarizine 0.01%</td>
<td>7.02 ± 0.25</td>
<td>7.02 ± 0.20</td>
<td>7.02 ± 0.20</td>
<td>7.00 ± 0.25</td>
<td>7.00 ± 0.20</td>
<td>7.00 ± 0.20</td>
<td>7.00 ± 0.20</td>
</tr>
<tr>
<td>Flunarizine 0.05%</td>
<td>6.95 ± 0.25</td>
<td>6.85 ± 0.30</td>
<td>6.90 ± 0.25</td>
<td>6.95 ± 0.30</td>
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<td>6.95 ± 0.30</td>
<td>6.95 ± 0.30</td>
</tr>
<tr>
<td>Flunarizine 0.1%</td>
<td>6.90 ± 0.10</td>
<td>6.90 ± 0.10</td>
<td>6.90 ± 0.10</td>
<td>6.90 ± 0.10</td>
<td>6.90 ± 0.10</td>
<td>6.95 ± 0.10</td>
<td>6.95 ± 0.10</td>
</tr>
</tbody>
</table>

Fig. 3. Levels of flunarizine (expressed as μg/g of wet tissue) in rabbit iris-ciliary body homogenates after topical instillation of a 0.05% solution of flunarizine. Mean ± S.E.M. of three rabbits.

[3H]NE Release in Iris-Ciliary Body. In perfusion experiments of the iris-ciliary body, flunarizine and (+)-pentazocine caused a dose-related inhibition of [3H]NE release elicited by electrical stimulation. Figure 4 reports a typical experiment showing the magnitude of suppression of [3H]NE release by increasing doses (1.0–100 nM) of flunarizine (panel A) and (+)-pentazocine (panel B). Figure 5 shows the mean percentage of ratios of the area under the curve of six experiments; consecutive electrical stimulations (S1–S4) evoked [3H]NE release and gave relatively constant ratios (S2/S1, S3/S1, and S4/S1). Flunarizine and (+)-pentazocine at 1 nM only minimally reduced [3H]NE release (respectively, 6 and 10%). The inhibition induced by flunarizine or (+)-pentazocine at 10 nM (62 and 77%) and 100 nM (80 and 91%) was significantly different from vehicle (Fig. 5). IC50 values for inhibition of [3H]NE release for flunarizine and (+)-pentazocine were 5.96 and 3.81 nM. NE-100 pretreatment (1.0 μM) reduced, but did not completely prevent, the suppression of [3H]NE release by the higher concentrations of flunarizine and (+)-pentazocine (Fig. 5).

Accumulation of cAMP in the Iris-Ciliary Body. Epithelial cells of the ciliary processes contain substantial amounts of σ1 recognition sites (Ola et al., 2001) and may represent a potential postjunctional site of action of σ1 ago- nists. We investigated the effects of flunarizine and (+)-pentazocine on cAMP accumulation in the isolated iris-ciliary body. Isoproterenol (1.0 μM), a β2-adrenergic receptor agonist, was used to stimulate adenylyl cyclase and caused a 2.2-fold increase in cAMP from basal levels (Fig. 6). Flunarizine and (+)-pentazocine (1.0 nM to 1.0 μM) reduced the accumulation of cAMP induced by isoproterenol in a concen-

TABLE 2
Effect of topical flunarizine (50 μl/eye) on ipsilateral PD in ocular normotensive rabbits
Each value is mean ± S.E.M. of six or seven animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PD before Treatment</th>
<th>PD (mm) at Various Time Intervals after Drug Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
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<td>6.85 ± 0.30</td>
</tr>
<tr>
<td>Flunarizine 0.1%</td>
<td>6.90 ± 0.10</td>
<td>6.90 ± 0.10</td>
</tr>
</tbody>
</table>
The action of (+)-pentazocine (IC_{50} = 27.76 nM) was significantly reduced by the σ_{1} antagonist NE-100 (1 μM), whereas flunarizine (IC_{50} = 6.64 nM) was more potent than (+)-pentazocine, and its effect was only partially reversed by NE-100 (Fig. 6 shows only one concentration of each drug). NE-100 did not change basal or isoproterenol-induced accumulation of cAMP (Fig. 6). Moreover, both compounds (10.0 nM to 1.0 μM) did not modify cAMP levels in the absence of stimulation by isoproterenol (Fig. 7).

**Discussion**

We previously ascertained the presence of σ_{1} and σ_{2} recognition sites in rabbit iris-ciliary body homogenates by receptor-binding assay (Bucolo et al., 1999). We characterized σ_{1} sites using the dextrorotatory benzomorphan (+)-pentazocine, which is considered a preferential ligand for these sites (DeHaven-Hudkins et al., 1992). We have now determined the affinity of flunarizine, a mixed L- and T-type calcium channel blocker (Holmes et al., 1984) and sodium channel antagonist (Pauwels et al., 1991), for these sites in competition binding experiments using (+)-[3H]pentazocine and [3H]DTG, respectively. Flunarizine bound only to σ_{1} sites expressed in the iris-ciliary body with moderate affinity (K_{i} = 68 nM). These results are in agreement with data obtained by DeHaven-Hudkins and Fleissner (1992) in guinea pig brain homogenates and by Basile et al. (1992) in guinea pig cerebellar homogenates, showing that flunarizine or cinnarizine, a diphenylpiperazine related to flunarizine (Holmes et al., 1984), bound to σ_{1} sites.

Topical flunarizine caused a significant dose-related reduction of IOP in ocular normotensive albino rabbits and lowered the elevated IOP of rabbits treated with chymotrypsin. This model is very responsive to topical ocular hypotensive agents and is useful for screening compounds for ocular hypotensive activity (Sears and Sears, 1974). Unilateral instillation of flunarizine did not affect IOP in the contralateral eye, meaning that the drug’s ability to lower IOP...
were much less responsive to topical flunarizine, a 0.1% solution reducing IOP by only 2 mm Hg at 60 min.

Ocular hypotension elicited by flunarizine was blocked by NE-100, which by itself, had no such effect. The present findings are consistent with the data suggesting that this compound acts as a σ1-site antagonist (Tanaka et al., 1995). Taken together, they provide further evidence that flunarizine probably acts as a σ1-site agonist and mimics the topical action of (+)-pentazocine on IOP (Bucolo et al., 1999). These findings are in agreement with the fact that flunarizine reduced IOP in humans (Cellini et al., 1997) and in the rabbit (Osborne et al., 2002).

Unlike topical cholinergic (Kaufman et al., 1984) and adrenergic agents (Sears, 1984), flunarizine did not affect PD; various drugs that reduce IOP, for instance several α- and β-adrenergic blockers, have been shown not to alter pupil size of rabbits (Lotti et al., 1984). Moreover, flunarizine also appeared to be well tolerated because it caused no ocular inflammatory response.

Detection of flunarizine in iris-ciliary body homogenates from rabbits given this compound topically showed that it adequately penetrates the rabbit eye. Thus, 30 min after treatment, a significant amount reaches the iris-ciliary body; the effect is not yet metabolized and may bind to σ1 sites in this tissue because its concentration is in excess of the binding sites [in a previous study (Bucolo et al., 1999), the Bmax of (+)-[3H]pentazocine was 212 fmol/mg of protein, or approximately 14 fmol per iris-ciliary body, assuming that it contains 68 μg of protein].

In this study we employed the isolated iris-ciliary body to evaluate the effects of flunarizine and (+)-pentazocine on electrically stimulated release of [3H]NE. This model is particularly useful for locating presynaptic drug effects (Jumblatt and North, 1986; Ogidigben et al., 1994; Russell and Potter, 2001) and may provide clues to cellular sites and mechanisms of action of compounds intended to reduce IOP. The iris and ciliary body are innervated by the sympathetic and parasympathetic autonomic nervous systems, which control aqueous humor formation and drainage (Kaufman et al., 1984; Sears, 1984). Several drugs used to control ocular hypertension, which is frequent in glaucoma (Leopold and Duzman, 1986), act presynaptically by affecting neurotransmitter release from nerve terminals. Flunarizine and (+)-pentazocine acted at a presynaptic site in the iris-ciliary body, inhibiting the release of [3H]NE in a concentration-related manner. This effect was blocked partially, but significantly, by the purported σ1 antagonist NE-100. Thus both compounds may interact with a common σ1 recognition site, possibly on sympathetic nerve endings as well as acting through additive mechanisms.

This would be in agreement with findings obtained in other tissues. σ1 agonists inhibited the release of [3H]NE from rat hippocampal slices (Gonzalez-Alvear and Werling, 1995) and of [3H]dopamine from rat striatal slices (Gonzalez and Werling, 1997), through presynaptic mechanisms. Since intracellular calcium is essential in neurotransmitter release, this inhibiting effect of σ1 agonists on catecholamine release could be a consequence of action on intracellular Ca2+ levels ([Ca2+]i). Hayashi et al. (2000), in fact, observed that (+)-pentazocine, acting through σ1 sites, inhibited depolarization-induced increase in cytosolic free Ca2+ and potentiated the bradykinin-induced increase of [Ca2+]i. Brent et al.

**Fig. 6.** Effect of flunarizine and (+)-pentazocine on isoproterenol-induced stimulation of cAMP levels in the iris-ciliary body. Isoproterenol (ISO; 1.0 μM) caused a 2.2-fold increase in cAMP levels. Flunarizine (flun.) and (+)-pentazocine (pent., 100 nM) significantly reduced isoproterenol-stimulated cAMP accumulation. Pretreatment with NE-100 (1.0 μM) significantly antagonized the effect of (+)-pentazocine but only partially that of flunarizine. NE-100 did not modify basal or isoproterenol-induced cAMP accumulation. Each bar indicates the mean ± S.E.M. of six experiments performed in duplicate. *** p < 0.001 versus isoproterenol; †† p < 0.001 versus flunarizine.

**Fig. 7.** Effect of flunarizine (flun.) and (+)-pentazocine (pent.) on CAMP accumulation in the iris-ciliary body. Each bar indicates the mean ± S.E.M. of six experiments performed in duplicate. *** p < 0.001 versus the vehicle.
(1996) reported that σ agonists inhibited the rise in [Ca\(^{2+}\)]\(_i\) levels produced by depolarizing agents in rat forebrain synaptosomes; moreover, flunarizine may act as a calcium channel blocker (Holmes et al., 1984) to reduce electrically evoked release of \([^{3}H\)NE], and σ-site ligands may block high voltage-activated Ca\(^{2+}\) channels (Church and Fletcher, 1995). Therefore, both compounds could interact with different sites influencing neuronal depolarization.

This presynaptic action of flunarizine and (+)-pentazocine is common to other compounds like the κ opioid receptor agonists (Moore and Potter, 2001) or imidazoline and α\(_2\)-adrenoceptor agonists (Ogidigben et al., 1994) that inhibit the release of \([^{3}H\)NE] in the iris-ciliary body by prejunctional receptors. Recently, Aydar et al. (2002) reported that the σ\(_1\) receptors serve as auxiliary subunits to voltage-gated K\(^+\) channels, suggesting a novel modulatory pathway of cell depolarization.

Flunarizine and (+)-pentazocine reduced the accumulation of cAMP induced by isoproterenol in iris-ciliary body preparations in a concentration-related manner. The action of flunarizine was more potent than (+)-pentazocine, and NE-100 only partially reversed its effect. Neither compound affected cAMP levels in the absence of stimulation by isoproterenol.

In the iris-ciliary body the formation of cAMP by isoproterenol is due to the enzyme adenylyl cyclase, which is also activated through G\(_s\) proteins coupled to the β-adrenergic receptor (Simonds, 1999). The inhibition of cAMP production by several mediators may require, in the autonomic nervous system, the previous activation of β-adrenergic receptors, whereas these compounds, per se, do not influence significantly adenylyl cyclase activity (Endoh, 1995). In the iris-ciliary body preparation, flunarizine and (+)-pentazocine behave as the κ opioid agonists (Moore and Potter, 2001) or naphazoline (Ogidigben et al., 2002) and moxonidine (Ogidigben et al., 1994), which have no effect on basal cAMP production.

Adenylyl cyclase can respond to the extracellular mediators either directly interacting with subunits of membrane-anchored G proteins or indirectly as a consequence of mediator-induced alteration of the intracellular ionic compartment and kinase activity, or both (Simonds, 1999). In previous studies, the σ\(_1\) site was linked to pertussis toxin-sensitive G\(_{16}\) proteins (Su and Junien, 1994). However, other studies have indicated that this receptor is not likely to be directly coupled to G proteins (Hong and Werling, 2000). Therefore, σ\(_1\) agonists may only indirectly influence the activity of isoproterenol on cAMP production. Morin-Surun et al. (1999), for instance, reported that the activation of σ\(_1\) sites results in the recruitment of a calcium-dependent phospholipase C/protein kinase C cascade that also involves membrane-bound G proteins.

Finally, as previously mentioned, σ\(_1\) agonists may affect [Ca\(^{2+}\)]\(_i\) (Hayashi et al., 2000). Therefore, these compounds may activate several components in plasma mem-}

\[\sigma_1\] sites. Moreover, flunarizine acts as a calmodulin antagonist (Santos et al., 1994).

Intracellular Ca\(^{2+}\) modulates diverse physiological responses; thus, it is not surprising that a Ca\(^{2+}\) signal regulates the enzyme activity of adenylyl cyclases (Hanoune and Defer, 2001) and activation of calmodulin by Ca\(^{2+}\) can increase cAMP levels in ciliary cells (Mishima et al., 1995).

Interestingly, Hirata et al. (1998) have observed that [Ca\(^{2+}\)]\(_i\) signaling in isolated intact ciliary epithelial bilayers depends on β adrenergic receptor activation, and isoproterenol increases gap junction conductance in the cell bilayer. This compound facilitates cell-to-cell spreading of [Ca\(^{2+}\)]\(_i\) signaling and has a "priming" action on epinephrine-induced [Ca\(^{2+}\)]\(_i\) signaling. Therefore, as suggested by the above results, flunarizine’s action on cAMP production may be due to its Ca\(^{2+}\) blocking activity and may require the presence of isoproterenol.

We are carrying out further studies to better support this hypothesis.

In conclusion, in the present study, flunarizine showed a moderate affinity for σ\(_1\) sites in the rabbit iris-ciliary body, an ocular structure associated with aqueous humor production and drainage. When applied topically, the drug lowered IOP in ocular normotensive albino rabbits and in the α-chymotrypsin model of ocular hypertension, by virtue of a local effect that was prevented by the blockade of ocular σ\(_1\) sites. In vitro studies on the isolated rabbit iris-ciliary body showed that flunarizine and (+)-pentazocine inhibit 1 \([^{3}H\)NE] release in a concentration-related manner, acting through ocular σ\(_1\) sites and 2) isoproterenol-induced cAMP accumulation. These actions were sensitive to NE-100 which, however, only partially prevented their effect on \([^{3}H\)NE] release and cAMP accumulation. Therefore, σ\(_1\) agonists may act pre- and postjunctionally on the iris-ciliary body to influence aqueous humor formation and drainage. These findings cast some light on their IOP-lowering action and indicate that these compounds may offer promise for the control of ocular hypertension.

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