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LOCALIZATION AND MOLECULAR SIGNALING PATHWAYS OF PROSTAGLANDIN RECEPTOR SUBTYPES IN THE EYE

by

Todd Lee Anthony

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)
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For the Degree of
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In the Graduate College
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1998
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Todd Lee Anthony entitled Localization and Molecular Signaling Pathways of Prostaglandin Receptors in the Eye and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director John W. Regan
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The eicosanoids are derived from membrane fatty acids. Prostaglandins (PGs), produced from the metabolism of arachidonic acid, are members of the eicosanoid family. Prostaglandins exert a broad range of biological effects by interacting with plasma membrane-bound receptors that are coupled to guanine nucleotide-binding proteins (G proteins). The topical application of prostaglandins results in a long-lasting reduction in intraocular pressure (IOP) in mammalian eyes, including humans. The distribution and function of the prostaglandin receptor subtypes within the eye are not clearly understood. The studies presented in this dissertation are focused at addressing three specific aims which have been designed to test the following hypothesis; that prostaglandin receptors are involved in the maintenance of intraocular pressure through the modulation of aqueous humor production and in the regulation of aqueous humor outflow pathways. Subtype selective antibodies to the individual prostanoid receptors have been generated to enable the study of these receptors at the tissue, cellular and molecular level. Utilization of the antibodies and a series of pharmacological and molecular techniques have identified specific prostanoid receptor subtypes in areas of the eye which are involved in the regulation of IOP. The functional responses obtained in primary cultures of human trabecular meshwork and bovine ciliary epithelium provide evidence for the involvement of selective prostanoid receptor subtypes in the regulation of both aqueous humor production and aqueous humor outflow.
CHAPTER ONE

General Introduction, Hypothesis and Aims

1.1 General Introduction

Anatomically, the eye can be divided into two distinct segments, the posterior segment located behind the lens of the eye and the anterior segment located in front of the lens of the eye. The anterior segment is further divided into the posterior chamber (area between the lens and the iris) and the anterior chamber (in front of the iris). The ophthalmic artery and the anterior ciliary veins provide the optic circulation, but some tissues of the eye, such as the lens and cornea, are not vascularized and rely on the diffusion of nutrients from the aqueous humor. The absence of vascular blood supply to the cornea and the lens allows for an unobstructed pathway for light information to reach the retina to be processed. Aqueous humor is produced and secreted by the cells of the ciliary epithelium, which line the outermost portion of the ciliary body in the posterior chamber of the eye. The production of aqueous humor in human subjects occurs at a rate of 0.24 ml/min.mm Hg⁻¹. From the posterior chamber it flows over the surface of the lens around the iris entering the anterior chamber where it baths the cells of the cornea. The aqueous humor leaves the eye through two characterized pathways. The majority of the aqueous humor (~ 85%) leaves by the so-called “conventional pathway” filtering through the trabecular meshwork regaining access to the systemic circulation via Schlemm’s canal. The remaining fluid (~ 15%) filters through the iris, ciliary body, and/or choroid entering the systemic circulation. This outflow route is collectively referred to as the “uveoscleral” pathway.
In the human eye, the balance between aqueous humor inflow (through the ciliary epithelium) and the rate of outflow (through the conventional or uveoscleral pathways) determines the intraocular pressure (IOP). The normal range for IOP in the human eye is between 12 and 18 mm Hg. Changes that may occur which alter the inflow or the outflow of aqueous humor can have serious pathological consequences. Sustained elevations in intraocular pressure ultimately leads to the disease state known as glaucoma. If left untreated, glaucoma eventually leads to an irreversible blindness due to the damage sustained to the optic nerve. The large majority of pharmacological approaches, which are used to treat glaucoma, have focused on the ciliary body including the cells of the ciliary epithelium. The ciliary epithelial cells are the active site where the formation of aqueous humor occurs in the eye. Consequently, these cells are often targets in the pharmacological management of glaucoma. Agents such as timolol, a β-adrenergic antagonist and pilocarpine, a muscarinic agonist, are quite effective in lowering IOP but are limited by either the development of tolerance or by systemic side effects. Recently, several experimental studies using topically applied prostaglandin analogues, including PGE\textsubscript{2} and PGF\textsubscript{2α}, produce sustained and significant decreases in IOP without detectable ocular or systemic side effects in several mammalian species, including humans. The tissue distribution of the prostanoid receptors and the molecular mechanisms by which these prostaglandin analogues produce their hypotensive actions in the eye are unclear and are the subject of this dissertation.
1.2 General Hypothesis

The principal mechanisms as it concerns the ocular pharmacology of the prostaglandins leads to my working hypothesis which is, that prostaglandin receptors are involved in the maintenance of intraocular pressure through the regulation of both the aqueous humor production and aqueous humor outflow pathways.

1.3 Specific Aims

This hypothesis will be addressed by the following specific aims. **Specific aim 1:** to generate subtype specific antibodies to the EP and FP prostanoid receptors and to characterize the expression of these prostanoid receptor subtypes in the trabecular meshwork and the cells of the ciliary body from human and bovine eyes, respectively.

**Specific aim 2:** will characterize the prostanoid receptor subtypes in the primary conventional outflow pathway of the eye. Cells of the trabecular meshwork will be examined using the subtype specific antibodies generated in specific aim 1 along with molecular biological techniques to determine the functional responses of the identified prostanoid receptors within the trabecular meshwork cells.

**Specific aim 3:** will determine the cellular location of the prostanoid receptor subtypes in the aqueous humor producing cells (ciliary epithelial cells). The non-pigmented (NPE) and pigmented (PE) ciliary epithelial cells of the bovine eye will be examined. Various functional assays will be performed to corroborate the receptor subtypes identified using immunocytochemistry.
A summary of the research literature is presented in chapter 2, the results of this work are presented in chapters 3-5, and a discussion of future experiments and significance of this work is provided in chapter 6.
CHAPTER TWO

Literature Review

2.1 The Structure of the Eye

The eye is a specialized organ that is constantly processing sensory information from the environment without our conscious effort. This specialized sensory organ is relatively secluded from the systemic access by blood-retinal, blood-aqueous, and blood-vitreous barriers (RAVIOLA, G., 1977). The eye is anatomically divided into the anterior and posterior segments. The anterior segment is composed of the cornea, limbus, anterior and posterior chambers, trabecular meshwork, Schlemm’s canal, iris, lens and the ciliary body. The anterior segment can be further subdivided into the anterior and posterior chambers (figure 2.1b). The posterior chamber of the anterior segment comprises the ciliary body (site of aqueous humor production), lens, and the iris. The anterior chamber of the anterior segment comprises the cornea, limbus, trabecular meshwork, and Schlemm’s canal. The sclera, retina, vitreous and optic nerve are contained in the posterior segment (figure 2.1a). The structures of the anterior and posterior chambers are actively involved in the production of aqueous humor and in the regulation of intraocular pressure (IOP). Intraocular pressure is a result of a complex interaction of aqueous humor dynamics. The rate of aqueous production, the resistance to outflow, and the venous pressure within the episcleral veins are especially important in regulation of IOP.
Figure 2.1: Schematic representations of the human eye. Cross-section of the human eye representing the anterior and posterior segment (A). Panel B, represents the anterior segment illustrating the anterior and posterior chambers (adapted from Anatomical Chart Co., Chicago, Illinois).
2.2 Aqueous Humor Dynamics

The ciliary epithelium produces aqueous humor through a combination of ultrafiltration and active secretion of blood plasma obtained from the ciliary capillaries in the stroma of the ciliary body. Aqueous humor is the product of three membrane transport characteristics of the ciliary epithelium. Simple diffusion of lipid soluble substances through the cellular membranes of the ciliary epithelium accounts for a small portion of aqueous humor content. Ultrafiltration of water and water-soluble substances (limited by size and charge) between the cells in response to osmotic gradient and the active transport of solutes (primarily sodium ions) over the doubled layer of the ciliary epithelium provide the remaining processes by which aqueous humor is produced. The composition of the aqueous humor, except for ascorbate, is virtually identical to that of plasma. The comparison of the components found in the aqueous humor versus blood plasma of the rabbit is presented in table 2.1.

Table 2.1: Comparison of the chemicals contained in the aqueous humor vs. plasma.

<table>
<thead>
<tr>
<th></th>
<th>Aqueous humor (mm/Kg H₂O)</th>
<th>Plasma (mm/Kg H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>143.5</td>
<td>151.5</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.25</td>
<td>5.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.78</td>
<td>1.0</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>109.5</td>
<td>108</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>33.6</td>
<td>27.4</td>
</tr>
<tr>
<td>Lactate</td>
<td>7.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.66</td>
<td>0.22</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.96</td>
<td>0.02</td>
</tr>
<tr>
<td>Urea</td>
<td>7.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.17</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Aqueous humor is continuously produced and is continuously leaves the eye. The rates of aqueous humor formation reported in the literature vary depending on the method of measurement. The normal range of aqueous humor formation is 1.8-4.3 μL/min with a mean value of 2.75 ± 0.63 μL/min (BRUBAKER, R. F., 1991). The volume of aqueous humor in the anterior segment of the human eye is approximately 0.25 mL. The importance of this circulating fluid is at least threefold. Circulating aqueous humor provides the necessary metabolic substrates and removes the metabolites from the avascular structures of the eye (cornea, lens and trabecular meshwork). The aqueous humor is involved in the local immune responses, and is essential in establishing the intraocular pressure needed for maintaining the optical properties of the eye. The normal range of intraocular pressure is 12 to 20 mm Hg with a mean value of 15 mm Hg. The major inflow and outflow pathways of aqueous humor is depicted in figure 2.2. The aqueous humor enters the posterior chamber from the ciliary epithelium between the iris and the lens. The bulk of aqueous humor flows through the pupil into the anterior chamber and leaves the eye through the trabecular meshwork and Schlemm’s canal (the conventional outflow pathway). There is no epithelial barrier that exists between the anterior chamber and the ciliary muscle and, therefore, aqueous humor can pass between the muscle cells into the suprachoroidal spaces, where it is drained through the sclera. The fluid then returns to the systemic circulation through the lymphatic vessels of the orbit (the uveoscleral outflow pathway).
**Figure 2.2:** Schematic diagram of the primary aqueous humor inflow and outflow pathway in the human anterior segment. The blue arrows depict the flow of aqueous humor from the ciliary epithelium through the iris and out through the conventional route (i.e. the trabecular meshwork and Schlemm’s canal). Abbreviations: TM, trabecular meshwork, SC, Schlemm’s canal, CP, ciliary process and CM, Ciliary muscle (adapted from Anatomical Chart Co., Chicago, Illinois).
Increase in the resistance of the outflow pathways will increase IOP. Sustained elevations in IOP result in the death of retinal ganglion cells and a distinctive type of optic atrophy characterized by cupping of the optic disk. These characteristics are the hallmarks of clinical glaucoma.

2.3 Overview of Glaucoma

Glaucoma is the leading cause of blindness throughout the world. Worldwide, glaucoma accounts for 13.5% of blindness which represents approximately 5.1 million people (SHIELDS, M. B., 1998). In the United States, glaucoma is the second leading cause of blindness among Caucasians, with the most frequent accounts occurring in the African American population. Glaucmatous optic neuropathy is associated with the progressive loss of a person’s visual field ultimately leading to a total and irreversible blindness. The glaucomas are traditionally divided into primary and secondary forms. This division is somewhat arbitrary, since all of the glaucomas are secondary to some type of abnormality. The “primary” glaucomas (e.g. open-angle, angle-closure and congenital) are confined to the anterior chamber or the conventional outflow pathways with little or no contributions from other ocular or systemic disorders. These forms of glaucoma are typically bilateral and potentially contain a genetic factor. The “secondary” glaucomas are in contrast to the “primary” glaucomas due to a partial understanding of the ocular or systemic effects leading to their development. Overall, the division of the glaucomas is more a reflection of a lack of understanding of the pathophysiological events that ultimately lead to the glaucomatous eye. The development of glaucomas can
be divided into five stages: (1) initiating events; (2) structural alterations; (3) functional alterations; (4) optic nerve damage; and (5) visual loss. The first three stages can be related to the etiology of glaucoma and are partially pressure-independent. The last two stages are due to mechanistic alterations of the anterior chamber angle (table 2.2). One common denominator to all glaucomas is a characteristic optic neuropathy, which is derived from a number of common risk factors the most common being a sustained increase in intraocular pressure.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Definitions</th>
<th>Pressure-Related</th>
<th>Pressure-Independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initiating events</td>
<td>Events that may lead to glaucoma</td>
<td>Genetic; acquired</td>
<td>Genetic? (not understood)</td>
</tr>
<tr>
<td>2. Structural alterations</td>
<td>Tissue changes leading to glaucoma</td>
<td>Aqueous humor outflow alterations</td>
<td>Optic nerve head alterations</td>
</tr>
<tr>
<td>3. Functional alterations</td>
<td>Physiological changes leading to glaucoma</td>
<td>Increased IOP</td>
<td>Reduced vascular perfusion</td>
</tr>
<tr>
<td>4. Optic nerve damage</td>
<td>Axonal loss leading to glaucoma</td>
<td></td>
<td>Glaucomatous optic neuropathy</td>
</tr>
<tr>
<td>5. Visual loss</td>
<td>Progressive loss of visual field</td>
<td></td>
<td>Glaucomatous visual field loss</td>
</tr>
</tbody>
</table>

Glaucomatous optic neuropathy is associated with the progressive loss of a person's visual field. Intraocular pressure and aqueous humor dynamics are the two most common and best-understood causative risk factors for glaucoma. Presently, they are the only two...
factors that can be managed clinically in attempts to delay the progressive optic neuropathy.

2.4 *Aqueous Humor Formation*

2.4.1 *The Pigmented and Nonpigmented Ciliary Epithelium*

The ciliary epithelium consists of a double layer of cells. The layer of cells which face the posterior chamber is called the nonpigmented epithelium (NPE), and is in direct contact with the aqueous humor in the posterior chamber. Next to the NPE is a layer of pigmented cells appropriately called the pigmented epithelium (PE). The NPE cells are columnar in shape and are derived from the forward continuation of the neuroepithelium from which the retinal cells are derived. The PE cells are cuboidal in shape and comprise the outer layer adjacent to the stroma. The PE represents the forward continuation of the retinal pigment epithelium (figure 2.3). The cells of the PE and NPE are secretory polarized epithelium, which oppose each other at their apical membranes. The unique orientation of this bilayer occurs as a result of embryological enfolding of the optic cup and is partially responsible for some of the difficulties in understanding how the ciliary epithelium regulates aqueous humor secretion (GREEN, K., 1984).

Along the ocular ciliary epithelium, several distinct anatomical regions have been identified and defined based on the morphological characteristics of the NPE and PE cells. Anatomical studies of the entire ciliary epithelium demonstrate a complex integration of cells that vary in different areas of the ciliary process. The *pars plicata*, found at the most anterior region of the ciliary epithelium, is distinguished by numerous
Figure 2.3: Drawings of the ciliary process and ciliary epithelium of the human eye. Panel A represents a cross-section of a single ciliary process. Plasma is filtered through the blood capillaries within the ciliary stroma (CS) and is filtered by the pigmented (PE) and nonpigmented epithelial (NPE) cells. The zonal fibers (ZF) attach the tips of the ciliary processes to the lens capsule (LC). A drawing of the entire ciliary process is shown in panel B. The arrows represent the two regions of the ciliary epithelium. Abbreviations: anterior chamber, AC, posterior chamber, PC, basement membrane, BM, apical membrane, AM, and tight-junction, TJ.
folding processes which provide a large surface area. The *pars plana*, or middle region, is relatively flat and extends to the *ora serrata*, which delineates the junction between the PE cells and the retinal pigment epithelium (COCA-PRADOS, M., 1998). Although the length of the ciliary epithelium differs in different species, the regional separations are consistent. Common to both cell types of the ciliary epithelium is the expression of membrane receptor proteins, membrane transport proteins and membrane channel proteins. Studies have demonstrated the presence of functional $\alpha_2$-adrenergic receptors in both the anterior segment and the retina (WIKBERG-MATSSON, A., 1996). The pharmacological significance of these receptor proteins will be discussed later in this chapter. Both cell types of the ciliary epithelium maintain an intimate cell-to-cell communication through numerous gap junction proteins primarily located at their apical boundaries (COCA-PRADOS, M., 1992). The presence of the gap junctions allows for the passage of ions and low molecular weight molecules to occur between the PE and NPE cells and enables the bilayer to function physiologically as a syncytium. The ciliary epithelial cells also express a number of membrane transport proteins. The expression of these proteins is known to occur in a differential fashion along the length of the ciliary epithelium. Examples of this differential gene expression are the Na\(^+\)-K\(^+\) exchange pump with its multiple $\alpha$- and $\beta$-subunits. The Na\(^+\),K\(^+\)-ATPase and isoforms present in the NPE cells which are differentially expressed in an anterior to posterior gradient along the layer corresponding to distinct regions of the ciliary epithelium (COCA-PRADOS, M., 1998). Other secretory proteins known to be present in the ciliary epithelium are a bumetanide-

A unique feature located near the basolateral surface of the NPE cells is the presence of a zonal occludens membrane protein. The expression of this protein creates a tight-junction between adjacent NPE cells and prevents the free passage of substances across the ciliary epithelium (NOSKE, W., 1994; STEVENSON, B. R., 1986). This membrane protein constitutes part of the blood-aqueous humor barrier between the underlying PE cell layer and the posterior chamber of the eye. The nonpigmented cells are considered to be the primary source of aqueous humor formation. They contain many mitochondria and have extensive invaginations in their basal surfaces serving primarily to increasing their surface area. The pigmented epithelial cells also contain numerous invaginations on their basolateral surfaces. Another abundant membrane protein present in the ciliary epithelium is aquaporin-1. Aquaporin-1 protein is a member of a family of proteins that function as water channels. Characterization of this protein by immunocytochemical and functional studies has localized the aquaporin-1 protein to the NPE cells (FRIGERI, A., 1995; STAMER, W. D., 1994b). These membrane proteins contained within the pigmented and nonpigmented epithelium along with a variety of intracellular second messenger pathway work concurrently in a manner that has not been fully elucidated to provide the anterior segment with a continuous supply of aqueous humor. In order to maintain a normal pressure homeostasis within the anterior segment, the aqueous must have a mechanism of removal.
2.5 *Aqueous Humor Outflow*

As mentioned earlier, aqueous humor leaves the eye through two distinctively different routes. The primary outflow of aqueous humor occurs through the trabecular meshwork and Schlemm’s canal (conventional pathway, *figure 2.2*). It is estimated that the conventional pathway constitutes 84% to 96% of the aqueous outflow in human eyes (NILSSON, S. F. E., 1997). To enter Schlemm’s canal, aqueous humor must pass through the cells of the trabecular meshwork that line the front of the canal. The trabecular meshwork is divided into three parts with characteristically different ultrastructures. The innermost portion is the *uveal meshwork*, the trabeculae making up the lamellae here are finer than those of the outer or *corneoscleral meshwork* where the meshwork is larger (TORIS, C. B., 1997). The third portion, lying immediately adjacent to the canal is the *endothelial meshwork*. The resistive characteristic of the trabecular meshwork was first characterized using trabeculotomized monkey eyes (ELLINGSEN, B. A., 1971). In this study, they were able to demonstrate the resistance to outflow through the conventional pathway, was virtually eliminated (83-97% reduction) after the trabeculotomy.

The secondary outflow pathway for aqueous humor occurs through the muscle bundles of the ciliary body where the fluid enters the supraciliary and suprachoroidal spaces and is drained through the sclera (uveoscleral pathway). This pathway accounts for the IOP-independent aqueous humor outflow. Studies using indirect measurements in human eyes have estimated that the uveoscleral routes drain less than 15%. Although, the contribution of the uveoscleral outflow accounts for only a small percentage of the total outflow, it has been suggested to be the primary site of action for the hypotensive action
of the prostaglandins (GABELT, B. T., 1989; NILSSON, S. F. E., 1989). The principle determinants of IOP are aqueous inflow, resistance to outflow, and episcleral venous pressure. Pharmacologic intervention in glaucoma therapy is directed towards these specific areas in the anterior segment of the eye.

2.6 Ocular Pharmacology

Topically applied drugs constitute the first line of therapy, and are the most frequently used agents in the treatment of glaucoma. Drugs that fall into this category include the cholinergic agonist, pilocarpine, which lower IOP by increasing aqueous humor outflow presumably through the contraction of the ciliary muscle. The $\alpha_2$-adrenergic agonist, apraclonidine, the beta-adrenergic antagonist, timolol, both act to lower IOP by decreasing aqueous humor production in the ciliary epithelium. The PGF$_{2\alpha}$ analogue, latanoprost, is hypothesized to lower IOP by increasing uveoscleral outflow pathways. The therapeutic endpoint in the treatment of glaucoma is to lower IOP.

2.6.1 Cholinergic Agents

Acetylcholine (ACh) is the postganglionic physiologic mediator of the parasympathetic nervous system. Drugs that mimic the actions of ACh are referred to as parasympathomimetics or cholinergic stimulators. These compounds may act directly, by stimulating the receptor, or by an indirect action which enhances the action of the endogenous mediator. The two receptors involved in the parasympathetic nervous system
are the nicotinic and muscarinic receptors. Muscarinic receptors and their associated G-proteins interact primarily with two second-messenger systems: adenylate cyclase and phospholipase C (PLC). Molecular biology studies have demonstrated five separate genes, \( m_1, m_2, m_3, m_4, \) and \( m_5 \) that code for the individual muscarinic receptors (HULME, E. C., 1990). In general, \( M_2 \) and \( M_4 \) receptor subtypes are coupled to the inhibition of adenylate cyclase (HULME, E. C., 1990), while the \( M_1, M_3 \) and \( M_5 \) receptor subtypes preferentially couple to the activation of PLC. *In situ* hybridization studies have demonstrated that mRNA for \( M_2, M_3 \) and \( M_5 \) are expressed in human ciliary muscle (ZHANG, X., 1995). Expression of \( M_3 \) mRNA was also demonstrated in bovine iris-sphincter muscle and ciliary process (HANKANEN, R. E., 1990).

In the eye, muscarinic antagonists such as atropine, inhibit cholinergic-mediated contraction of the iris-sphincter and ciliary muscle. The response to topically applied atropine increases the diameter of the pupil (mydriasis) and relaxes the ciliary muscle (cycloplegia). In people with narrow anterior chambers, the pupillary dilation may occlude the trabecular meshwork and produce an acute rise in intraocular pressure (angle-closure glaucoma).

The parasympathomimetic agents (muscarinic agonists) posses some beneficial affects. Methacholine, carbachol and bethanechol, all act like ACh but differ due to their individual resistances to the enzymatic activities of the cholinesterases. Carbachol is used clinically as a mitotic agent following cataract surgery and in the treatment of glaucoma. The ability of cholinergic agonists to reduce IOP results from the contraction of the ciliary muscles. This increases the tension on the scleral spur changing the shape
of the trabecular meshwork. The change in the structure of the TM results in an increase outflow facility (i.e. decreased resistance) ultimately reducing IOP (KAUFMAN, P. L., 1984b). The natural alkaloids pilocarpine, muscarine and arecoline have profiles similar to the choline esters. Pilocarpine is the primary direct acting cholinergic agent used in ophthalmology. Application of pilocarpine is effective in reducing IOP in monkeys (MIICHI, H., 1983). Pilocarpine’s mechanism of action is similar to that of carbachol. The ocular hypotensive actions of pilocarpine are additive to other hypotensive agents such as the β-adrenergic antagonists. The muscarinic agonists (which stimulate contraction of the ciliary muscle and iris sphincter muscle) are quite effective in lowering IOP but are limited by either the development of tolerance or by systemic side effects. These can include drug-induced myopia (result from ciliary muscle contraction), miosis, an accompanying decrease in vision under low light. They also are known to produce several systemic side effects such as, bronchoconstriction, salivation and abdominal cramps.

2.6.2 Adrenergic agents

Epinephrine and norepinephrine are the endogenous modulators of the sympathetic (adrenergic) nervous system. Compounds that mimic the effects of epinephrine and norepinephrine are commonly referred to as sympathomimetics. The sympathomimetic compounds interact with cell surface adrenergic receptors. The adrenergic receptors were originally classified into three distinct groups; α₁-, α₂- and β-
adrenergic receptors (AHLQUIST, R. P., 1948). Pharmacologic and molecular studies have identified individual subtypes of adrenergic receptors within each group. The $\alpha_1$-adrenergic receptors contain three subtypes; $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$, the $\alpha_2$-adrenergic receptors contain four subtypes; the $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$ and $\alpha_{2D}$ and the $\beta$-adrenergic receptors contain three subtypes; the $\beta_1$, $\beta_2$ and $\beta_3$. The adrenergic receptors within the membrane form complexes with heterotrimeric G-proteins initiating a variety of cellular responses. Activation of phospholipase C (PLC) results from the stimulation of $\alpha_1$-adrenergic receptors. The $\alpha_2$-adrenergic receptors are typically coupled to inhibitory G-proteins ($G_i$) to inhibit adenylate cyclase (HOFFMAN, B. B., 1996), but have demonstrated the ability to stimulate PLC through the activation of members of the $G_q$ family of G-proteins. The involvement of more than one type of G-protein, or from the ability of the same G-protein to interact with more than one effector system has been suggested (POTTER, D. E., 1997). All $\beta$-adrenergic receptors activate stimulatory G-proteins ($G_s$) resulting in the activation of adenylate cyclase (HOFFMAN, B. B., 1996).

Activation of each of the adrenergic receptor subtypes produces a variety of physiological responses throughout the body, including apparent influences on aqueous humor dynamics. Epinephrine is synthesized and released from chromaffin cells of the adrenal medulla into the circulation, thereby functioning primarily as a circulating neurohormone. Tissue effects of epinephrine are the combined result of the activation of the $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\beta_3$-adrenergic receptor subtypes. Norepinephrine produces its biological effects through the activation of the $\alpha_1$, $\alpha_2$ and $\beta_1$-adrenergic receptors. Norepinephrine released from sympathetic nerve endings is one of the principle means of
altering peripheral vascular resistance. Changes in systemic blood pressure effects the regional blood flow to various parts of the eye. In addition to regulating ocular blood flow, norepinephrine has important roles in aqueous humor production and iris function.

The response to adrenergic activation in the eye varies from one species to the other (POTTER, D. E., 1981). These differences may be due to the distribution of adrenergic receptor subtypes among the individual species examined. A considerable amount of information on the adrenergic regulation of IOP has been obtained from studies using epinephrine, a nonselective catecholamine with both α- and β-agonist properties. In normal human eyes, the effects of short applications of high doses of epinephrine include a lowering of IOP, and an increase in the outflow facility. Acute applications of epinephrine can result in increases in aqueous humor formation in human eyes that can be reduced with the β₂-adrenergic antagonist timolol. The β₂-adrenergic receptor antagonists are among the most widely used agents for the treatment of open-angle glaucoma. These agents are presumed to lower IOP primarily by inhibiting aqueous humor production (ERICKSON, K., 1994; MIICHI, H., 1983; SHAHIDULLAH, M., 1995). The effects at the cellular level are presumed to be through a cAMP-dependent mechanism. Functional studies examining adenylate cyclase stimulation in ocular tissue support the role for the involvement of cAMP in aqueous humor dynamics (BHATTACHERJEE, P., 1993; NEUFELD, A. H., 1975; SEARS, M. L., 1985). Direct stimulation of adenylate cyclase by forskolin has significant effects on lowering intraocular pressure by decreasing aqueous humor inflow in rabbits, monkeys and humans (BARTELS, S.P., 1987; CAPRIOLI, J., 1984; CAPRIOLI, J., 1984). Recently,
prostaglandins have been introduced as a new class of compounds in the management of glaucoma. The exact mechanism of action is still under investigation, but part of their IOP-lowering effects appear to be mediated in a similar fashion as that of the $\beta_2$-adrenergic antagonists.

2.6.3 *Prostaglandins*

Prostaglandins are ubiquitous local hormones that represent part of the family of arachidonic acid derivatives, or eicosanoids. The release of arachidonic acid results in a cascade of events in which a cyclooxygenase pathway leads to the synthesis of stable prostaglandins and labile products such as thromboxane A$_2$ (TXA$_2$) and prostacyclin (PGI$_2$), while a lipoxygenase pathway leads to the production of leukotrienes (*figure 2.4*). In common with other biologically active compounds, prostaglandins elicit cellular responses through the stimulation of distinct receptors. The prostanoid receptors were first characterized based on the responses of smooth muscle preparations, PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, PGI$_2$ and thromboxane A$_2$ (COLEMAN, R. A., 1994). The receptors were termed DP, EP, FP, IP and TP, respectively. To date, the only prostanoid receptors to possess subtypes are the TP, EP and FP receptors. The EP prostanoid receptor contains four known subtypes, EP$_1$, EP$_2$, EP$_3$ and EP$_4$, respectively. In the EP prostanoid receptors, the only one known to contain isoforms from alternative splicing is the EP$_3$ prostanoid receptor. The FP prostanoid receptor contains two isoforms as a result of alternative splicing, FP$_A$ and FP$_B$, respectively (PIERCE, K. L., 1997). Like the adrenergic and
muscarinic receptors, the prostaglandin receptors contain seven-transmembrane domains and are coupled to different second messenger signaling pathways (table 2.3).

Table 2.3: Signal transduction pathways of the prostanoid receptors

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>G-protein</th>
<th>Second Messenger</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Gs</td>
<td>Adenylyl cyclase, increase cAMP, K-channel opening,</td>
<td>Platelets, smooth muscle</td>
</tr>
<tr>
<td>DP</td>
<td>Gs</td>
<td>Adenylyl cyclase, increase cAMP, increase NO</td>
<td>Platelets, bovine coronary arteries</td>
</tr>
<tr>
<td>EP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Go</td>
<td>Increase Phospholipase C, increase Ca</td>
<td>Guinea pig ileum</td>
</tr>
<tr>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Gs</td>
<td>Adenylyl cyclase, increase cAMP</td>
<td>Trachea relaxation, circular ileum relaxation</td>
</tr>
<tr>
<td>EP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Gi</td>
<td>Adenylyl cyclase, decrease cAMP</td>
<td>Gastric acid secretion, neurotransmitter release, uterus contraction</td>
</tr>
<tr>
<td>EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Gs</td>
<td>Adenylyl cyclase, increase cAMP</td>
<td>Smooth muscle relaxation, pig saphenous vein</td>
</tr>
<tr>
<td>FP</td>
<td>Go</td>
<td>Increase Phospholipase C, Increase [Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Vascular smooth muscle, corpus luteum</td>
</tr>
<tr>
<td>TP</td>
<td>Go</td>
<td>Increase Phospholipase C, Increase [Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Smooth muscle, platelets</td>
</tr>
</tbody>
</table>
Figure 2.4: The prostaglandin side of the eicosanoid biosynthetic pathway. The metabolic enzymes are in italics.
Prostanoids exert a wide range of biological effects and are found in almost every tissue and body fluid examined. The ocular tissues are no exception, they respond in various ways to endogenous and synthetic prostaglandins depending on the type and dose of the prostaglandin and the animal species. Some of the responses include, vasodilation, increased permeability of the blood-aqueous barrier and corneal neovascularization (BHATTACHERJEE, P, 1997; MACRI, F. J., 1980; TORIS, C. B., 1997). Studies have indicated that smaller amounts of prostaglandins actually produced beneficial ocular hypotensive effects (RUSUL, B., 1997) leading to investigations of these agents for the medical treatment of glaucoma.

Studies in human eyes have demonstrated specific binding sites for both PGF$_{2\alpha}$ and PGE$_2$ in areas of the iris sphincter and ciliary muscles (MATSUO, T., 1992). Binding sites for PGE$_2$ and PGF$_{2\alpha}$ have also been demonstrated in porcine and bovine ocular tissues (BHATTACHERJEE, P., 1990; CSUKAS, S., 1993; SANO, N., 1993) suggesting that prostaglandins could modulate uveoscleral outflow by binding to receptors in the ciliary muscle. Recently, a PGF$_{2\alpha}$ analog, latanoprost, was approved for clinical use for the treatment of glaucoma. Although one study of the human eye suggested that improved conventional outflow might account for the hypotensive actions of latanoprost (ZIAI, N., 1993), the majority of the evidence supports the hypothesis that the primary mechanism of IOP reduction by PGF$_{2\alpha}$ analogs is improved uveoscleral outflow (PATEL, S. S., 1996; RULO, A. H., 1996; STJERNSCHANTZ, J., 1995). Functional studies in the ciliary muscle of the cat suggest that the ocular hypotensive
actions of PGF$_{2\alpha}$ may be mediated in part from the activation of DP or other prostanoid receptors and not by FP receptors (CHEN, J., 1992).

Several experimental studies using topically applied PGE$_2$ demonstrate a sustained and significant fall in IOP without detectable ocular or systemic side effects in several mammalian species, including humans (FLEISHER, L. N., 1996; JUMBLATT, M. M., 1994; WOODWARD, D. F., 1995). PGE$_2$ actions have been attributed to the modulation of adenylate cyclase and the formation of cAMP. Studies have demonstrated that IOP can be lowered by a number of agents that alter the level of cAMP in tissues of the anterior eye (SEARS, M. L., 1985). The mechanism in which cAMP potentially effect IOP has been postulated to involve the transport properties of the ciliary epithelial cells (CIVAN, M. M., 1994; CIVAN, M. M., 1996; DELAMERE, N. A., 1992; DELAMERE, N. A., 1997). These and other mechanisms for the hypotensive actions of prostaglandins in ocular tissue are still under investigation.

Understanding the cellular mechanisms involved in modulating IOP and aqueous humor dynamics, which regulate pressure, is critical towards determining the factors involved in the pathophysiology of glaucoma. Determining the cellular locations of the various membrane receptors involved in the regulation of aqueous humor dynamics is one step in understanding their mechanism of action. Understanding the cellular signaling pathways of these receptors can provide useful information for the development of more selective compounds. The goal of this dissertation was to provide some of this missing information by identifying the cellular location and the cellular signaling pathways of the prostanoid receptor subtypes in the eye. Molecular biological techniques were used to
generate subtype specific antibodies to the EP prostanoid receptor subtypes and the FP$_A$ receptor subtype. The cellular distribution and functional characterizations of the prostaglandin receptor subtypes were performed in tissues obtained from human and bovine eyes. These studies provide evidence for the existence of prostanoid receptors in ocular tissues. In chapter three, I provide the molecular methods used in generating subtype selective antibodies for the EP$_3$ and the FP$_A$ prostanoid receptor isoforms. In chapter four, I provide additional evidence for the involvement of PGF$_{2\alpha}$ in the trabecular meshwork (conventional outflow pathway) cells of the human eyes. In chapter five, I have characterized the distribution of the EP prostanoid receptor subtypes in both tissue sections and primary cultures of the bovine nonpigmented and pigmented epithelial cells. Additionally, I have provided evidence for a modulatory role of the EP$_3$ prostanoid receptor subtype in cAMP formation in the cells of the ciliary epithelium. In chapter six, I address the significance of these studies and provide a discussion of future experiments that could ultimately strengthen our understanding of the hypotensive actions of the prostanglandins in the eye.
CHAPTER THREE

Generation and Characterization of Prostanoid Receptor Antibodies

3.1 Introduction

Since the cloning of the individual prostanoid receptors, several subtypes and splice variants have been identified. Many of the prostanoid receptors have similar, if not, identical second messenger signaling pathways. The lack of selective agonists and antagonists for the prostanoid receptors makes the determination of the specific receptor subtypes difficult at best. Determining the tissue and/or cellular distribution of an individual membrane receptor subtype involved in a given biological response has many advantages. Therapeutically, more selective compounds could be synthesized, which ultimately would produce a more selective response. In addition, a more selective compound would potentially have a greater potency, in turn, allowing for less of the compound to be administered. Development of more selective compounds could potentially eliminate any unwanted side effects from the compound. Antibodies directed to unique regions of receptor proteins is one way of determining specific receptor distributions in tissues and in cells. Polyclonal antibodies have been generated in other laboratories to synthetic peptides which correspond to the carboxyl tail of the mouse EP1, EP3 and EP4 subtypes (ZHAO, C., 1995).

For our studies, we have generated subtype selective antibodies to the human EP1, EP2, EP3 and EP4 prostanoid receptor subtypes and to the sheep FP alpha prostanoid receptor subtype. Using recombinant techniques, we prepared fusion proteins to specific regions of the individual prostanoid receptor subtypes which were then used to generate
antibodies. Previously in our lab, antibodies have been generated and used successfully in *in vitro* and *in situ* experiments to the three individual $\alpha_2$-adrenergic receptor subtypes and to the aquaporin-1 channel protein (HUANG, Y., 1995; STAMER, W. D., 1996). For this section of the dissertation, I will discuss the procedure used to generate subtype selective antibodies for a variant of the human EP$_3$ prostanoid receptor and to the sheep FP$_A$ receptor. The antibodies directed towards the EP$_1$, EP$_2$ and EP$_4$ receptors were done in a similar manner by others in Dr. Regan’s laboratory.

3.2 Materials and Methods

3.2.1 Construction of the Fusion Proteins (pGEX/EP$_{3D}$ and pGEX/FP$_A$)

Polymerase chain reaction (PCR) was used to generate a 407 base pair product of the sheep FP$_A$ carboxyl terminus and a 350 base pair product of the human EP$_{3D}$ carboxyl terminus. The primer set used for the PCR reaction for the sheep FP$_A$ included the sense primer corresponding to nucleotides 1038-1065 and the antisense primer corresponded to nucleotides 1419-1445. The amplified PCR product using these primers encodes a 46 amino acid portion of the carboxyl tail of the sheep FP$_A$ receptor protein. The primer set used for the PCR reaction for the human EP$_{3D}$ included the sense primer corresponding to nucleotides 1124-1151 and the antisense primer corresponded to nucleotides 1440-1466. The amplified PCR product using these primers encodes a 29 amino acid portion of the carboxyl tail of the human EP$_{3D}$ receptor protein. Below are the nucleotide sequences of the individual primers used in the PCR reactions. Identified in lowercase italics are the
restriction sites for BamHI and EcoRI that were introduced in the primers for cloning purposes:

FP<sub>A</sub> carboxyl sense 5' - GAA Tgg atc cGT GTG TAC CAG ACG CTG T - 3'
FP<sub>A</sub> carboxyl antisense 5' - TAT Tga att cAG TTT GAC TCC TAT AG - 3'
EP<sub>JD</sub> carboxyl sense 5' - GTT Tgg atc cGT AGC AAA TGC TGT CTC C - 3'
EP<sub>JD</sub> carboxyl antisense 5' - TCA Cga att cAG CTA CAA TGG CAG ACT - 3'

Each of the PCR products were digested with BamHI and EcoRI and cloned in-frame behind the gene for glutathione-S-transferase (GST) in the expression vector pGEX2T, yielding the plasmids pGEX/FP<sub>A</sub> and pGEX/EP<sub>JD</sub>. E. coli strain, XL-1 Blue (Gibco/BRL,) was used to express the fusion proteins as described below.

3.2.2 Polymerase chain reaction and purification

The primer sets used to amplify the carboxy tail region of both the sheep FP<sub>A</sub> and the human EP<sub>JD</sub> receptors were used in the following reaction. The PCR (final volume 50 µl) contained 24 µl of sterile H<sub>2</sub>O, 5 µls of each of the following; 10X PCR buffer (Perkin-Elmer, Norwalk, CT), dimethylsulfoxide (DMSO), and dNTPs (2.5 mM), 2.5 µl each of the sense and antisense primers (20 µM), and 0.5 µl taq polymerase (Perkin Elmer, 2.5 U ml<sup>-1</sup>). The PCR program consisted of an initial step at 95°C for 7 min, followed by 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final step at 72°C for 7 minutes. Products were analyzed by electrophoresis in 1% agarose
gels. The PCR products were isolated from the agarose gel using a Gene clean kit (Gibco/BRL). Restriction digestion of the isolated PCR products was performed using the restriction endonucleases BamHI and EcoRI at 37°C for 1 hour.

3.2.3 Ligation reaction

The concentration of the Gene-cleaned PCR products of the digested pGEX2T plasmid and the FP_A and EP_3D fragments were determined using ultraviolet (260/280 nm) spectrometry. The ligation (final volume 10 µl) contained restricted insert and vector DNA (3:1 concentration ratio), 2 µl ligase buffer, 1 µl T4 DNA ligase (Gibco/BRL) and the volume was brought to final volume with sterile H2O. Each ligation was run for 3 hours at 16°C. Half of the ligation mixture (5 µl) was transformed into XL-1 blue competent E. coli cells.

3.2.4 Transformation into Bacterial Cells

The transformation of competent E. coli cells was performed using the following procedure. Room temperature SOC media (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 25 mM KCl, 20 mM Mg²⁺, 20 mM glucose, pH 7.0) was filtered through a 0.2 µm filter prior to its use. Luria broth (LB) plates containing ampicillin (100 µg/ml) were placed in a 37°C humidified incubator for a minimum of 1 hour. Ice cold Falcon #2059 tubes (10 ml) containing 50 µl of competent cells were inoculated with 5 µl of each of the ligation mixtures. The tubes were incubated on ice for 30 min. Heat shocked for 45
seconds at 42°C and immediately chilled on ice for 2 minutes. SOC media (950 μl) was added to each tube and placed in a floor shaker (250 rpm, 37°C) for 1 hour. The transformation mixture (100 and 250 μl) was spread onto 10 cm LB-ampicillin/agarose plates and incubated over-night at 37°C.

3.2.5 *Plasmid Mini-Prep*

Conformation of positive colonies was performed using the following procedure. Colonies (10-20) were picked from the LB-ampicillin plates using sterile tooth-picks, placed in 3 ml of LB containing ampicillin (100 μg/ml) and allowed to grow for 12-16 hours (250 rpm, 37°C). Aliquots (1.5 ml) were placed in microfuge tubes and centrifuged to pellet the bacterial cells. The supernatant was discarded and 100 μl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0) was added and allowed to incubate for 5 minutes at 22°C. 200 μl of solution II (0.2 N NaOH, 1% SDS) was immediately added and the tube was mixed by inversion and placed on ice for 5 minutes. 150 μl of solution III (3 M KOAc, 5 M glacial acetic acid) was added, mixed by inversion and placed on ice. Mixture was centrifuged for 15 min and the supernatant was transferred (~200 μl) to clean microfuge tubes. Ethanol (1 ml, 100%) was added to the supernatant and the tubes were placed in the freezer (-80°C) for 1 hour. The solution was centrifuged (1200 rpm) for 15 min, supernatant was discarded and the pellet was washed with ice cold 70% ethanol and respun. The DNA pellet was suspended in 40 μl of H₂O. Restriction digest
was performed on 6.5 μl DNA using the restriction endonucleases *BamHI* and *EcoRI* and visualized on a 1% agarose gel.

### 3.2.6 Fusion protein induction and purification

Samples of the expression plasmids were used to inoculate 25 mL of LB containing 100μg/ml ampicillin (LB-amp). The cultures were grown over-night in a floor shaker (250 rpm) maintained at 37°C. The over-night culture was transferred (12.5 mL/flask) to Erlenmeyer flask containing 250 mL of LB without ampicillin. Cultures were grown for approximately 2 hours (until optical density at 600 nm = 0.6). 60 mg of IPTG was added to one flask and the cultures were grown for an additional 4 hours (37°C, 250 rpm). Each sample was transferred to 250 mL polymer tubes and centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was discarded and the pellets resuspended in 10 mL of 100 mM Tris-HCl, pH 8.0 and 1% Triton X-100. The solutions were transferred to 50 mL polymer tubes and sonicated for 30 seconds. Samples were frozen in ETOH/dry ice bath and thawed in a 37°C water bath. This was repeated an additional 2 times. The samples were centrifuged at 18,000 rpm for 15 min at 4°C and the supernatant was collected (soluble, S2 fraction). The remaining pellet was resuspended in 10 mL of Tris-HCl, pH 8.0 and 0.5% SDS, sonicated for 30 seconds and centrifuged at 18,000 rpm for 15 minutes at 4°C. The supernatant was collected (insoluble, S3 fraction). 20 μL of the S2 and S3 fractions were then visualized on 12% SDS-PAGE gels. Soluble fusion proteins (S2 fraction) were purified by column affinity chromatography. Fusion proteins
contained in the insoluble fraction (S3) were purified by over-night elution from SDS-PAGE gels.

3.2.7 *GST subtraction and antibody purification*

Antibodies were purified from crude IgY samples using affinity chromatography. Two columns were prepared (affinity column and GST-subtraction column) using AmminoLink resin as manufacture suggested. Briefly, a 50% slurry of resin was added to a 3 ml column (Pierce, Rockford, NJ). The column was drained and washed 3 times with 0.1 M NaH$_2$PO$_4$ buffer, pH 7.0. Fusion protein (3 mg) was added to the column along with 150 μl of 1M sodium cyanoborohydride (NaCNBH$_3$) and allowed to rotate for 2 hours at 22°C. At the end of the 2 hours the rotation was stopped and the column was incubated for an additional 4 hours at 22°C. The solution was drained by vacuum and the column was washed with 3 ml of 1M Tris/HCl, pH 7.4. After washing, 1.5 ml of 1M Tris/HCl was added along with 150 μl of 1M NaCNBH$_3$ and the column was rotated for 30 min at 22°C. Column was drained and washed with 10 bed volumes (30 mls) of 1 M NaCl. Column was drained and washed with 10 bed volumes (30 ml) of 0.05% NaN$_3$. The column was stored at 4°C in 0.1M NaH$_2$PO$_4$ (pH 7.0) containing 0.05% NaN$_3$ until use.

3.2.8 *Immunoblot analysis*

Purified fusion proteins were separated on a 12 % SDS-PAGE gels with 0.1% SDS (Sodium Dodecyl Sulfate) and transferred to nitrocellulose using the Transblot
system (BioRad, Hercules, CA). The blots were incubated in Tris buffered saline, containing 0.2% Tween and 5% non-fat milk (TBS-T) over-night at 4°C. The blots were rotated for 20 minutes at 22°C and then probed with affinity purified GST/FP \( \alpha \) or GST/EP \( \beta \) antibodies (1:500 dilution) for 2 hours. The blots were washed in TBS-T for 2 hours at 22°C, then probed for 1 hour with rabbit-anti-chicken peroxidase (Sigma, St.Louis, MO) secondary (1:1000 dilution). Blots were then washed in TBS-T for 1 hour and visualized using chemiluminescent SuperSignal\textsuperscript{®} substrate (Pierce, Rockford, IL) per manufacture suggestion.

3.2.9 Transfection of COS-7 cells

Plasmids encoding either the FP \( \alpha \) or EP \( \beta \) were transiently transfected into COS-7 cells with the following procedure. COS-7 cells were plated in 10 cm culture plates (Falcon #3003) and allowed to reach 50% confluency. Culture media (Dulbecco's modified Eagle media, 5% fetal bovine serum, 1% penicillin-streptomycin) was removed and the cells were washed once with 20 ml phosphate buffered saline (PBS), pH 7.3. The cells were incubated (20 minutes) at 37°C in PBS solution (10 ml total volume) containing, 15 \( \mu \)g of plasmid DNA, 9.5 ml of phosphate buffered saline, pH 7.2 and 500 \( \mu \)L DEAE-dextran (500 \( \mu \)M). DNA/Dextran solution was removed and replaced with culture media (10 mL) containing 1 mM chloroquine and continued incubating for 2 hours. The chloroquine solution was removed and 10 ml of culture media containing 10% DMSO was added for 3 min at 22°C. This solution was aspirated and 15 ml of
culture media was added. The cells were maintained in a humidified incubator 95% air/5% CO₂ for 2 days prior to use.

3.3 Results

3.3.1 Construction of bacterial expression vectors

Bacterial expression plasmids were constructed using sense and antisense oligonucleotide primers designed to flank regions of the carboxyl tail of the sheep FPₐ and the human EP₃D receptor protein. Each primer included restriction sites for BamHI and EcoRI enabling the PCR products to be inserted in-frame behind a portion of the glutathione-S-transferase (GST) gene. The templates for each of the PCR reactions were the pBC/FPₐ \{Pierce\} and the pBC/EP₃D (REGAN, J. W. et al., 1994) plasmids. The amplified PCR products (figure 3.1A) and the bacterial expression vector, pGEX2T (figure 3.1B), were digested with BamHI and EcoRI and ligated together to form pGEX-FPₐ and pGEX-EP₃D expression plasmids. Following the ligation reaction, a restriction digest using the endonucleases, BamHI and EcoRI, in order to confirm the presence of the insert. After a one hour digestion with the enzymes, the appropriate size fragments could be visualized on a 1% agarose gel (figure 3.1B).
Figure 3.1: Ethidium-stained agarose gels of the amplification of the PCR products and the restriction products of the GST-FP \textsubscript{A} and GST-EP\textsubscript{3D}. The amplified PCR products obtained using primers specific for the carboxyl tail regions of the human plasmid encoding the EP\textsubscript{3D} receptor protein (lanes 1-2, panel A), and the sheep plasmid encoding the FP\textsubscript{A} receptor protein (lanes 3-4, panel A) are shown. As predicted, 350 bp (EP\textsubscript{3D}) and a 407 bp (FP\textsubscript{A}) PCR products were obtained. Plasmid DNA, transformed with pGEX2T-EP\textsubscript{3D} or pGEX2T-FP\textsubscript{A}, were restricted for one hour at 37° C with BamHI and EcoRI and the results are shown in (panel B). The appropriate 350 bp insert (EP\textsubscript{3D}) and 407 bp insert (FP\textsubscript{A}) were obtained after the restriction digest.
3.3.2 *Expression and purification of fusion proteins*

Competent bacteria, *E. coli*, were transformed with each of the constructs, plated and grown on ampicillin containing agarose plates. Colonies were picked to inoculate lura broth containing ampicillin (100 μg/ml) and grown over night. Plasmid DNA was isolated from the cultures and confirmed with restriction enzymes to identify positive colonies. If confirmation occurred, a portion of the mini-prep culture was used to inoculate large culture preparations. Large cultures were induced with IPTG (isopropylthio-β-D-galactoside) to initiate the expression of the fusion protein. Cultures were then checked for expression of the fusion protein by SDS-polyacrylamide gel electrophoresis. *Figure 3.2* shows the results of crude bacterial cultures transformed with pGEX-FPλ (panel A) and pGEX-EP3D (panel B). The first lane in each panel (S) are the molecular weight markers. Lane 1 in each panel is purified GST. Lane 2 (panel A) represents the induced S2 fraction of the GST/FPλ fusion protein. Lane 3 (panel A) is the induced S3 fraction of the GST/FPλ fusion protein. Since the GST/FPλ fusion protein resided in the S3 fraction of the bacterial cultures, extra steps were taken to obtain purified fusion protein ("*Materials and Methods*’). The last lane in panel A is the purified GST/FPλ fusion protein after large-scale SDS-PAGE purification.
Figure 3.2: Comassie-stained, SDS-polyacrylamide gel after induction and protein purification of GST/FP$_A$ and GST/EP$_{3D}$ fusion proteins. *E. Coli* were transformed with pGEX-2T (encoding GST, lanes 1 panel A and B). Lane 2 and 3 (panel A) are the induced S2 and S3 fraction of the GST/FP$_A$ fusion protein, respectively. Last lane (far right, panel A) is purified GST/FP$_A$ fusion protein as indicated. Lane 2 and 3 (panel B) are the uninduced and induced S2 fraction of the GST/EP$_{3D}$ fusion protein, lanes 4 and 5 (panel B) are the uninduced and induced S3 fraction of the GST/EP$_{3D}$ fusion protein respectively. Last lane (far right, panel B) is purified GST/EP$_{3D}$ fusion protein as indicated.
SDS-PAGE purification of crude and purified GST/EP\textsubscript{3D} fusion protein is shown in figure 3.2 (panel B). Lanes 2 and 3 (panel B) are the uninduced and induced S2 fractions of bacterial cultures expressing the GST/EP\textsubscript{3D} fusion construct. Lanes 4 and 5 (panel B) protein are the uninduced and induced S3 fractions of the bacterial culture expressing the GST/EP\textsubscript{3D} fusion construct. Since the GST/EP\textsubscript{3D} fusion protein resided in the soluble fraction, the protein was capable of being purified using a glutathione-agarose column. The purified GST/EP\textsubscript{3D} fusion protein is shown in the far right lane of figure 3.2 (panel B). As expected the apparent molecular sizes of the GST/FP\textsubscript{A} and the GST/EP\textsubscript{3D} were \(\sim 30,000\) and \(32,000\) dalton, respectively (table 3.1). Once enough of each of the purified fusion proteins were obtained, they were sent away (Covance, Inc.) to be inoculated into chickens.

<table>
<thead>
<tr>
<th>Ab Name</th>
<th>Nucleotides</th>
<th>Amino Acids</th>
<th>FSP name</th>
<th>Sizes:</th>
<th>Insert</th>
<th>Total</th>
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<tr>
<td>FP\textsubscript{A}-tail</td>
<td>1038-1444</td>
<td>46</td>
<td>GST/FP\textsubscript{A}</td>
<td>27,500</td>
<td>5,060</td>
<td>32,560</td>
</tr>
<tr>
<td>EP\textsubscript{3D}-tail</td>
<td>1134-1455</td>
<td>29</td>
<td>GST/EP\textsubscript{3D}</td>
<td>27,500</td>
<td>3,190</td>
<td>30,690</td>
</tr>
</tbody>
</table>

3.3.3 Initial Determination of Antigenicity and Purification of the antibodies

Covance, Inc. (Pennsylvania, PA) performed the immunizations of the chickens with each of the purified fusion proteins. After approximately one month chicken serum was analyzed by protein dot-blot to determine if antibodies were being produced.
Figure 3.3: Dot-blot analysis of GST/EP3D and GST/FPA fusion proteins with pre-immunized (PRE) versus post-immunized (POST) chicken serum. Chicken sera were diluted (1:20) with hybridization buffer (Tris buffered saline (TBS) containing 0.2% Tween-20 and 5% non-fat dry milk) incubated with nitrocellulose strips blotted with 1 μg, 100 ng and 1 ng of fusion protein as indicated. Blots were incubated with sera for 2 hr at 22°C, washed with TBS (3 times, 10 min), probed with alkaline phosphatase-conjugated secondary antibody in hybridization buffer for 1 hr at 22°C. Blots were visualized with nitro blue and tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.
Chicken sera prior to the immunizations were compared with sera collected 6 weeks after the initial injection. The reactivity to each of the fusion proteins is shown in figure 3.3. Each of the post-immunized bleeds of each chicken demonstrated positive reactions with their respective fusion proteins. Chicken serum was diluted (1:20) in hybridization buffer (Tris buffered saline (TBS) containing 2% Tween-20 and 5% non-fat dry milk). Reactivity was visualized to 1 ng of the GST/EP$_{30}$ (figure 3.3, panel A) and to the GST/FP$_{A}$ (figure 3.3, panel B), respectively.

Crude IgY was purified and concentrated for each of the antibodies ("Materials and Methods"). Antibodies were purified from the concentrated crude IgY by immunoaffinity chromatography. Initial purification was performed by application of the crude IgY to affinity columns coupled with either the GST/EP$_{30}$ or the GST/FP$_{A}$ fusion proteins. Examples of the antibody elution profiles are shown in figure 3.4. Ultraviolet spectroscopy was used to monitor the protein content of each elution. Each column underwent 5-7 washes (3 ml each) after the columns had been saturated, followed by two salt washes (333 mM NaCl) to remove any unbound antibodies. Each antibody was eluted from the columns using glycine (100 mM, pH 2.5). The eluted fractions (indicated by the arrows, figure 3.4) were subsequently applied to GST-subtraction columns for removal of GST-specific antibodies. The purified antibodies were then tested for specificity and optimal dilutions.
Figure 3.4: Antibody elution profiles. Antibodies were purified from concentrated crude IgY by immunoaffinity chromatography and antibodies to GST were removed by GST-subtraction. Ultraviolet spectroscopy (290 nm) was used to monitor the protein content of the elution. Elution profiles for the affinity purification of the anti-EP_{1D} antibodies (A) and the anti-EP_{A} antibodies (B) is shown. Arrows indicate the fraction obtained for application to the GST-subtraction column.
Figure 3.5: SDS-polyacrylamide gel electrophoresis and immunobloting of FP₄ and EP₃D fusion proteins. Panel A shows the reactivity of antibodies against the FP₄ carboxyl tail (anti-FP₄) to 100 ng, 30 ng, and 10 ng of GST/FP₄ fusion protein (lanes 2-4) and to 100 ng, 30 ng, and 10 ng of a second GST/FP₄-short fusion protein (lanes 5-7). Lane 1 is GST alone (1 µg). Panel B shows the reactivity of antibodies against the EP₃D carboxyl tail (anti-EP₃D) to 300 ng, 180 ng, 90 ng, and 40 ng of GST/EP₃D fusion protein (lanes 2-5). Lane 1 is GST alone (1 µg).
3.3.4 Characterizations of purified prostaglandin antibodies

The purified antibodies were characterized by immunoblot analysis and by immunofluorescent labeling of transiently transfected COS-7 cells. The sensitivity was determined for the FP\textsubscript{\textalpha} and EP\textsubscript{3D} antibody preparations and to the amount of antibodies to the GST fusion protein. A high concentration (100 ng) of GST fusion protein and serial dilutions of the GST/FP\textsubscript{\textalpha} and GST/EP\textsubscript{3D} were run on SDS-PAGE and analyzed on immunoblots using affinity purified antibodies. In panel A, (figure 3.5) antibodies to the carboxyl tail of the FP\textsubscript{\textalpha} receptor recognized 10 ng of GST/FP\textsubscript{\textalpha} and a GST/FP\textsubscript{\textalpha}-truncated fusion proteins (lane 4 and 7, respectively). This is in comparison to no recognition of the 100 ng of GST fusion protein (lane 1, panel A), suggesting that this antibody has a minimum of 10 fold sensitivity to FP\textsubscript{\textalpha} portion of the fusion protein. In panel B, (figure 3.5) antibodies to the carboxyl tail of the EP\textsubscript{3D} receptor recognized 40 ng of GST/EP\textsubscript{3D} fusion protein (lane 5). Again, there was no recognition of 100 ng of GST fusion protein (lane 1, panel B), suggesting a 4 fold sensitivity of this antibody.

Immunofluorescent microscopy on COS-7 cells transiently transfected with plasmids encoding either the human EP\textsubscript{3D}, the sheep FP\textsubscript{\textalpha}, or the sheep FPB receptors and were labeled for immunofluorescence microscopy using antibodies raised against the GST/EP\textsubscript{3D} and GST/FP\textsubscript{\textalpha}-carboxyl terminal fusion proteins. Figure 3.6, shows photomicrographs of cells transfected with the human EP\textsubscript{3D} receptor and examined for the fluorescence following incubations with the primary and secondary fluorescein antibodies.
Figure 3.6: Immunofluorescence microscopy of COS-7 cells transiently transfected with plasmids encoding either the human EP$_{3D}$ or sheep FP$_{A}$ receptors and labeled with antibodies to GST/EP$_{3D}$ or GST/FP$_{A}$ fusion proteins. Antibody purification and COS-7 cell labeling were done as described in "Materials and Methods". Cells transfected with the human EP$_{3D}$ receptor were labeled with either primary antibody alone (panel A) or with antibody that had been preincubated with fusion protein (panel B). Cells transfected with the sheep FP$_{A}$ receptor (panel C) or the sheep FP$_{B}$ (panel D) were labeled with primary alone. Secondary antibody was FITC-rabbit anti-chicken IgG. Bar = 20 μm.
(panel A). Strong labeling was present over the cell surface that could be specifically blocked by preincubation of the antibodies with the GST/EP<sub>3D</sub>-carboxyl terminal fusion protein (panel B). Omission of the primary antibody (data not shown) resulted in a low level of background labeling that was comparable to preincubation of the antibody with the fusion protein. An analogous series of experiments with COS-7 cells that were transfected with the sheep FP<sub>A</sub> and FP<sub>B</sub> receptors produced essentially identical results figure 3.6 (panels C and D), showing that the antibodies were specific and could be blocked with preincubation of their corresponding GST-fusion proteins. Preincubation of the antibodies with GST alone or with fusion proteins containing portions of other prostaglandin receptors did not block labeling (data not shown).

### 3.4 Discussion

The cellular localization of membrane receptor provides useful information that potentially can lead to an understanding of their function. Studies using radioligand binding techniques can provide such information, but is limited to membrane preparations of tissues. This technique does provide information to the amount of receptor expressed in a given tissue, but does not provide any information to the potential subcellular distribution within a given tissue. The use of receptor subtype selective antibodies provides researchers with a valuable tool in determining the exact distribution of the receptor in question. In this chapter, I have provided experimental characterizations of the FP<sub>A</sub> and EP<sub>3D</sub> prostanoid receptor antibodies. The antibodies
generated are subtype specific. They do not cross react with other prostanoid receptors and they have been successfully used to identify receptor subtypes in various tissues.

The positive immunoreactivity to the GST-fusion proteins (figure 3.5) indicates that the purified antibodies are specific to the protein to which they were made. However, this does not necessarily mean that the antibodies will recognize the expressed receptor. Therefore, experiments were performed using transiently transfected COS-7 cells expressing either the sheep FP\textsubscript{A} receptor or the human EP\textsubscript{3D} receptor.

One method in determining specificity of the antibodies is by preincubating the primary antibody with its corresponding fusion protein. The interaction of the individual antibodies with the fusion protein to which they were generated was demonstrated by the immunoreactivity seen in figure 3.5. Preincubation with the GST fusion protein will occupy the free antibodies making them unavailable. Labeling with primary antibody alone versus antibody preincubated with the fusion protein is one way of demonstrating selectivity. An alternative method would be to have a receptor with a similar sequence homology to the receptor that the antibody was made. Fortunately this was the case for the sheep FP receptor.

Recently, an alternative splice variant of the sheep FP receptor was cloned and identified (PIERCE, K. L. et al., 1997). This splice variant identified as the FP\textsubscript{B} receptor is identical to the sheep FP\textsubscript{A} receptor from the amino-terminus through the seven transmembrane domains. The difference between the two receptors exists at the carboxy tail. The FP\textsubscript{B} carboxy tail stops nine amino acids into the carboxyl tail, whereas the FP\textsubscript{A} receptor continues for an additional 49 amino acids. The GST/FP\textsubscript{A} antibody was made to
this portion of the tail that is not contained in the FP\textsubscript{b} receptor. This makes the sheep FP\textsubscript{b} splice variant an ideal control for testing the GST/FP\textsubscript{A} antibody. Immunofluorescent labeling of COS-7 cells expressing the sheep FP\textsubscript{A} and the human EP\textsubscript{3D} receptors is represented in figure 3.6 (panels C & D). This experiment demonstrates that the affinity purified FP\textsubscript{A} and EP\textsubscript{3D} antibodies are specific and recognize their respective endogenous receptor proteins.

The goal of this chapter was to generate specific antibodies to a subset of prostanoid receptor subtypes. Using molecular biology techniques, individual fusion proteins were constructed to specific regions to the EP\textsubscript{3D} and FP\textsubscript{A} prostanoid receptor subtypes. The antibodies obtained and purified from the yolk of the chicken eggs were shown to react specifically to the fusion protein from which they were made and to the endogenous receptor expressed in transiently transfected COS-7 cells.
CHAPTER FOUR

Prostaglandin F\textsubscript{2\alpha} Receptors in Human Trabecular Meshwork

4.1 Introduction

The trabecular meshwork (TM) is the major route for aqueous outflow in the anterior chamber of the human eye. As such, the TM has an important role in the regulation of intraocular pressure (IOP) and is frequently a target in the pharmacological management of glaucoma. Cells of the TM and of Schlemm’s canal are involved with aqueous humor outflow but the details of their involvement, at least at the molecular level, are not clearly understood. In particular, specific receptors that may be present in these cells, and which may mediate some of the IOP lowering effects of drugs, are largely unknown.

Pharmacological management of glaucoma has included a variety of drugs acting at different receptors and locations in the eye. For example, \(\alpha\)\textsubscript{2}-adrenergic agonists and \(\beta\)-adrenergic antagonists lower IOP by acting on their respective receptors in the ciliary epithelium to decrease the secretion of aqueous humor (BRUBAKER, R. F., 1991; SUGRUE, M. F., 1989). Muscarinic agonists, on the other hand, act on the muscarinic acetylcholine receptors in the ciliary smooth muscle to cause contraction and thereby decrease resistance of aqueous outflow through the TM (KAUFMAN, P. L., 1984a). The recently introduced prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}) analogue, latanoprost (13,14-dihydro-15R-17-phenyl-18,19,20-trinor-PGF\textsubscript{2\alpha}-isopropyl ester, PhXA41), is believed to act through FP prostanoid receptors in the ciliary muscle to enhance uveoscleral outflow.

One of the limitations to understanding the ocular effects of PGF$_2\alpha$ and its analogues has been our knowledge of the FP receptors themselves. FP prostaglandin receptors are members of the seven transmembrane families of receptors and are coupled to activation of guanine nucleotide binding proteins (G-proteins). cDNAs encoding FP prostanoid receptors were first cloned in 1994 and recently a novel alternative mRNA splice variant has been identified (PIERCE, K. L., 1997). The original FP receptors have been termed FP$_{A}$ and the splice variant, which contains a truncated carboxyl terminus relative to the FP$_{A}$, has been termed the FP$_{B}$. Stimulation of both FP receptor isoforms results in the activation of phospholipase C-mediated phosphoinositide turnover (THIERAUCH, K. H., 1994). FP prostanoid receptors are known to be present in the eye but their specific localization and cellular functions are not well characterized. Recently, however, in situ hybridization and immunohistochemical studies have indicated that FP receptors are present in the corneal, conjunctival, and iridial epithelia and in the ciliary muscle and ciliary process of the monkey eye (OCKLIND, A., 1996). Radioligand binding and autoradiography suggest localization of the FP receptors in the ciliary muscle and iris sphincter muscle of the human eye (MATSUO, T., 1992).

The present study was initiated to determine if FP receptors might be present in cells of the trabecular meshwork where their activation might influence aqueous outflow. The results of this study indicate that FP$_{A}$ prostanoid receptors are present and that they are functionally coupled to the activation of phosphoinositide hydrolysis.
4.2 Materials and Methods

4.2.1 HTM Cell Culture

Human trabecular meshwork (HTM) cells were isolated following collagenase digestion of human TM explants exactly as described by Stamer, (STAMER, W. D., 1995b). They were characterized with respect to their growth characteristics, morphology, presence of a cell surface receptor for low density lipoproteins, and have been previously used for the characterization of aquaporin-1 (STAMER, W. D., 1995a) and \( \alpha_2 \)-adrenergic receptors (STAMER, W. D., 1996) which are present in these cells. HTM cells were maintained on 10 cm\(^2\) plates with Medium 199 containing 15% fetal bovine serum, 10 \( \mu \)g/ml endothelial growth supplement (Sigma), 5 mM HEPES, 1.7 mM \( l \)-glutamine in an humidified incubator (95% air/5% CO\(_2\)) at 37\(^\circ\)C.

4.2.2 Fluorescence Microscopy

Antibodies to the sheep FP\(_A\) receptor were generated in chickens using a recombinant fusion protein consisting of glutathione-S-transferase (GST) and a portion of the carboxyl terminus of the receptor consisting of amino acids 317 to 362. Preparation of the fusion protein and antibody purification was done as described in Chapter 3.

Initial characterization of the antibodies was done as previously described in Chapter 3 using COS-7 cells transfected with a plasmid encoding the sheep FP\(_A\) receptor. For immunolabeling, cells were cultured for 3-4 days on 1% gelatin-coated coverslips and media was removed and the cells washed twice with phosphate buffered saline
(PBS). The cells were placed in 4% paraformaldehyde/PBS for 15 minutes at room temperature, washed once with PBS, and then placed in 300 mM glycine solution for 20 minutes. Cells were washed again in PBS and were permeabilized with 30 mM sodium chloride/300 mM sodium citrate (SSC) containing 0.1% Triton-X 100 for 1 hour. After an overnight incubation at 4°C with the primary antibody (0.6 μg/ml), the cells were washed with SSC and incubated for 2 hours at room temperature with secondary antibody (rabbit-anti-chicken/FITC, Sigma) at a dilution of 1:1000. Coverslips were washed and mounted on glass slides for viewing. For labeling of human eye tissues, eyes were fixed in 4% paraformaldehyde 3-5 hours and transverse sections were embedded in paraffin and serially sectioned (10 μm thickness). Fluorescent immunolabeling was performed as described above with the following addition. After an overnight incubation at 4°C with affinity purified primary antibody (0.6 μg/ml), the cells were washed with SSC and incubated for 1 hour at room temperature with secondary antibody (rabbit-anti-chicken/CY-5, Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:750. Eye sections were washed and mounted under glass coverslips for confocal imaging.

4.2.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primers were chosen to amplify a unique 1118 nucleotide (nt.) of the Human FP<sub>A</sub> receptor. The sense primer (nt. 170-193) corresponds to a position 61 nucleotides upstream of the translational start site and the antisense primer (nt. 1333-1356) corresponds to a position 39 nucleotides downstream of the stop codon in the human FP<sub>A</sub>
sequence. Both PCR primers were 100% homologous with the reported cloned sequence of the human FP<sub>a</sub> receptor. The sense and antisense primers were used for RT-PCR as previously described with total RNA isolated from primary cultures of HTM cells (STAMER, W. D. et al., 1995a). The PCR (final volume 20 µl) contained 5 µl of the RT reaction, 5 µl 10X PCR buffer (Perkin Elmer, Norwalk, CT), 5 µl dimethylsulfoxide (DMSO), 2.5 µl each of the sense primer and antisense primers (20 µM), and 0.5 µl Taq polymerase (Perkin Elmer, 2.5 U ml⁻¹). The PCR program consisted of an initial step at 95°C for 7 min, followed by 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final step at 72°C for 7 minutes. Products were analyzed by electrophoresis in 1% agarose gels.

4.2.4 Inositol Phosphate Assay

HTM cells were grown in 12-well plates in Medium 199 containing 15% fetal bovine serum, 10 µg/ml endothelial growth supplement (Sigma), 5 mM HEPES, 1.7 mM L-glutamine and were allowed to grow to 80% confluency in a humidified incubator (95% air/5% CO₂) maintained at 37°C. Medium was removed 24 hours prior to assay and replaced with serum free medium containing 0.2 µM myo-[³H]inositol. After 24 hours at 37°C, medium was removed and replaced with 1 ml of fresh serum free medium and placed back into the incubator for 1 hour. Lithium chloride (10 mM) was added and allowed to equilibrate for 10 min, at which time PGF<sub>2α</sub> (endogenous FP receptor ligand) was added and placed back into the incubator for 1 hour. Plates were placed on ice and
immediately aspirated and washed once with fresh medium. Cold methanol was added (0.5 ml/well), the wells were scraped, and contents added to 1.5 ml of chloroform/H₂O (2:1). The solutions were vortexed and centrifuged at 2100 rpm for 15 minutes an aliquot (0.9 ml) of the aqueous phase was added to 2 ml of H₂O, and then applied to a 2.5 ml anion exchange column (AG1-X8 resin formate form, BioRad). The columns were washed three times with H₂O (5 ml/wash), twice with 5 mM sodium tetraborate/60 mM sodium formate (5 ml/wash). The columns were then eluted with 2 ml of 0.2M ammonium formate/0.1M formic acid and the radioactivity determined by liquid scintillation counting.

4.2.5 Intracellular Calcium Measurements

HTM cells were grown to confluence in 15 cm² culture plates. Cells were trypsinized, counted and resuspended at a concentration of 3 X 10⁶ cells/ml in serum free media. Fura2-AM (Sigma; 1 mM, 1µl) was added per ml of cells. The cells were submerged in a 37°C water bath and incubated with slow shaking for 45 min to 1 hour. After the incubation, 40 ml media was added, cells were centrifuged at 1200 RPM for 5 min. The supernatant was aspirated and the cells were resuspended in media (2 X 10⁶ cells/ml). HTM cells were distributed to microcentrifuge tubes in 500 µl aliquots. Just prior to use, cells were centrifuged for 1 min (2000 RPM) in a microcentrifuge. The supernatants were aspirated, and the cells were resuspended in recording buffer (120 mM NaCl, 6 mM KCl, 1.4 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 1mg/ml glucose, 1
mg/ml sodium pyruvate, pH 7.4). Recording and analysis were performed using a Perkin Elmer L5-50 fluorescence spectrometer (WOODWARD, D. F., 1994). The cells were lysed with 0.1% Triton X-100 to obtain maximum fluorescence, and EGTA (final concentration, 6 mM) and NaOH (final concentration, 20 mM) were added successively to obtain minimum fluorescence.

4.3 Results

4.3.1 Cross-reactivity of FP<sub>λ</sub> Receptor Antibody in COS-7 Cells

COS-7 cells were transiently transfected with plasmids encoding the human FP<sub>λ</sub> receptor and were labeled for immunofluorescence microscopy using antibodies raised against a sheep GST/FP<sub>λ</sub>-carboxyl terminal fusion protein. Figure 1, panel A shows photomicrographs of cells transfected with the human FP<sub>λ</sub> receptor and examined for the fluorescence following incubations with the primary and secondary fluorescein antibodies. Strong labeling was present over the cell surface which could be specifically blocked (panel B) by preincubation of the antibodies with the GST/FP<sub>λ</sub>-carboxyl terminal fusion protein. Omission of the primary antibody (data not shown) resulted in a low level
Figure 4.1: Immunofluorescence microscopy of COS-7 cells transiently transfected with plasmids encoding human FP<sub>A</sub> prostanoid receptors and labeled with antibodies to a GST/FP<sub>A</sub> fusion protein. Antibody purification and COS-7 cell labeling were done as described in “Materials and Methods”. Cells transfected with the human FP<sub>A</sub> receptor were labeled with either primary antibody alone (panel A) or with antibody that had been preincubated with fusion protein (panel B). Secondary antibody was FITC-rabbit anti-chicken IgG. Bar = 20 μm.
of background labeling that was comparable to preincubation of the antibody with the fusion protein (panel B). This data confirms that the FP₄ antibodies could cross react with the carboxyl terminus of the human FP₄ receptor. The homology between the human and sheep FP₄ receptors in this region is approximately 70% and is consistent with the observed cross reactivity. Preincubation of the antibodies with GST alone or with fusion proteins containing portions of other prostanoid receptors did not block labeling (data not shown).

4.3.2 Immunofluorescent Microscopy of Prostanoid FP₄ Receptor Subtype in Primary Cultures of Human Trabecular Meshwork Cells

Figure 2 shows the fluorescein fluorescence of HTM cells following labeling with antibodies to the GST/FP₄ receptor fusion protein. Similar to the labeling observed with FP₄ transfected COS-7 cells, the HTM cells showed strong immunoreactivity when incubated with both the primary and FITC- labeled secondary antibodies (panel A). In contrast to the labeling of the COS-7 cells, however, all the HTM cells showed immunoreactivity, which is consistent with the expression of an endogenous receptor as opposed to the heterologous expression in transfected cells. Pre-incubation of the primary antibody with the GST/FP₄ carboxyl terminal fusion protein blocked the labeling of the HTM cells (panel B). Omission of the primary antibody yielded a low level of background fluorescence (data not shown). To see if FP₄ receptor immunoreactivity was present in the TM itself 10 µm sections of human eyes (n = 2 from separate donors) were labeled and examined by confocal microscopy. As shown in figure 3, immunoreactivity
was observed in the TM (panels A and C) and was blocked pre-incubation of the primary antibody with the GST/FP\textsubscript{A} carboxyl terminal fusion protein (panels B and D).

4.3.3 Expression of FPA Receptor mRNA in Cultured Human Trabecular Meshwork Cells

To confirm that the HTM cells contained mRNA encoding a prostanoid FP\textsubscript{A} receptor, RT-PCR was done with primers that were predicted to yield an FP\textsubscript{A} specific product of 1268 base pairs. Figure 4 shows an ethidium-stained agarose gel with the PCR products obtained from cDNA prepared from total RNA isolated from two different donor populations of HTM cells (lanes 1 & 3). To confirm specificity of the PCR products, the products were incubated with the restriction endonuclease HIND II for 1 hour at 37°C. As expected, a single cut was obtained yielding the appropriate two bands of 900 bp and 368 bp (lanes 2 and 4). A negative control is also shown in which the PCR reaction contained everything except a DNA template (lane 5). As shown, a product of the expected size was obtained with the RNA isolated from both populations of donor cells. Since the primers were chosen to span an intron, the PCR products did not result from the amplification of genomic DNA and are consistent with the presence of mRNA encoding a human prostanoid FP\textsubscript{A} receptor.
**Figure 4.2:** Immunofluorescence labeling of human trabecular meshwork (HTM) cells with antibodies to a GST/FP\textsubscript{A} fusion protein. HTM cells were cultured as described in Methods and were labeled with either primary antibody alone (panel A) or primary antibody preincubated with fusion protein (panel B). Secondary antibody was FITC-rabbit anti-chicken IgG. Bar = 20 μm.
Figure 4.3: Confocal microscopy of human trabecular meshwork (TM) after labeling with antibodies to a GST/FP\(_x\) fusion protein. Fixation and labeling were as described in "Materials and Methods". The panels represent serial sections (10 μm) of the anterior chamber labeled with either primary antibody alone (panels A and C) or with primary antibody preincubated with fusion protein (panels B and D). The arrows indicate positive immunoreactivity. SC, Schlemm’s canal; C, cornea and TM, trabecular meshwork. Bar = 20 μm.
Figure 4.4: Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from human trabecular meshwork cells and amplified with specific primers for the prostanoid FP_A receptor. Lanes 1 and 3, products obtained after RT-PCR using total RNA isolated from two populations of donor human trabecular meshwork cells, respectively. Lanes 2 and 4, expected products (825 and 361 bp) obtained after restriction digestion with BamHI. Lane 5 is a negative control without template. Standards (lane S) are λ-phage and φX174 DNA cut with HindIII and HaeIII, respectively, and are shown in kilobase pair units. The predicted size of the uncut human FP_A receptor PCR product (1186 bp) was obtained from both populations of donor human trabecular meshwork cells.
4.3.4 Stimulation of Inositol Phosphate Formation in Cultured Human Trabecular Meshwork Cells

Stimulation of prostanoid FP receptors by agonists is known to produce intracellular signaling through an increase in phosphoinositide hydrolysis and a release of intracellular calcium (THIERAUCH, K. H. et al., 1994). The ability of PGF$_{2\alpha}$ to stimulate phosphoinositide turnover in HTM cells was examined and the results are presented in figure 5. As shown, PGF$_{2\alpha}$ produced a dose-dependent increase in [$^3$H]-inositol phosphate accumulation with an EC$_{50}$ approximately 100 nM. Using HTM cells prepared from a second donor, a similar dose-response curve was obtained with a maximal stimulation of [$^3$H]-inositol phosphate accumulation that was 3 fold over basal. In both series of experiments the stimulation of [$^3$H]-inositol phosphate accumulation was statistically significant (ANOVA, p < 0.01).

4.3.5 Effect of PGF$_{2\alpha}$ on Intracellular Calcium Levels in Cultured Human Trabecular Meshwork Cells

The ability of PGF$_{2\alpha}$ to stimulate the release of intracellular calcium was examined and the results are represented in figure 6. PGF$_{2\alpha}$ produced a dose-dependent increase in the overall change in intracellular calcium with an EC$_{50}$ approximately 60 nM. Human trabecular meshwork cells used for the calcium measurements were obtained from the same human donors used in the phosphoinositide hydrolysis assays.
Figure 4.5: Stimulation of $[^3\text{H}]$-inositol phosphate formation by PGF$_{2\alpha}$ in cultured human trabecular meshwork cells. Incubation conditions and assay of inositol phosphates were as described in "Materials and Methods". Final concentrations of PGF$_{2\alpha}$ are indicated. Data represent the mean of two independent experiments each done in duplicate using cells from one donor. The experiment was repeated two more times in duplicate with HTM cells from a second donor. Asterisks indicates significant difference from basal (ANOVA, followed by multiple range testing; $p < 0.01$).
Figure 4.6: Stimulation of intracellular calcium by PGF$_{2\alpha}$ in cultured human TM cells. Conditions of the assay were as described in the "Materials and Methods". Final concentrations of PGF$_{2\alpha}$ are indicated. Data represents the values from a population of cells obtained from a single donor eye.
These results are consistent with the activation of a FP prostanoid receptor that demonstrates a downstream effect from the stimulation of phosphoinositide turnover in the human trabecular meshwork cells.

### 4.4 Discussion

Previous studies utilizing *in situ* hybridization, radioligand binding and immunohistochemistry have identified FP prostanoid receptors in several regions of the human and primate eyes. Some of the areas identified in which FP receptors were present included the corneal, conjunctival and iridal epithelia as well as the iris sphincter and ciliary smooth muscles (MATSUO, T., 1992; OCKLIND, A., 1996). These studies did not report, however, on the presence of FP receptors in the trabecular meshwork (TM) nor did they establish the nature of the FP receptor isoforms that may be present or the second messenger pathways of these receptors. In the present study, immunohistochemistry and the PCR were used to identify the presence of the FP$^\alpha$ receptor isoform in cultured cells from the human TM. It was also determined that the activation of these receptors by PGF$_{2\alpha}$ stimulated phosphoinositide turnover in these cells.

The physiological and pharmacological significance of the prostanoid FP receptors in the human TM remains to be established. As it concerns the treatment of glaucoma, it is generally acknowledged that the IOP lowering effects of PGF$_{2\alpha}$, and its analogues, occurs by way of an increase in uveoscleral outflow. However, these agents may have actions in other regions of the eye that influence these effects on IOP (ZIAI, N.,
For example, in a recent clinical study of the interaction between PhXA41 (13,14-dihydro-15R-17-phenyl-18,19,20-trinor-PGF$_{2\alpha}$-isopropyl ester) and pilocarpine, it was determined that reduction in IOP could not be explained solely by increased uveoscleral outflow (FRISTROM, B., 1993). Among the possibilities suggested was enhanced outflow through the conventional trabecular pathway. In addition, the effect of PGF$_{2\alpha}$ on IOP may differ under normal physiological conditions as compared with pathophysiological conditions, therefore, the potential effect of PGF$_{2\alpha}$ on the trabecular meshwork needs to be understood.

The role of inositol phosphates, particularly inositol 1,4,5-trisphosphate (IP$_3$), in initiating the release of intracellular calcium is well established (BERRIDGE, M. J., 1993). In this study, we have found that PGF$_2$ can stimulate the turnover of inositol phosphates (EC$_{50}$ 100 nM) in HTM cells. The EC$_{50}$ value reported for the stimulation of inositol phosphates in the HTM was within the range of values reported by others for PGF$_{2\alpha}$ stimulation (RUSUL, B., 1997; YOUSUFZAI, S. Y. K., 1996). We hypothesized that this would result in a release of intracellular calcium. We found in the same population of HTM cells examined, that stimulation with PGF$_{2\alpha}$ resulted in a dose-dependent increase in intracellular calcium release. Taking these data together, we conclude that activation of the prostanoid FP receptors present on the HTM cells results in an accumulation of inositol phosphate ultimately resulting in the release of intracellular calcium. Interestingly, it has been found that TM cells contain contractile filaments similar to those found in smooth muscle (DE KATER, A. W., 1990). In addition, HTM cells have been shown in electrophysiological studies to exhibit a nifedipine sensitive
electrical behavior characteristics of smooth muscle cells (LEPPLE-WIENHUES, A., 1994). From our results, it is possible, that the activation of FP prostanoid receptors in the TM resulting in the release of intracellular calcium, in turn, could modify the shape and/or size of the cells through a smooth-muscle like contractile event. This could potentially affect the resistive properties of the TM cells and thereby influence aqueous outflow. Obviously, more work is needed to establish this possibility, but at least some of the elements are in place. Furthermore, whether or not a contractile event is taking place, PGF$_{2\alpha}$ does appear to have an effect in the HTM and it is reasonable to predict that PGF$_{2\alpha}$ and its analogs mediate their effects through FP$_{A}$ receptors on HTM cells.
CHAPTER FIVE

Functional Expression and Characterization of Prostanoid EP Receptor Subtypes in Bovine Ciliary Epithelial Cells

5.1 Introduction

Prostaglandins (PGs) can both indirectly and directly affect intraocular pressure (IOP) in animal and human eyes. For example, the hypotensive effects of topically applied epinephrine was shown to be blocked by indomethicin, suggesting that prostaglandins may be involved (DELAMERE, N. A., 1997). Furthermore, topically applied prostaglandin E$_2$ (PGE$_2$) in low doses demonstrated a sustained and significant fall in IOP without detectable side effects (LEE, P. P., 1984; VILLUMSEN, J., 1992). Recent studies showed that PGs such as PGF$_{2\alpha}$, at low concentrations, decreased IOP in several mammalian species including humans (CAMRAS, C. B., 1995; CAMRAS, C. B., 1987; RULO, A. H., 1996). Although the mechanism of the hypotensive effect is not clearly understood, it was hypothesized to occur through an increase in uveoscleral outflow (CAMRAS, C. B., 1995; POYER, J. F., 1992). The pigmented (PE) and nonpigmented (NPE) cells of the ciliary epithelium are responsible for the generation of aqueous humor but the details of their involvement in producing the aqueous humor, at least at the receptor and molecular level, have yet to be fully clarified.

PGE$_2$ and its analogues bind to four EP prostanoid receptor subtypes identified as EP$_1$, EP$_2$, EP$_3$ and EP$_4$ (COLEMAN, R. A., 1994). Functional binding studies have described the presence of EP$_2$ receptors in the bovine iris-ciliary body (BHATTACHERJEE, P., 1990) and two binding sites for PGE$_2$ in membranes from NPE
of the pig (SANO, N., 1993). Prostanoid induced relaxation of precontracted cat ciliary muscle is mediated by EP<sub>2</sub> and DP receptors (CHEN, J., 1992). EP prostanoid receptor stimulation results in the generation of several second messengers depending on the individual receptor subtype involved. Stimulation of adenylyl cyclase and increases in intracellular cAMP occur in the iris-ciliary body of rabbits, cats and cows following treatment with PG agonists (BHATTACHERJEE, P., 1993). In ciliary epithelial cells, prostanoid receptor activation and stimulation of adenylyl cyclase has been attributed to changes in aqueous humor formation. In addition, PGE<sub>2</sub> stimulates adenylyl cyclase in SV-40 transformed nonpigmented epithelial cells (BHATTACHERJEE, P., 1996; JUMBLATT, M. M., 1994) and stimulation of adenylyl cyclase by forskolin, or by cholera toxin, has been shown to decrease intraocular pressure in rabbits, monkeys and humans (BARTELS, S.P., 1987; CAPRIOLI, J., 1984; GREGORY, D., 1981). Thus, determining the distribution of the individual EP prostanoid receptor subtypes within the ciliary epithelium is a necessary step toward understanding the actions of PGE<sub>2</sub> and its analogues in regulating aqueous humor production.

The present study was initiated to determine the expression of the EP prostanoid receptor subtypes in the cells of the bovine ciliary epithelium at the tissue and cellular level. The results of this study indicate that EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> prostanoid receptors are present and are functionally coupled to both the activation of phosphoinositide hydrolysis and stimulation of cAMP formation. In addition to the stimulatory effects previously reported and confirmed in this study, we demonstrate the existence of a
functionally coupled EP3 prostanoid receptor in bovine ciliary epithelial (BCE) cells, which inhibits cAMP formation through pertussis toxin sensitive inhibitory G-proteins.

5.2 Materials and Methods

5.2.1 Bovine Ciliary Epithelial Cell Culture

Bovine ciliary epithelial (BCE) cells were isolated by the following procedure. Bovine eyes were obtained from a local slaughterhouse 1-2 hours after death and transported to the laboratory on ice. The eyes were surgically cleaned of connective tissue and muscle and rinsed once in Hank's balanced salt solution (HBSS) containing penicillin/streptomycin. The eyes were enucleated and placed cornea side down in a dissecting dish. The lens was removed by cutting around the zonal fibers. The remaining lens capsule was dissected and removed. The tips of the ciliary processes were collected from 2-4 eyes and placed in HBSS. Ciliary tips were rinsed with 0.05% Trypsin-EDTA, then incubated in 6 ml (0.05% Trypsin-EDTA) for 10 minutes at 37°C. The ciliary tips were triturtated using a fire polished glass pipette and were centrifuged for 10 minutes (1000 rpm at 4°C). Supernatant was removed and the cells were resuspended (10 ml of 0.05% Trypsin-EDTA) for an additional 20 minutes at 37°C. The tissue pellet was again tritutated, and 1 ml (10%) fetal bovine serum was added to stop the digestion. Cells were collected by centrifugation for 1 minute (1000 rpm, 4°C), resuspended in culture media then plated in 10 cm² culture dishes. Cultures were maintained in humidified incubator (95% air/5% CO₂) at 37°C.
5.2.2 Fluorescence Microscopy

Antibodies to the human EP₁, EP₂, EP₃, and EP₄ prostanoid receptors were generated in chickens using recombinant fusion proteins consisting of glutathione-S-transferase (GST) and a portion of the carboxyl terminus specific for human EP₃ and EP₄ receptors and the third intracellular loop of the human EP₁ and EP₂ receptors. Procedures for preparation of the fusion protein and antibody purification are described in a previous study (HUANG, Y., 1995). Initial characterization of the antibodies was done as previously described (ANTHONY, T. L., 1998) using COS-7 cells transfected with plasmid DNA encoding the individual human EP receptor subtypes.

For immunolabeling, cells were cultured for 3-4 days on glass coverslips. The media was removed and the cells washed twice with phosphate buffered saline (PBS). Cells were placed in 4% paraformaldehyde/PBS for 10 minutes at room temperature, washed once with PBS, and then placed in 300 mM glycine solution for 20 minutes. Cells were washed again in PBS and permeabilized with 30 mM sodium chloride/300 mM sodium citrate (SSC) containing 0.1% triton-X100 for 1 hour. After an overnight incubation at 4°C with the primary antibody (0.5-1.0 mg/ml), the cells were washed with SSC and incubated for 1 hour at room temperature with secondary antibody (rabbit-anti-chicken/FITC, Sigma) at a dilution of 1:1000. Coverslips were washed and mounted on glass slides for viewing. For labeling of bovine tissue sections, tissues were fixed in 4% paraformaldehyde and snap-frozen in O.C.T. (Tissue-Tek®, Miles Inc.). Sections (10 µm thick) were obtained and prepared for confocal immunofluorescent microscopy.
5.2.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primers were chosen to amplify unique regions within the human EP receptor subtype sequences. The sense and antisense primers for each of the EP receptor subtypes was used for RT-PCR as previously described (STAMER, W. D., 1995a) with total RNA isolated from primary cultures of BCE cells. The PCR (final volume 20 µl) contained 5 µl of the RT reaction, 5 µl 10X PCR buffer (Perkin-Elmer, Norwalk, CT), 5 µl dimethylsulfoxide (DMSO), 2.5 µl each of the sense primer and antisense primers (20 µM), and 0.5 µl taq polymerase (Perkin Elmer, 2.5 µ ml⁻¹). The PCR program consisted of an initial step at 95°C for 7 min, followed by 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final step at 72°C for 7 minutes. Products were analyzed by electrophoresis in 1% agarose gels and confirmed by either restriction enzyme mapping or nucleotide sequencing.

5.2.4 Cyclic Adenosine-3'-5' Monophosphate Assay

Primary cultures of BCE cells taken from the same passage as those used for immunofluorescence microscopy were plated in 24-well culture plates and allowed to grow to confluence. Twenty-four hours prior to the assay, media was replaced with serum-free media. On the day of the assay cells were washed once with fresh serum-free media and incubated for 2 minutes with serum-free media containing 0.1 mg/ml isobutyl-methyl xanthine (IBMX). Cells were then treated with IBMX/media with or without drug for 15 minutes at 37°C. Media was removed, cells were placed on ice and 150 µl
(50 mM Tris/10 mM EDTA) was added. Cells were scraped, and the cell homogenate was transferred to microfuge tubes and boiled for 10 minutes. Tubes were placed on ice, centrifuged, and 50 µl of the supernatant from each sample was added to new tubes containing 50 µl ([³H]-cAMP, NEN) and 100 µl protein kinase A (100 μg/ml). This mixture was vortex and incubated on ice for 2 hours at 4°C. After the incubation, 100 µl of charcoal/BSA was added, vortexed and centrifuged to pellet the charcoal mixture. Radioactivity in the supernatant (200 µl) was determined by liquid scintillation counting.

5.2.5 Inositol Phosphate Assay

BCE cells were grown in 12-well plates in DMEM containing 10% fetal bovine serum, 10 μg/ml endothelial growth supplement (Sigma), 5 mM HEPES, 1.7 mM l-glutamine and were allowed to grow to 80% confluency in a humidified incubator (95% air/5% CO₂) at 37°C. Medium was removed 24 hours prior to assay and replaced with serum-free medium containing 0.2 μM myo-[³H]inositol. After 24 hours, the medium was removed and replaced with 1 ml of fresh serum free medium and incubated at 37°C for 1 hour. Lithium chloride (10 mM) was added and allowed to equilibrate for 10 min, at which time PGE₂ was added and allowed to incubate for 1 hour at 37°C. Plates were placed on ice and immediately aspirated and washed once with fresh medium. Cold methanol was added (1.0 ml/well), the wells were scraped, and contents added to 1.5 ml of chloroform/H₂O (2:1). The solutions were vortexed and centrifuged at 2100 rpm for 15 minutes, then an aliquot (0.9 ml) of the aqueous phase was added to 2 ml of H₂O,
vortexed, then applied to a 2.5 ml anion exchange column (AG1-X8 resin formate form, BioRad). The columns were washed three times with H₂O (5 ml/wash), twice with 5 mM sodium tetraborate/60 mM sodium formate (5 ml/wash). The columns were eluted with 2 ml of 0.2M ammonium formate/0.1M formic acid and the radioactivity determined by liquid scintillation counting.

5.3 Results

5.3.1 Characterization of EP Receptor Subtype Selective Antibodies

Transiently transfected COS-7 cells were used to characterize the EP prostanoid receptor antibodies. Specific labeling was determined by indirect immunofluorescent microscopy using antibodies preincubated with its corresponding fusion protein, or with preincubation with fusion proteins constructed for a different EP prostanoid receptor subtype sequence. Figure 5.1 demonstrates the specificity of the individual EP receptor antibodies in transfected COS-7 cells. Panels (A-C) are COS-7 cells transfected with the human plasmid for the EP₁ receptor labeled with primary antibody (A), with primary antibody preincubated with its corresponding fusion protein (B), and primary antibody preincubated with the EP₂ fusion protein (C).
Figure 5.1: Immunofluorescence microscopy of COS-7 cells transiently transfected with plasmids encoding human EP₁ (A-C), EP₂ (D-F), EP₃ (G-I) and EP₄ (J-L) receptors and labeled with antibodies to GST-EP subtype receptor fusion proteins. COS-7 cells expressing the human prostanoid receptors to EP₁ (A), EP₂ (D), EP₃ (G) and EP₄ (J) were labeled with primary antibody followed by an FITC-conjugated secondary antibody. Panels (B, E, H, and K) are cells labeled with primary antibody that had been preincubated with their corresponding fusion proteins. Panels C, F, I and L are cells labeled with primary antibodies that had been preincubated with non-corresponding fusion proteins.

Panels (D-F) are COS-7 cells transfected with the human plasmid for the EP₂ receptor labeled with primary antibody (D), with primary antibody preincubated with its corresponding fusion protein (E), and primary antibody preincubated with the EP₁ fusion protein (F). Panels (G-I) are COS-7 cells transfected with the human plasmid for the EP₃ receptor labeled with primary antibody (G), with primary antibody preincubated with its corresponding fusion protein (H), and primary antibody preincubated with the EP₄ fusion protein (I). Panels (J-L) are COS-7 cells transfected with the human plasmid for the EP₄ receptor labeled with primary antibody (J), with primary antibody preincubated with its corresponding fusion protein (K), and primary antibody preincubated with the EP₁ fusion protein (L). The labeling was consistent with an heterologous expression system. Specific immunoreactivity was blocked only when the primary antibody was preincubated with its respective fusion protein. Preincubating the antibodies with fusion
proteins generated to other EP prostanoid receptors was ineffective in blocking the specific labeling.

5.3.2 Characterization of Bovine Ciliary Epithelial Cells in Tissue Sections and Primary Cultures.

Cells were characterized with respect to their growth characteristics, morphology, presence of previously characterized membrane receptor proteins and with tight-junction specific monoclonal antibody raised to rat zonal occludens (ZO-1) (STEVenson, B. R., 1986). The data obtained in primary cultures were compared with the immunofluorescent labeling performed on frozen thin sections of bovine ciliary processes. Tissue sections of bovine ciliary processes (10 μm) and primary cultures of BCE cells were examined with antibodies to aquaporin-1 and viewed by confocal microscopy (figure 5.2). Positive immunoreactivity was primarily localized to the nonpigmented cells of the ciliary epithelium in tissue sections (A) and on a subset of cells in primary culture (C). These results suggest that bovine ciliary NPE cells express the aquaporin-1 membrane channel protein and are consistent with results obtained with human eye sections (STAMER, W. D., 1994a). A separate set of experiments were performed to examine the distribution of the α₂-adrenergic receptor expression in primary cultures of BCE cells (figure 5.3). Previous studies using tissue sections of human ciliary epithelium demonstrated that α₂-adrenergic receptor labeling was primarily localized to the nonpigmented cells (HUANG, Y., 1995). In primary cultures of BCE cells, positive labeling was detected to the α₂-A (figure 5.3A) and to the α₂-B (figure 5.3B) membrane receptor proteins. The α₂-C adrenergic receptor was not expressed in primary cultures of BCE cells (figure 5.3C). A
final characterization of the BCE cells was performed using a monoclonal antibody to the tight-junction (zonal occludens) protein which has previously been shown to be present exclusively on NPE cells (STEVenson, B. R., 1986). Tight-junction specific labeling was seen in both tissue sections (figure 5.4A) and on the outer cell membrane of a subset of BCE cells in primary culture (figure 5.4B). All of the data presented in this section demonstrate that bovine ciliary epithelial cells express membrane proteins similar to those seen in other mammalian species including humans. The data also support the idea that BCE cells continue to maintain the phenotypical expression of these proteins in primary cultures.
Figure 5.2: Confocal microscopy of aquaporin-1 expression in bovine ciliary epithelium. Bovine tissue sections (10 μm) were labeled with primary antibodies to a glutathione-S-transferase-AQP-1 fusion protein (A) or with primary antibody preincubated with fusion protein (B). Primary cultures of bovine ciliary epithelial cells were labeled with primary antibody alone (C) or with primary antibody preincubated with fusion protein (D). Immunoreactivity was localized to the NPE cells in both tissue sections and in primary culture. NPE, non-pigmented epithelium, PE, pigmented epithelium. Arrows indicate specific immunoreactivity.
Figure 5.3: Immunofluorescence microscopy of primary cultures of BCE cells after labeling with antibodies to GST-α₂ adrenergic receptor fusion proteins. Cells were labeled with either primary antibodies to the GST-α₂A (A), GST-α₂B (C) or GST-α₂C (E) fusion proteins or with antibodies that had been preincubated with their corresponding fusion proteins (B, D and F), respectively. The secondary antibody was fluorescein isothiocyanate-rabbit anti-chicken immunoglobulin G. Bar = 20 μm.
Figure 5.4: Confocal microscopy of tight junction (zonal occludens) labeling in bovine ciliary epithelium. Bovine tissue sections (10 μm) were labeled with rat anti-ZO-1 monoclonal antibody (MAB1520) specific to tight junction membrane proteins (A) or with primary antibody omitted (B). Primary cultures of bovine ciliary epithelial cells were labeled with rat anti-ZO-1 monoclonal antibody (C). The secondary antibody was fluorescein isothiocyanate-goat anti-rat immunoglobulin G. Panel D is a differential interference contrast (DIC) image of BCE cells in culture. Immunoreactivity is outlined with arrows. NPE, non-pigmented epithelium, PE, pigmented epithelium.
5.3.3 Immunofluorescence Microscopy of EP Prostanoid Receptor Subtypes in Primary Cultures of Bovine Ciliary Epithelial Cells

The distribution of EP prostanoid receptor subtype expression was examined in frozen thin sections and in primary cell cultures of bovine ciliary process. Thin sections (10 μm) were examined with antibodies to each of the EP prostanoid receptor subtypes and viewed by confocal microscopy (figure 5.5). Positive immunoreactivity was observed in tissue sections labeled with antibodies to the EP₁ (panel A), EP₂ (panel B), EP₃ (panel C) and EP₄ (panel D) prostanoid receptors. Although all the EP prostanoid receptor subtypes were present their distribution appeared to differ among the specific cell types within the ciliary epithelium. For example, EP₁ receptor expression was primarily localized to the NPE cells, as was the EP₄ receptor expression (panels A and D), whereas, EP₂ receptor expression was localized to the pigmented epithelial cells (panel B). EP₃ receptor expression on the other hand was present in both cell types of the ciliary process (panel C). Positive immunofluorescence was blocked when the primary antibodies were preincubated with their corresponding GST-fusion proteins: GST-EP₁ fusion protein (panel E), GST-EP₂ fusion protein (panel F), GST-EP₃ fusion protein (panel G) and GST-EP₄ fusion protein (panel H).

EP prostanoid receptor expression was also examined in primary cultures of BCE cells. Indirect immunofluorescent microscopy was performed on freshly dissociated BCE cells or on cells that were maintained in primary culture for 4-6 weeks (2-3 passages). Figure 5.6 shows the fluorescence of primary cultures of bovine epithelial cells after labeling with antibodies to the GST-EP₁ (A), the GST-EP₂ (C), the GST-EP₃ (E) or the
GST-EP₄ (G) receptor fusion proteins. Positive immunofluorescence labeling was observed with the EP₁, EP₃, and EP₄ prostanoid receptor antibodies. There was no specific immunoreactivity in primary cultures of BCE cells with the EP₂ prostanoid receptor antibody.

5.3.4 Expression of EP Prostanoid Receptor mRNA in Cultured Bovine Ciliary Epithelial Cells

To confirm that the ciliary epithelial cells contained mRNA encoding the EP prostanoid receptors, RT-PCR was done with primers that were predicted to yield an EP₁ specific product of 322 base pairs, an EP₂ specific product of 607 base pairs, an EP₃ specific product of 479 base pairs, and an EP₄ specific product of 727 base pairs. Figure 5.7 shows an ethidium-stained agarose gel with the PCR products obtained from cDNA prepared from total RNA isolated from four different preparations of BCE cells (lanes 2, 5, 8 and 11). Human plasmid DNA encoding the individual EP receptor subtypes was used as positive control (lanes 1, 4, 7 and 10). A negative control for each condition is also shown in which the PCR reaction contained everything except the cDNA template (lanes 3, 6, 9 and 12).
Figure 5.5: Confocal microscopy of cryosections (10 μm) of bovine ciliary processes after labeling with antibodies to GST-EP subtype receptor fusion proteins. Positive immunoreactivity was seen with primary antibodies to EP₁ (panel A), EP₂ (panel B), EP₃ (panel C) and EP₄ (panel D). The primary antibodies were conjugated to an FITC-conjugated secondary antibody. Panels E, F, G and H represent the corresponding sections after labeling with primary antibodies that had been preincubated with their individual GST-fusion proteins. PE, pigmented epithelium, NPE, non-pigmented epithelium. Arrows indicate NPE cell layer.
Figure 5.6: Immunofluorescence microscopy of primary cultures of BCE cells after labeling with antibodies to GST/EP receptor fusion proteins. Bovine ciliary epithelial cells were cultured as described in “Materials and Methods”, and were labeled with either primary antibodies to the GST/EP₁ fusion protein (A), GST/EP₂ fusion protein (C), GST/EP₃ fusion protein (E) or GST/EP₄ fusion protein (G) or with antibodies that had been preincubated with their corresponding fusion proteins (B, D, F and H, respectively). The secondary antibody was fluorescein isothiocyanate-rabbit anti-chicken immunoglobulin G. Bar = 20 μm.
Figure 5.7: Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from bovine ciliary epithelial cells and amplified with specific primers for the human EP prostanoid receptor subtypes. Lanes 1, 4, 7 and 10 show the products obtained from human plasmid DNA after PCR using specific primers for EP₁, EP₂, EP₃ and EP₄, respectively. Lanes 2, 5, 8 and 11 are the products obtained from BCE RNA after PCR using specific primers for EP₁, EP₂, EP₃ and EP₄, respectively. Lanes 3, 6, 9 and 12 are negative controls (no added template). Standards (S) are λ-phage and φX174 DNA cut with HindIII and HaeIII, respectively, and are shown in kilobase pair (kb) units.
5.3.5 Stimulation of Inositol Phosphate Formation in Primary Cultures of Bovine Ciliary Epithelial Cells

The presence of a functional inositol phosphate second messenger pathway has been demonstrated in cultured human nonpigmented ciliary epithelium (CROOK, R. B., 1992). Activation of the EP₁ prostanoid receptor by agonists is known to produce intracellular signaling through the increase in phosphoinositide hydrolysis (BHATTACHERJEE, P., 1996). We examine the ability of the endogenous ligand PGE₂ to stimulate phosphoinositide turnover in cultured bovine ciliary epithelial cells. PGE₂ produced a dose-dependent increase in [³H]-inositol phosphate formation (figure 5.8) with an EC₅₀ value of approximately 1.7 μM. The stimulation of [³H]-inositol phosphate formation by PGE₂ was statistically significant compared to basal at each concentration examined (Student’s t-test, p < 0.05). These data are consistent with the expression of an inositol phosphate stimulatory EP₁ receptor in BCE cells.

5.3.6 Effects of Prostanoid Agonists on Intracellular cAMP Levels in Cultured Bovine Ciliary Epithelial Cells

The affect of prostanoid agonists on the formation of cAMP in cultured bovine ciliary epithelial cells was examined, and as shown in figure 5.9, PGE₂ produced a dose-dependent increase in cAMP accumulation with an EC₅₀ of approximately 1 μM. The data represents the mean from three separate experiments each done in quadruplicate. The cells used for the analysis of cAMP accumulation were obtained from separate
Figure 5.8: Stimulation of [$^3$H]-inositol phosphate formation by PGE$_2$ in cultured BCE cells. Incubation and assay conditions are described in “Materials and Methods”. Final concentrations of PGE$_2$ are indicated. Data represents the mean of three separate experiments, each performed in quadruplicate. The asterisks indicate a significant difference from basal (Student’s t-test, p<0.05).
Figure 5.9: Stimulation of cAMP formation in primary cultures of BCE cells. Final concentrations of PGE$_2$ are indicated. Data represents the mean of three separate experiments done in quadruplicate. The asterisks indicate significance over basal (Student’s t-test p<0.05). Forskolin (5 µM) was a positive control.
preparations of BCE cells and were performed at the same passages as those used for the inositol phosphate assays. Application of PGE$_2$ resulted in a dose-dependent stimulation of cAMP. The stimulation of cAMP formation was statistically significant above basal for each of the PGE$_2$ concentrations (Student’s t-test, $p< 0.05$). Forskolin (5 $\mu$M) was applied as a positive control to insure that a functional adenylyl cyclase system was present in the cells. These data are consistent with the presence of a EP$_2$ and/or EP$_4$ adenylyl cyclase stimulatory receptors.

The immunofluorescent data presented in figures 5.5 and 5.6 indicated the presence of a EP$_3$ prostanoid receptor subtype, therefore, we examined BCE cells in culture for a functional EP$_3$ prostanoid receptor response. Activation of the EP$_3$ prostanoid receptor is known to inhibit the activity of adenylyl cyclase in other systems (HASEGAWA, H., 1996). The effect of the EP$_3$ prostanoid receptor agonist sulprostone on cAMP accumulation is shown in figure 5.10. In order to determine the potential inhibitory action of sulprostone on cAMP accumulation in cultured ciliary epithelial cells, the cells were first stimulated with the $\beta$-adrenergic agonist isoproterenol (isoproterenol alone produced a dose-dependent stimulation of cAMP formation with an EC$_{50}$ value of approximately 2 $\mu$M, data not shown). The cells were treated with 2 $\mu$M of the isoproterenol for 5 minutes prior to the addition of sulprostone and was present throughout the experiment. As shown in figure 5.10, sulprostone produced a dose-dependent attenuation of isoproterenol-stimulated cAMP formation. Each value is
**Figure 5.10**: Sulprostone inhibits isoproterenol-stimulated cAMP formation in primary cultures of BCE cells. Isoproterenol (2 μM) was used at a concentration that would give a half-maximal response, final concentrations of sulprostone are indicated. Data represent the mean of three separate experiments done in quadruplicate. The asterisks indicate a significance difference as compared with isoproterenol alone (Student’s t-test, p<0.05).
Figure 5.11: Pertussis toxin attenuates sulprostone inhibition of isoproterenol-stimulated cAMP formation in BCE cells. The attenuation of sulprostone by pertussis toxin indicates involvement of a G_i/G_o mediated pathway. Cells were pretreated for four hours with pertussis toxin 100 ng/ml and then treated with a half-maximal concentration of isoproterenol (2 μM) with or without increasing concentrations of sulprostone. Data represents the mean of three separate experiments done in quadruplicate.
expressed as the mean of three separate experiments done in quadruplicate. Significant
difference from control was observed at the three highest doses of sulprostone (p < 0.05).
The data confirm that a functional EP₃ subtype receptor is present and also demonstrate
that there exists a β-adrenergic cAMP stimulatory system in primary cultures of BCE
cells. To determine whether the modulation of cAMP by sulprostone was mediated
through a Gₛ/Go-coupled pathway (COLEMAN, R. A., 1994), a similar set of
experiments was performed after pretreatment with pertussis toxin and the results are
shown in figure 5.11. Pertussis toxin is known to ADP-ribosylate and inactivate the
inhibitory G-proteins (FIELDS, T.A., 1997). Pertussis toxin pretreatment (4 hours with
100 ng/ml) appeared to inhibit the actions of sulprostone on isoproterenol-stimulated
cAMP in cultured bovine ciliary epithelial cells, at least at the higher concentrations of
sulprostone. This data provides additional support for the presence of a functional EP₃
prostanoid subtype receptor in bovine ciliary epithelial cells.

5.4 Discussion

Studies utilizing radioligand binding and functional assays have identified various
prostanoid receptor subtypes in several regions of the mammalian eyes, including
humans. Some of the prostanoid receptors identified within the iris-ciliary body or in
cultured SV40 transformed cell lines include EP₁, EP₂, DP, TP and FP
(BHATTACHERJEE, P., 1990; BHATTACHERJEE, P., 1996; CSUKAS, S., 1993;
JUMBLATT, M. M., 1994). These studies did not report, however, on the presence of
EP₃ receptors in the ciliary body nor were they able to distinguish as to whether the EP₂ and the EP₄ receptor subtypes were actually responsible for the cyclase stimulatory effects of PGE₂. In the present study, immunohistochemistry and PCR were used to identify the presence of EP₁, EP₂, EP₃, and EP₄ receptor subtypes in frozen thin sections of bovine ciliary epithelial process and EP₁, EP₃, and EP₄ in primary cultures of BCE cells. It was also determined that there is a functional modulation of intracellular cAMP formation from the activation of EP₃ and EP₂ and/or EP₄ receptors.

The physiological significance of this diversity of EP prostanoid receptors in the ciliary process and its significance as it concerns glaucoma, remains to be established. It is generally acknowledged that prostanoid receptors couple to adenylyl cyclase in the iris-ciliary body (BHATTACHERJEE, P., 1993) and that cAMP formation is involved to some extent in the hypotensive actions of the adrenergic and prostaglandin compounds (CAPRIOLI, J., 1984; GREGORY, D., 1981; NEUFELD, A. H., 1975). The involvement of cAMP in the cellular function of the ciliary epithelium indicates that intracellular signaling by adenylyl cyclase participates in the regulation of transepithelial transport systems involved in the formation of aqueous humor (DELMER, N. A., 1992; MITTAG, T. W., 1986; SEARS, M. L., 1985).

A concern with investigations using functional data obtained from primary isolated cells is the extrapolation of what is observed in cultured cells to the intact tissue. The immunoreactivity observed with the aquaporin-1 labeling was consistent with what had been reported previously in humans (STAMER, W. D., 1994a). The labeling was localized to only the NPE cells in tissue sections of bovine ciliary epithelium, and was
observed on a subset of cells in primary culture. It is generally acknowledged that cells of the ciliary epithelium express functional $\alpha_2$-adrenergic receptors (HUANG, Y., 1995; JIN, Y., 1994; SERLE, J.B., 1991). In this study, the expression of the $\alpha_2$-adrenergic receptors was confirmed by immunofluorescent microscopy. The adrenergic receptor labeling obtained in the primary cultures of BCE cells was observed to the $\alpha_2$-A and the $\alpha_2$-B receptor protein, but not to $\alpha_2$-C receptor protein. We also examined the tight-junction (zonal occludens) membrane protein in both tissue sections and primary cultures. This protein has been previously characterized and shown to be expressed exclusively on the nonpigmented ciliary epithelial cells (STEVenson, B. R., 1986). The labeling observed in bovine tissue sections was consistent with the expression of the tight-junction protein. Expression of the tight-junction protein was also observed in primary cultures of BCE cells. All of the immunofluorescent data suggest that bovine ciliary epithelial cells express membrane proteins similar to what is seen in other mammalian species including humans and that they maintain the phenotypical expression of these proteins in primary culture.

Functional evidence for the presence of an inositol phosphate second messenger pathway in ciliary epithelial cells exits (CROOK, R. B., 1992). Stimulation of the cultured BCE cells with PGE$_2$ resulted in a dose-dependent inositol phosphate formation consistent with the presence of an EP$_1$ receptor. Another prostanoid receptor that is also coupled to the IP second messenger pathway is the FP prostanoid receptor. Using subtype specific antibodies (ANTHONY, T. L., 1998) we examined the expression of FP prostanoid receptors in primary cultures of BCE cells. Under no circumstances was
immunoreactivity detected in BCE cells (data not shown). Our findings do support the presence of a functional adenylate cyclase system in the ciliary epithelium. PGE₂ can stimulate the formation of cAMP (EC₅₀ 1 μM) in bovine ciliary epithelial cells (figure 5.9). The EC₅₀ value reported for the stimulation of cAMP in the BCE cells was within the range of values previously reported for both human and rabbit ciliary epithelium (BHATTACHERJEE, P., 1996; JUMBLATT, M. M., 1994). Forskolin was also a potent activator of the adenylate cyclase system in the BCE cells (figure 5.9).

In determining the potential cAMP-inhibitory actions of the EP₃ prostanoid receptor subtypes, we examined the effects of the selective EP₁/EP₃ agonist, sulprostone, on isoproterenol-stimulated cAMP formation in cultured BCE cells. From our results, we conclude that the activation of the EP₃ receptor subtype in the BCE cells attenuates cAMP formation through the activation of a pertussis toxin sensitive G-protein. Activation of EP₃ receptors has been suggested to reduce intraocular pressure in rabbits (WATERBURY, L. D., 1990). The data presented suggests that the regulation of IOP by prostaglandins is likely to involve activation of cAMP messenger pathways. In a majority of the experiments performed, the predominant effect of the prostaglandin agonist appears to be on the adenylyl cyclase stimulatory receptors (EP₂/EP₄).
CHAPTER SIX
Summary and Future Directions

6.1 Summary

It is well established that topical application of prostaglandins and prostaglandin analogs produce significant and substantial ocular hypotensive responses. The exact mechanism of action and tissue distribution of the individual prostanoid receptor subtypes that are responsible for there affects on IOP is an area of active research. The experimental results presented in these studies provide a basis for understanding the involvement of the prostanoid receptor subtypes in the anterior segment of the mammalian eye. With the use of molecular biological techniques subtype specific antibodies were generated which enabled us to localize individual prostanoid receptor subtypes in the cells of the conventional outflow pathway (trabecular meshwork) and in the cells of aqueous humor producing pathway (ciliary epithelium). Understanding the tissue localization of the various prostanoid receptor subtypes in these two regions of the eye provides additional information towards determining their hypotensive effects in the eye.

Recently, the PGF$\text{_{2α}}$ analog latanoprost was demonstrated to significantly lower intraocular pressure in humans. It is now being used clinically in the treatment of glaucoma. Increased uveoscleral outflow is the proposed pharmacological effect of latanoprost. However, in chapter four, we provide evidence for the expression of the FP prostanoid receptor subtype in the human trabecular meshwork cells. Activation of this
receptor resulted in the dose-dependent production of inositol phosphate formation and an increase in intracellular calcium. The morphological characteristics of trabecular meshwork cells indicate the presence of contractile filaments similar to those in smooth muscle. We speculate that the activation of FP prostanoid receptors in the human trabecular meshwork could modify the trabecular meshwork cells through a contractile event and influence flow through the ciliary muscle as suggested by Wiederholt (1995). This data does not dispute the current opinion on the action of latanoprost, however, does provide an alternative explanation for the ocular hypotensive actions of latanoprost.

The functional significance and cellular distribution of the EP prostanoid receptor subtypes in the ciliary epithelium is slowly being elucidated. Using tissue samples obtained from bovine eyes, we have examined the cellular distribution of the EP₁, EP₂, EP₃ and EP₄ receptor subtypes using selective antibodies and functional studies. The cellular localization of the EP prostanoid receptor subtypes in tissue sections versus cells in primary culture was presented in chapter five. From the immunofluorescent data, we demonstrate that the cells of the ciliary epithelium express all four of the EP prostanoid receptor subtypes in tissue sections and EP₁, EP₃ and EP₄ prostanoid receptor subtypes were also shown to be present in primary cultures of these cells. The functional studies provide additional support for the receptor activation of cAMP formation through the stimulation of EP₂/EP₄ receptors. Additionally, we demonstrated that PGE₂ dose-dependently stimulated the formation of inositol phosphate in cultured epithelial cells of the bovine eye. The data also provide additional evidence for the inhibition of cAMP formation through a G₁/G₆-coupled EP₃ prostanoid receptor subtype. However, the
predominate effect of PGE$_2$ in the ciliary epithelium appears to be on the cAMP stimulatory receptor subtypes.

6.2 Future Studies

The presence of the FP prostanoid receptor subtype in trabecular meshwork cells suggests an alternative site of action for the PGF$_{2\alpha}$ analog latanoprost. One hypothesis as it concerns the presence of FP prostanoid receptor expression in the trabecular meshwork is the possibility that activation of FP receptors modulates uveoscleral outflow by contraction of the trabecular meshwork cells and a resulting effect on the ciliary muscle to increase uveoscleral. Anatomically, the trabecular meshwork is connected to the ciliary smooth muscle through the sclera spur. Contraction of the trabecular meshwork could result in an increase in the intracellular spaces between the smooth muscle cells of the ciliary muscle; thus, providing for the observed increase in uveoscleral outflow produced by latanoprost. Examination of the distribution of the FP prostanoid receptors other ocular tissues such as the ciliary muscle, are currently under investigation in our laboratory.

The inhibitory actions of sulprostone on cAMP formation in the ciliary epithelium cells provides a basis for additional studies. cAMP formation has been shown to be involved in the regulation of aqueous humor production and the ability of EP receptor activation to modulate the levels of intracellular cAMP in the ciliary epithelium, provides an interesting new area of study. Investigations in this area may lead to a better
understanding of the role of cAMP in aqueous humor production and to more selective compounds for the treatment of glaucoma.
REFERENCES


