Characterization of Ciliary Muscle Relaxation Induced by Various Agents in Cats
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Purpose. To understand the cellular mechanism underlying the relaxation of ciliary muscle, relaxation induced by prostaglandins (PGs) and some other agents was characterized in the cat.

Methods. Tone of isolated ciliary muscle was measured by means of a force-displacement transducer. Adenylate cyclase activity was determined with membrane fraction of ciliary muscle by measuring the formation of cyclic adenosine monophosphate (cAMP).

Results. The addition of various PGs and isoproterenol relaxed the ciliary muscle that had been precontracted with 3 x 10−6 M carbachol. The relaxation was dose dependent, with an EC50 of 2 x 10−7 M for PGE2. The rank order of potency by which PGs induced relaxation (PGE2 > D2 > F2a > I2) was identical with that reported for EP type prostaglandin receptor-mediated responses except for PGD2, which was more potent than expected. Agents that increased cellular cAMP, such as forskolin and IBMX, also relaxed the precontracted muscle. Nitric oxide donors, such as sodium nitroprusside and S-nitroso-N-acetyl-DL-penicillamine (SNAP), also caused dose-dependent relaxation. PGs and isoproterenol, but not nitroprusside, stimulated adenylate cyclase. The rank order of potency by which PGs stimulate adenylate cyclase was similar to that observed for muscle relaxation, suggesting that cAMP is the cellular second messenger for the PG-induced muscle relaxation and that PG receptors of EP2 and DP type are involved.

Conclusions. Relaxation of cat ciliary muscle is mediated by two independent mechanisms: a cAMP-dependent one, which includes β-adrenergic, EP2, and DP receptor-mediated responses, and a cAMP-independent one, which includes the nitric oxide-induced mechanism. Invest Ophthalmol Vis Sci. 1995;36:1188–1192.

The ciliary muscle not only controls accommodation, it also plays an important role in regulating aqueous humor dynamics. Contraction of the muscle reportedly inhibits uveoscleral outflow, probably by narrowing the spaces between the muscle fiber bundles, and it facilitates aqueous outflow through a conventional route by loosening the trabecular meshwork. Conversely, ciliary muscle relaxation facilitates uveoscleral outflow while it inhibits conventional outflow.

Recently, topically applied prostaglandin (PG) F2a, or its ester has been shown to reduce intraocular pressure (IOP) in many animal species.1–3 Further, analogues of PGF2α were demonstrated to have hypotensive effects in normal and glaucomatous human eyes without severe local side effects.4,5 Now these analogues have been recognized as a new class of antiglaucoma drugs. It is interesting that these compounds are thought to reduce IOP mainly by facilitating uveoscleral outflow.2,4,5 However, few reports have examined the direct or in vitro effects of PGs on the tone of ciliary muscle. Van Alphen et al6 have shown in several animal species that PGs such as PGE1, PGE2, and PGF2α reduce the tone of precontracted ciliary muscle. Recently, Chen and Woodward7 have reported from the rank order of potency of PG analogues that prostaglandin EP2 and DP receptors are involved in the PG-induced relaxation of cat ciliary muscle.

Cyclic adenosine monophosphate (cAMP)-dependent and cyclic guanosine 3',5'-monophosphate (cGMP)-dependent mechanisms are known to exist in the relaxation of smooth muscle preparations.8 In the present study, we investigated the cellular mechanism responsible for the relaxation of cat ciliary muscle in response to PGs and other agents, particularly the cAMP-dependent mechanism.

METHODS. Prostaglandins were purchased from Funakoshi (Tokyo, Japan). S-nitroso-N-acetyl-DL-penicillamine (SNAP) and Nω-nitro-L-arginine methyl ester hydrochloride (L-NNAME) were obtained from Wako Chemicals (Osaka, Japan). All other chemicals were obtained from Sigma (St. Louis, MO).

From eyes freshly obtained from adult cats, strips of ciliary muscle were prepared using the method reported.9 They were bathed in a 10-ml organ bath filled with Krebs–Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl2, 0.61 mM MgSO4, 1.18 mM KH2PO4, 25 mM NaHCO3, and 11.1 mM glucose) containing 10 μM indomethacin at 37°C, which was aerated with 95% O2 and 5% CO2. Contractile responses were measured isometrically by means of a force-displacement transducer (TB-661T; Nihon Koden, Tokyo, Japan). The tissues were allowed to equilibrate for 1 hour before stimulation was applied. The methods of crude membrane preparation and of adenylate cyclase assay were the same as previously reported.9 Protein was determined according to Lowry’s method using bovine serum albumin as the standard. These experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
RESULTS. At rest, ciliary muscle strips had no spontaneous contraction. The addition of carbachol (Cch) into the medium caused a dose-dependent contraction of ciliary muscle strips, which yielded an EC₅₀ value of 6.5 × 10⁻⁷ M. The contraction induced by 10⁻⁶ M Cch was completely inhibited by the pretreatment of 10⁻⁵ M atropin, indicating the involvement of a muscarinic receptor, as is generally known.

The addition of isoproterenol, an agonist of β-adrenergic receptor, caused no change in muscle tone. However, when the strip had been precontracted by Cch, the same treatment reduced muscle tone (Fig. 1a). In these experiments, the concentration of Cch was set at 3 × 10⁻⁶ M, so that approximately 80% of maximal contraction could be seen. The relaxing effect was concentration-dependent; the EC₅₀ was 10⁻⁷ M (Fig. 2). Maximal relaxation ranged from 55% to 50% of contraction induced by 3 × 10⁻⁶ M Cch.

The addition of PGs also relaxed precontracted muscle strips (Fig. 1b). No effect on basal muscle tone was seen with any PG tested. Among the PGs tested, PGE₂ was the most effective, with an EC₅₀ of approximately 2 × 10⁻⁷ M (Fig. 2). PGE₁ was also highly effective, with an EC₅₀ of approximately 3 × 10⁻⁷ M. These were followed in effectiveness by PGD₂, PGF₂α, and PGI₂ (Fig. 2). The potency of PGs to cause relaxation of ciliary muscle was identical in rank order with that reported previously⁷ and with that shown for responses mediated by an EP-type PG receptor.¹⁰ The relaxation induced by PGD₂, however, was greater than that expected for an EP receptor-mediated response. Similar to the effect of the β-adrenergic agonist, maximal relaxation produced by PGE₁ and PGE₂ was approximately 50% when the Cch concentration was 3 × 10⁻⁶ M.

When the strips were maximally relaxed by isoproterenol, further addition of PGs induced no change in the muscle tone (Fig. 1c). Similarly, the addition of isoproterenol induced no further relaxation when the strips were maximally relaxed by PGs (Fig. 1d). Thus, these agents appeared to share the same relaxation mechanism.

Because β-adrenergic and EP₂ receptor stimulation is known to elevate cellular cAMP, it is possible that the accumulation of cellular cAMP causes muscle relaxation. Therefore, we next examined the effects of agents that increase cellular cAMP on precontracted ciliary muscles. Forskolin (a direct adenylate cyclase stimulator) and IBMX (a cyclic nucleotide phosphodiesterase inhibitor) caused relaxation of Cch-precontracted ciliary muscle (Figs. 1e, 1f). The development of muscle relaxation was slightly slower with these agents than with receptor-stimulating agents. Forskolin produced approximately 50% maximal relaxation, which was similar to that produced by isoproterenol or a PG. However, IBMX produced almost complete relaxation. These results suggested that stimulation of adenylate cyclase increases cellular cAMP level and causes muscle relaxation.
Concentration (log [M])

FIGURE 2. Dose-dependent relaxation of ciliary muscle induced by isoproterenol and various PGs. Ciliary muscle was precontracted with 3 x 10^-6 M carbachol, and isoproterenol (Isop), PGE₂, PGE₁, PGD₂, PGF₂α, or PGI₂ at indicated concentrations was added to induce relaxation. Relaxation was expressed as the percentage of the relaxation induced by 10^-5 M isoproterenol. Each point shows a mean (±SE) of six determinations.

To demonstrate more directly the involvement of the cAMP system in PG- and isoproterenol-induced relaxation, we examined the effect of various PGs and isoproterenol on the adenylate cyclase of membrane fractions of cat ciliary muscle. When membrane fractions of ciliary muscle were incubated in the assay mixture, cAMP was formed in a time-dependent manner. The basal adenylate cyclase activity was 108 ± 10 nmol/minute per milligram of protein. The omission of guanosine triphosphate from the incubation medium reduced the basal adenylate cyclase activity by approximately 80%. The inability of this treatment to inhibit completely the activity is most likely due to the presence of endogenous guanosine triphosphate.

The addition of isoproterenol augmented in a dose-dependent manner the formation of cAMP to approximately 200% at 10^-5 M. With PGE₁ or PGE₂, the formation of cAMP was also facilitated in a dose-dependent manner; the maximal stimulatory effect was approximately 200% at 10^-5 M. Other PGs similarly stimulated adenylate cyclase activity, but the magnitude was less than that with PGE₂ or PGE₁ (Table 1). The order of potency of PGs in activating adenylate cyclase was similar to that observed in causing muscle relaxation.

Because IBMX produced stronger relaxation of ciliary muscle than the other agents examined, IBMX should have had an effect in addition to the accumulation of cAMP. In smooth muscle cells, it is known that the elevation of cGMP causes relaxation. Because IBMX is known to inhibit cGMP-dependent phosphodiesterase in addition to cAMP-dependent phosphodiesterase, we examined the effect of nitric oxide (NO) donors on the tone of precontracted ciliary muscle. Nitric oxide is known to stimulate guanylate cyclase directly and to increase cGMP in some smooth muscle cells.

The addition of sodium nitroprusside alone produced a slight and insignificant relaxation on resting ciliary muscle. In Cch-precontracted muscle, nitroprusside produced slowly developing relaxation (Fig. 1g), which was presumably due to the slower and sustained release of NO from the compound. The relaxation was seen from 10^-7 M, and at 5 x 10^-4 M this agent caused approximately 200% of the relaxation induced by isoproterenol (more than 80% relaxation of 3 x 10^-6 M Cch-induced contraction, Fig. 3). When muscle was maximally relaxed by isoproterenol or PGE₂, the addition of this agent caused further relaxation in an additive fashion (data not shown). No change in adenylate cyclase activity was observed with nitroprusside (Table 1). The other NO donor SNAP also caused relaxation of Cch-precontracted ciliary muscle (Fig. 1h). The relaxation was seen from 10^-7 M, and at 5 x 10^-4 M this agent caused approximately 70% of the relaxation induced by 10^-5 M isoproterenol (Fig. 3). All the relaxations induced by these NO donors were reversible, suggesting that the relaxation was not caused by the cytotoxic effect. The addition of the NO synthetase inhibitor, L-NAME (3 x 10^-5 M), caused no change in the resting muscle tone, suggesting no basal release of NO (data not shown). These observations suggest that NO donors cause functional relaxation of precontracted ciliary muscle. The relaxation induced by IBMX can be considered to be due to the combined effect of accumulated cAMP and NO-independent cGMP.

- TABLE 1. Effects of Various Agents on Adenylate Cyclase Activity

<table>
<thead>
<tr>
<th>Agents</th>
<th>Dose (μM)</th>
<th>Adenylate Cyclase Activity (% of control)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>PGE₂</td>
<td>10</td>
<td>201 ± 29</td>
</tr>
<tr>
<td>PGE₁</td>
<td>10</td>
<td>215 ± 20</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10</td>
<td>186 ± 21</td>
</tr>
<tr>
<td>PGD₂</td>
<td>10</td>
<td>131 ± 15</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>10</td>
<td>128 ± 17</td>
</tr>
<tr>
<td>PGI₂</td>
<td>10</td>
<td>151 ± 7</td>
</tr>
<tr>
<td>Forskolin</td>
<td>10</td>
<td>411 ± 85</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>100</td>
<td>105 ± 8</td>
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Adenylate cyclase activity was determined by measuring cyclic adenosine monophosphate formation during 10 minutes of incubation with membrane fraction of ciliary muscle and expressed as the percentage of control. Each value shows a mean (± SE) of more than four determinations.
FIGURE 3. Dose-dependent relaxation of ciliary muscle induced by various agents. Ciliary muscle precontracted with 3 × 10⁻⁶ M carbachol was added with forskolin (closed circles), IBMX (open circles), sodium nitroprusside (open squares), or SNAP (closed squares). Relaxation was expressed as the percentage of the relaxation induced by 10⁻⁵ M isoproterenol. Each point shows a mean (±SE) of four determinations.

DISCUSSION. In the present study, we found that the relaxation-inducing effect of PGs and isoproterenol correlated highly with their ability to stimulate adenylate cyclase of ciliary muscle. We also observed that forskolin (a direct adenylate cyclase stimulator) induced relaxation similar to that observed with receptor-stimulating agents. These findings suggested that PG-induced relaxation is mediated by cAMP as a cellular second messenger. In addition, we demonstrated that NO donors relaxed ciliary muscle. The mechanism by which NO donors induced relaxation appeared to be different from the cAMP-dependent system because the muscle maximally relaxed by cAMP-dependent agents was further relaxed by nitroprusside and because nitroprusside did not stimulate adenylate cyclase. In some smooth muscle preparations, NO released from surrounding endothelia or innervating nerve terminals is known to stimulate cytosolic guanylate cyclase directly and to cause relaxation. Thus, it is likely that cat ciliary muscle, similar to some other smooth muscles, possesses an NO-mediated and a cGMP-dependent mechanism for relaxation and a cAMP-dependent system. To demonstrate unequivocally the presence of NO- or cGMP-dependent relaxation of ciliary muscle, more extensive investigation is currently under way.

The rank order of potency of PGs to induce relaxation obtained in this study was similar to that reported previously and, except for the activity of PGD₂, fits well with the PGF₂α receptor (EP receptor) classified by Coleman et al. The activity of PGD₂ to induce relaxation is higher than that reported for the agonist specificity reported for EP receptor. We think, as previously reported, that ciliary muscle possesses PGD₂ receptor (DP receptor) in addition to EP receptors. The EP₂ receptor, but not EP₁ or EP₃, is known to be coupled with adenylate cyclase and has been reported in many muscle preparations in association with muscle-relaxing activity. These properties of EP₂ receptor were consistent with the present observation. Thus, we concluded that EP₂ and DP receptors are involved in the PG-induced relaxation in the cat ciliary muscle.

Topically applied PGF₂α or its analogues have reduced IOP in many animal species, including humans, by facilitating uveoscleral outflow. In this system, aqueous humor leaves the eye through the spaces between ciliary muscle bundles, and the widening of these spaces (or the relaxation of ciliary muscle) increases the facility of uveoscleral outflow. We found in the present study that PGF₂α was one of the weakest PGs in causing muscle relaxation in cats. At 10⁻⁶ M, which is hardly an obtainable concentration after topical application, PGF₂α caused only approximately 25% of maximal relaxation induced by PGE₂. Therefore, the large IOP reduction by PGF₂α or its analogues observed in cats may be difficult to explain by increased uveoscleral outflow caused by relaxation of ciliary muscle.

In some pathologic conditions accompanying inflammation, extremely low IOP can be seen. Breakdown of the blood–aqueous barrier and increased uveoscleral outflow are one of the causes for the low IOP. Breakdown of the blood–aqueous barrier may be caused by EP receptor-mediated response because PGE₂ is known to increase peripheral vascular permeability. The present observation raises possibilities that the increase of uveoscleral outflow is induced by both EP receptor and NO-mediated ciliary muscle relaxation. Leukocytes and some other inflammation-related cells are good candidates for the source of NO.

In conclusion, this study has shown that stimulation of β-adrenergic and EP₂- and DP-type PG receptors, by activating adenylate cyclase and increasing cellular cAMP, relaxes precontracted ciliary muscle. In addition, we think the NO- or cGMP-dependent relaxation mechanism may be found in ciliary muscle.

Key Words
ciliary muscle relaxation, prostaglandin receptor, cat, cAMP, cGMP

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Soluble Forms of the High-Affinity Fibroblast Growth Factor Receptor in Human Vitreous Fluid

Anne Hanneken and Andrew Baird

**Purpose.** Fibroblast growth factor-binding proteins (FGF-BPs) have been identified recently in blood and other biologic fluids and have been shown to be identical to a truncated form of the high-affinity cell surface FGF receptor. The authors examined the hypothesis that FGF-BPs also are present in human vitreous fluid.

**Methods.** Vitreous fluid obtained from 12 patients was incubated overnight with heparin-Sepharose, and wheat germ agglutinin (WGA)-Sepharose. Western blot analysis revealed that the proteins bound avidly to WGA-Sepharose but not by an antibody raised to the intracellular domain of the high-affinity FGF receptor. These naturally occurring FGF-BPs may sequester free FGF in the vitreous cavity and may modulate the biologic activity of FGF.

**Results.** A 70- to 85-kd FGF-BP was detected in the vitreous of each of the 12 eyes examined. A 55-kd FGF-BP was detected in six of the samples. Both the 70- to 85-kd and the 55-kd FGF-BPs were precipitated by FGF-2 heparin-Sepharose but not by heparin-Sepharose alone, suggesting that the interaction was dependent on the presence of FGF-2. The proteins bound avidly to WGA-Sepharose. Western blot analysis revealed that the proteins were recognized by an antibody raised to the extracellular domain of the high-affinity FGF receptor but not by an antibody raised to the intracellular domain of the FGF receptor, indicating they are likely to be truncated portions of the extracellular domain of the high-affinity FGF receptor.

**Conclusions.** Vitreous fluid contains 70- to 85-kd and 55-kd FGF-BPs that have biochemical and immunologic characteristics similar to the extracellular domain of the high-affinity FGF receptor. These naturally occurring FGF-BPs may sequester free FGF in the vitreous cavity and may modulate the biologic activity of FGF in vitreoretinal diseases.

Proliferative diabetic retinopathy leads to severe visual loss and blindness as a consequence of uncontrolled retinal neovascularization, vitreous hemorrhages, and retinal detachments. Like other wound repair responses, proliferative diabetic retinopathy is regulated by local growth factors and their modulators. The two leading candidates are vascular endothelial cell growth factor and basic fibroblast growth factor (FGF-2). Both proteins are potent angiogenic agents and are elevated in the vitreous fluid of patients with proliferative retinal diseases. Although the biologic activity of vascular endothelial cell growth factor appears to be specific to endothelial cells, FGF-2 is mitogenic for endothelial cells and other cells found in diabetic fibrovascular membranes, such as fibrous astrocytes and fibrocytes.