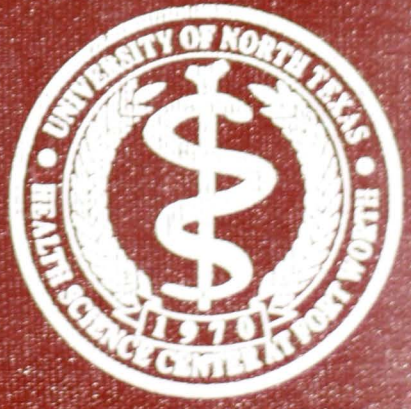
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Landry, Theresa A., Characterization of the Serotonin Receptors in the Long Posterior Ciliary Artery of the Bovine Eye. Doctor of Philosophy (Biomedical Science), August 2000, 114 pp., 5 tables, 29 illustrations, bibliography, 104 titles.

Vascular disease and vasospasm are implicated in the etiology of glaucoma. The long posterior ciliary (LPCA) is the major blood supply for the ciliary body including the ciliary processes that produce aqueous humor. Information about the pharmacological control of this vessel would be helpful in understanding its normal and pathologic function. Serotonin (5-HT) is a neurotransmitter that effectively constricts the LPCA. The objective of this research is to identify the serotonin receptor subtype responsible for the 5-HT induced vasoconstriction of the LPCA and to characterize the cellular mechanisms that mediate that contraction.

Ring segments of the LPCA were dissected from bovine eyes and mounted on tungsten triangles attached to a force transducer. Changes in vascular tension were measured and recorded using a physiograph recorder.

Dose response curves with 5-HT, 5-HT $_{1\text{-like}}$ agonist, 5-CT and 5-HT$_2$ agonist, α-methyl-5-HT, indicate that the 5-HT$_{1\text{-like}}$ receptor contributed about 15.13% to the contraction and the 5-HT$_2$ receptor contributed 61.61%. The EC_{50} for the three agonists were 283 nM (5-HT), 336 nM (5-CT), and 1.7 μM (α-methyl-5-HT). Inhibition curves with selective antagonists indicate that the IC_{50} is < 1 nM for both methiothepin (5-HT $_{1\text{-like}}$ antagonist) and ketanserin (5-HT$_2$ antagonist). Following incubation of the rings with diltiazem 10 μM or nifedipine 10 μM, the response to 5-HT was reduced 65.8% and 61.7%, respectively. Incubation in calcium free PB produced similar results. Ryanodine inhibited the 5-HT contraction by 58.1% and caffeine inhibited the response 100%. PKC inhibitors bisindolylmaleimide II 1 μM, bisindolylmaleimide II 10 μM, chelerythrine 25 μM and H-7 5 μM decreased the 5-HT response by 19.8%, 55.7%, 31.1% and 61.5%, respectively. Incubation of the ring segments with one of three PLC antagonists, 2-NCDC 70 μM, U73122 0.5 μM, or neomycin 5 mM, prior to the addition of 1 μM serotonin, significantly reduced the contraction of each vessel, $p<0.0001$.

The 5-HT-induced vasoconstriction of the LPCA of the bovine eye is mediated through activation of both 5-HT_2 and 5-$HT_{1\text{-like}}$ receptors. The contraction is dependent on the mobilization of calcium and is mediated in part through PLC activated intracellular calcium release from IP_3 sensitive stores.

CHARACTERIZATION OF THE SEROTONIN RECEPTORS IN THE LONG POSTERIOR CILIARY ARTERY OF THE BOVINE EYE

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CHARACTERIZATION OF THE SEROTONIN RECEPTORS IN THE LONG POSTERIOR CILIARY ARTERY OF THE BOVINE EYE

Dissertation

Presented to the Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

By

Theresa A. Landry, B.S.

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For Ted, Chris, Cara, Mom, and Dad

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Statement of the Problem

Glaucoma is not a single disease but a group of optic neuropathies that are characterized by nerve fiber layer defects, optic disc changes and visual field loss. There are still many questions concerning the pathogenesis of the disease. There is evidence of a mechanical component that would correlate the elevation of intraocular pressure with both the damage to the axons of the optic nerve and the reduced perfusion in the optic nerve head. There is further evidence that implicates a vascular component to glaucoma since there is a high prevalence of vasospastic disorders and systemic orthostatic hypotension in glaucoma patients, especially in individuals diagnosed with normal tension glaucoma (Flammer *et al.*, 1999). Clinically, the progression of the disease is monitored through intraocular pressure measurements, visual field evaluations and optic nerve assessments. In most cases, progressive glaucomatous changes are associated with elevated intraocular pressure. Therefore, the focus of current pharmacological and surgical therapies is to reduce intraocular pressure by either decreasing the production of aqueous humor or by increasing the outflow facility.

Serotonin is a neurotransmitter that produces vasoconstriction in some vascular beds. It has been determined that serotonin effectively constricts the long posterior ciliary artery which provides the major blood supply for the ciliary body including the ciliary processes that produce aqueous humor. This artery further branches to supply blood to the posterior part of the eye. A full characterization of the serotonin-induced vasoconstriction of the long posterior ciliary artery

could provide valuable information concerning the role that serotonin receptors play in the long, posterior ciliary artery.

Therefore, the objective of this research was to identify the serotonin receptor subtype responsible for the 5-HT induced vasoconstriction of the long posterior ciliary artery and to characterize the cellular mechanisms that mediate that contraction.

Discussion of the Disease

Glaucoma affects more than 67 million people worldwide and is the leading cause of preventable blindness. It is estimated that as many as 50% of the people with the disease, do not even know that they have it (Quigley, 1996). Glaucoma induced blindness is permanent and irreversible. At the time of Hippocrates, there was no clear differentiation between cataract and glaucoma. Both terms referred to blinding diseases. In time, the term cataract came to be used for curable blindness and glaucoma came to be used in reference to incurable blindness. The word *glaucoma* came from *glaukos* meaning green pupil or green star. This term was in reference to the shimmering, sea-colored green pupil that was distinctive of the disease (Mackenzie, 1835). Even without sophisticated instruments to measure the pressure inside the eye, clinicians knew that elevated intraocular pressure, as measured by the rigidity of the globe, was an indicator of the disease. With the invention of ophthalmic instrumentation, our understanding of the disease expanded from simply a disease of elevated intraocular pressure to a disease that causes optic nerve damage and visual field loss. For almost 300 years, scientists and clinicians have searched for the cause and for a cure for glaucoma.

Today, we know that there are many risk factors that would predispose an individual to the disease. We know that the disease is more prevalent in people over 40, African-Americans, diabetics, high myopics, and that individuals with a family history of the disease are at greater risk (Lee, 1999). Additionally, there is a high prevalence of systemic hypotension and vasospastic

diathesis among glaucoma patients. Only by understanding the pathogenesis of the disease, will it be possible to develop better therapies and in time a cure for glaucoma.

Treatment Options

Although the clinical progression of the disease can not be definitively ascribed to the elevated intraocular pressure, the efficacy of current therapies is measured by their ability to lower the intraocular pressure. Topical, ocular medication is usually the first line treatment prescribed. In more advanced stages of the disease or if intraocular pressure is not sufficiently reduced, more aggressive treatment options are employed which can include the addition of systemic medication, laser therapy or surgery. Numerous pharmacological classes of drugs have been researched for their hypotensive actions in the treatment of glaucoma. The pharmacological classes of topical compounds currently approved by the FDA to lower intraocular pressure include: 1) β-adrenergic antagonists, 2) adrenergic agonists, 3) cholinergic agonists, 4) carbonic anhydrase inhibitors, and 5) prostaglandins (Eid and Spaeth, 1999).

Table 1
Pharmacological Agents Prescribed To Treat Glaucoma

Pharmacological Drug Class	Examples of FDA Approved Medications	Mechanism of Action
β-adrenergic antagonists	Betoptic-S Ocupress Timoptic	↓ Production of aqueous humor
Non-selective adrenergic agonists	Epinephrine Dipivefrin	↑ Outflow through trabecular meshwork
α–adrenergic agonists	Alphagan* Iopidine	↑ Outflow through trabecular meshwork
Cholinergic agonists	Carbachol Ocuserts Pilocarpine	↑ Outflow through trabecular meshwork
Carbonic anhydrase inhibitors	Azopt Trusopt Diamox	↓ Production of aqueous humor
Prostaglandin	Xalatan	↑ Uveoscleral Outflow
Combination products (CAI inhibitor/β-blocker)	Cosopt	↓ Production of aqueous humor

Alphagan may also increase uveoscleral outflow.

These medications reduce intraocular pressure by either decreasing the production of aqueous humor or by increasing the outflow facility. The β-adrenergic antagonists and carbonic anhydrase inhibitors decrease the production of aqueous humor and the cholinergics, sympathomimetics and prostaglandins increase the outflow.

Historically, glaucoma is thought to be a disease caused by an impairment of the outflow facility as opposed to a pathological increase in aqueous humor formation. Current therapeutic agents are developed to reduce intraocular pressure to a level that will arrest progressive optic nerve damage.

Anatomy of the Eye

The eye is a hollow, spherical structure about 2.5 cm in diameter. The spaces within the eye are filled with fluids that provide support for the internal parts of the eye and help to maintain its shape. The anterior, transparent portion of the eye bulges forward and is called the *cornea* (see Figure 1, page 7). The cornea helps to focus light rays and serves as the window of the eye. While the cornea contains no blood vessels, it is well supplied with nerve fibers. These fibers are associated with numerous pain receptors that have a very low threshold for pain.

Along the circumference, the cornea is continuous with the sclera or the opaque, white portion of the eye. The sclera provides protection for the eye and is white in color due to the presence of many collagenous and elastic fibers. The sclera is pierced in the back of the eye by the optic nerve and a collection of blood vessels. The middle layer of the eye is a vascular tunic and is called the *uveal layer*. The uveal layer includes the choroid, ciliary body and the iris. The choroid is loosely joined to the sclera and is honeycombed with blood vessels that provide nourishment to the surrounding tissues.

The ciliary body extends forward from the choroid and forms an internal ring around the eye. Within the ciliary body there are many radiating folds called the *ciliary processes* and two distinct groups of muscle fibers that comprise the ciliary muscles. The transparent lens is held in position by suspensory ligaments or zonules that attach to the ciliary processes. When the tension on these ligaments is relaxed, the lens changes shape to focus the eye. The relaxation of the suspensory ligaments during accomodation is a function of the ciliary muscles.

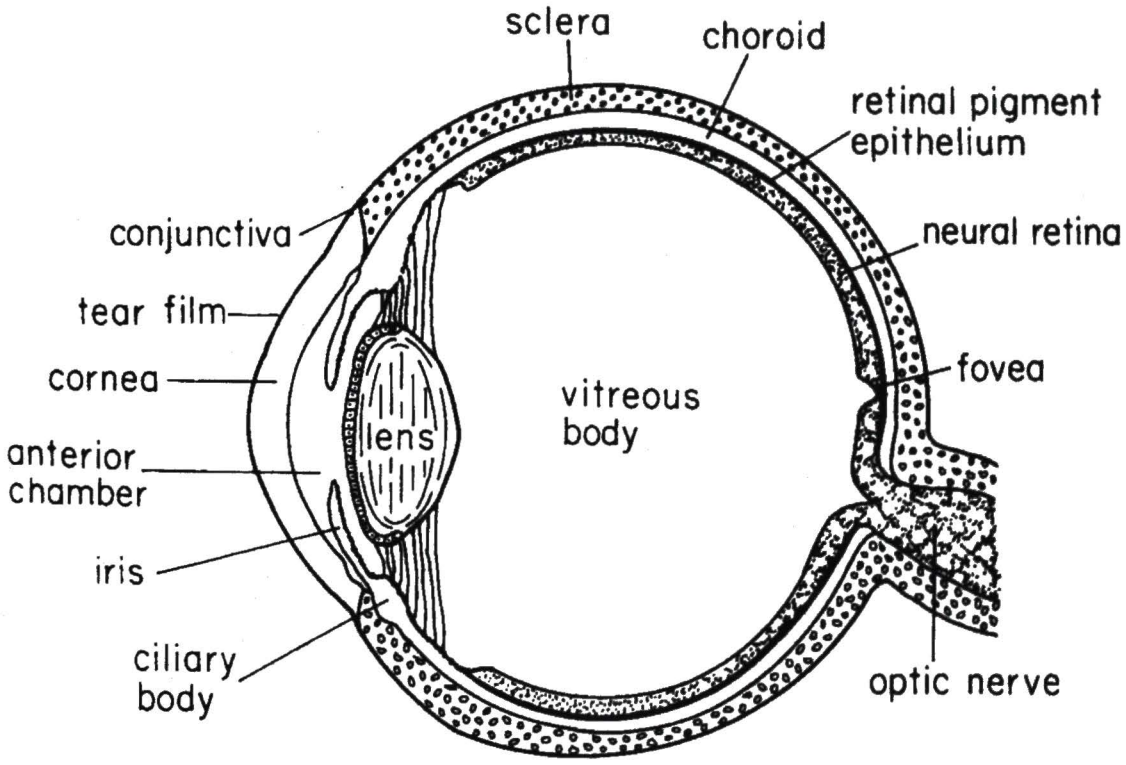
The iris is the thin muscular diaphragm that is the colored portion of the eye. It extends forward from the periphery of the ciliary body and lies between the cornea and the lens. The iris divides this part of the eye into the anterior and posterior chambers. There are two groups of

smooth muscle fibers in the iris, a circular set and a radial set. These muscles function to control the size of the pupil to regulate the amount of light entering the eye.

The inner layer of the back of the eye is the retina and it contains the receptor cells sensitive to light signals. The central region of the retina is called the *fovea centralis* and it is the area associated with the sharpest vision. Just medial to the fovea is the optic disc. Here the nerve fibers from the retina leave the eye and join to form the optic nerve. The compartment bounded by the lens, the ciliary body and the retina is filled with a clear, jelly-like fluid called *vitreous humor.*

Light waves entering the eye, pass through the cornea, the aqueous humor, the lens, the vitreous humor and several layers of cells in the retina to reach visual receptor cells. When the receptor cells are stimulated, impulses travel from the cell to the brain via the optic nerve. It is at this point that an image is perceived by the brain (Guyton, 1991).

Figure 1
Anatomy of the Eye



Vascular Supply to the Ciliary Body

In the human eye, ocular blood vessels are derived from the ophthalmic artery, which branches into the central retinal artery, two or three posterior ciliary arteries and several anterior arteries (Figure 2, page 9). The long posterior ciliary arteries enters the globe near the optic nerve and run forward to the ciliary body where they join with the anterior ciliary arteries to form the major arterial circle.

Figure 2
Vascular Supply to the Eye

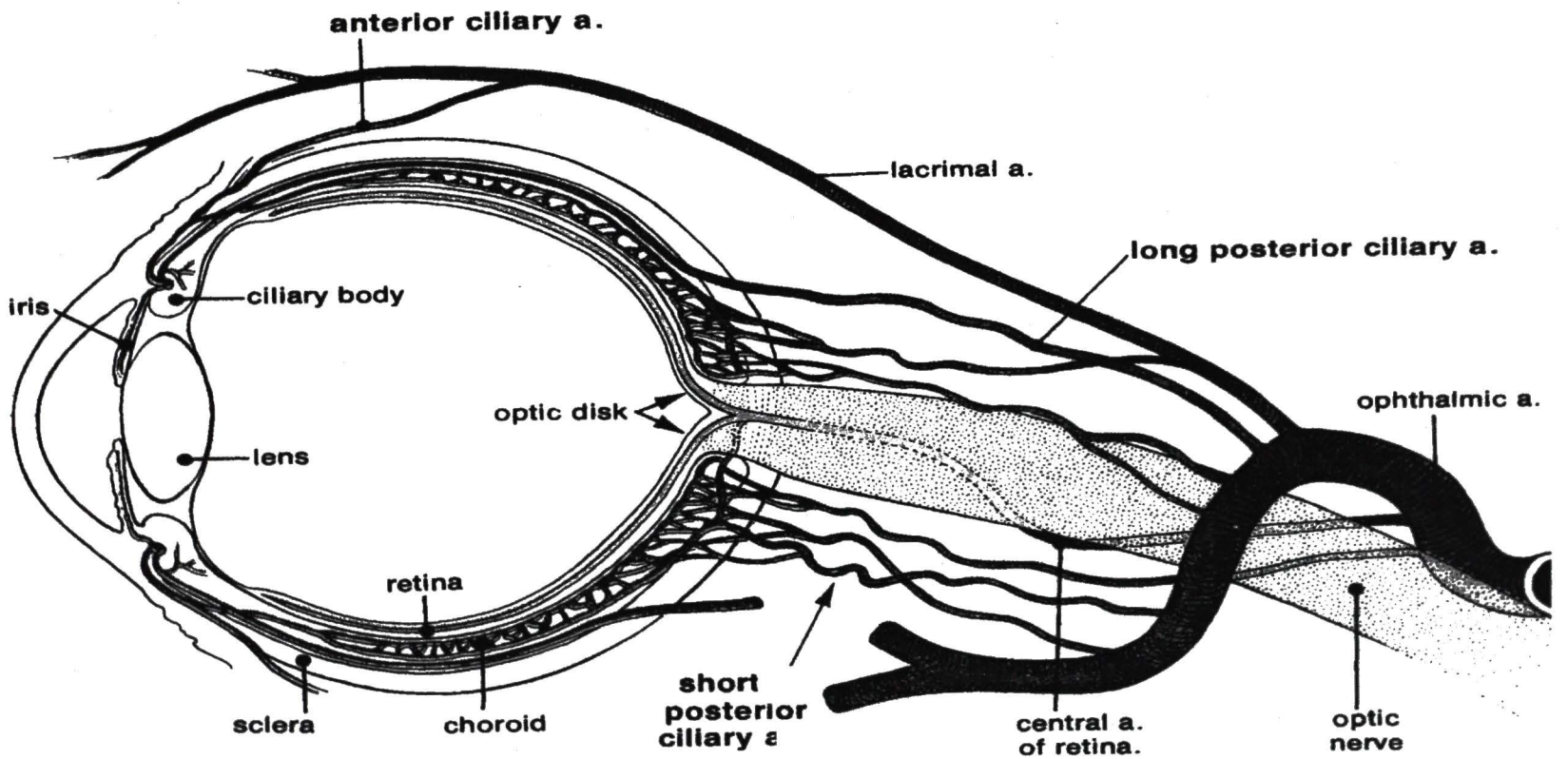
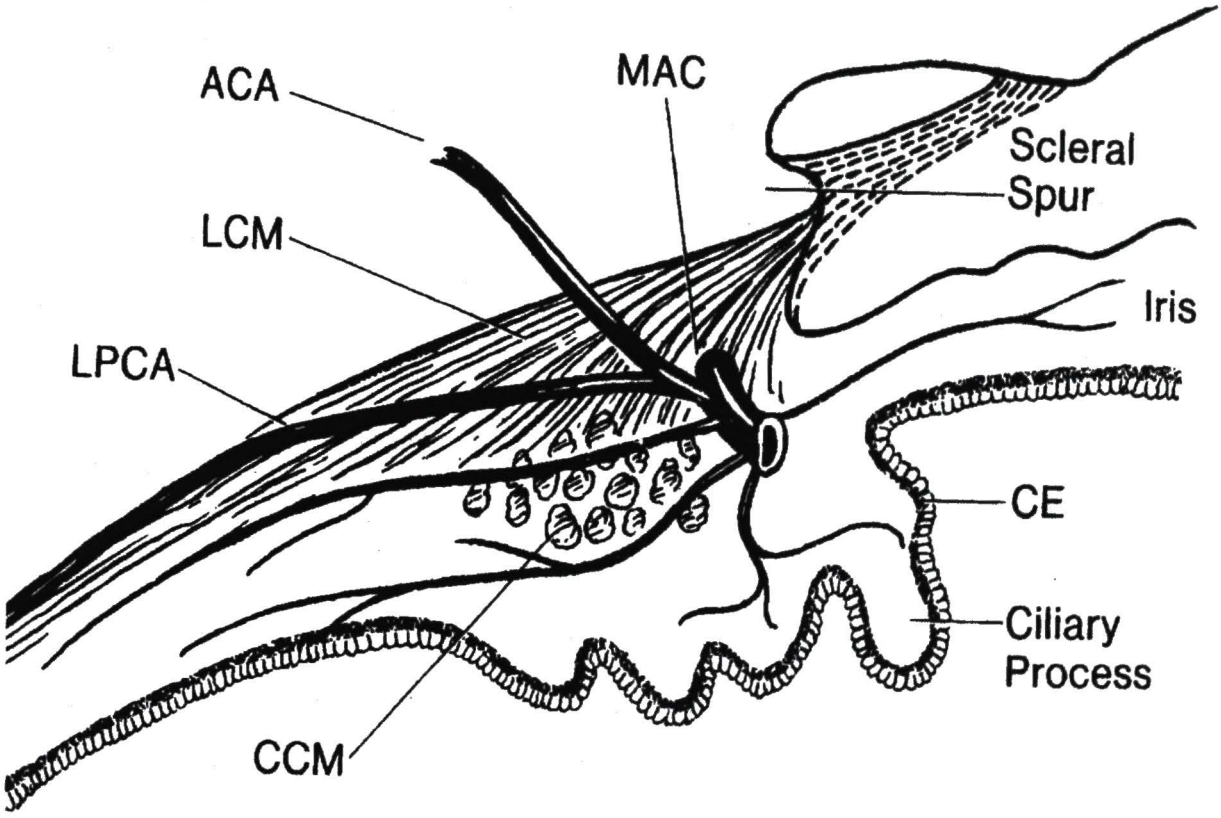


Diagram of the eye with the extraocular vasculature. The long posterior ciliary arteries branch from the ophthalmic artery and run forward to join the anterior ciliary arteries to form the major arterial circle supplying blood to the ciliary body. (Reprinted from Melloni *Illustrated Medical Dictionary*, 1979).

Branches of the major arterial circle provide the blood supply to the iris and ciliary processes (Woodlief, 1980). Each ciliary process is perfused by a precapillary arteriole branching from the major arterial circle. Sphincters are present on these arterioles which may play a role in the regulation of blood supply, aqueous humor formation and intraocular pressure. The precapillary arterioles further branch to form a plexus within each process. These capillaries have a fenestrated endothelium and are very leaky. Although a slight reduction in blood flow to the ciliary process may not have significant effect on the rate of aqueous humor production, a profound contraction of the vessels supplying blood to the ciliary processes may result in a decrease in aqueous humor formation and a decrease in intraocular pressure.

Figure 3
Vascular Supply to the Ciliary Body



Three major components of the ciliary body: 1) the ciliary muscle , composed of longitudinal (LCM) and circular (CCM) fibers, 2) the vascular system formed by branches of the anterior ciliary arteries (ACA) and the long posterior ciliary arteries (LPCA) which form the major arterial circle (MAC), 3) the ciliary epithelium (CE), composed of an outer pigmented and an inner nonpigmented layer. (Reprinted from Shields's *Textbook of Glaucoma*, 1992).

Vascular Supply to the Optic Nerve Head

The ciliary arteries and the central retinal artery are also branches of the ophthalmic artery and provide the blood supply for the posterior portion of the eye. The main central retinal artery provides branches to the optic nerve as far forward as the lamina cribrosa and then forms the vascular network in the inner layers of the retina. To some extent, this retinal circulation also supplies the most superficial layer of the optic nerve head. The prelaminar region of the optic nerve head as well as the area of the lamina cribrosa is principally supplied by branches from the large choroidal arteries and to a lesser extent, by branches arising directly from the short posterior ciliary arteries. In some eyes, the temporal part of the inner-most layer of the optic nerve head is also supplied by branches from the posterior ciliary arteries. The region immediately behind the lamina is supplied by vessels from the peripheral pial plexus, which in turn is supplied by branches from the posterior ciliary arteries. The vessels of the optic nerve and the optic nerve head have tight junctions and are not fenestrated. On the other hand, choroidal capillaries are fenestrated and allow passage of low molecular weight substances. The retinal pigment epithelium prevents the passage of these substances into the retina. However, there is no barrier between the choroid and the optic nerve head. Therefore, vasoactive substances can pass from the choroid into the optic nerve head and may under some conditions, provoke vasoconstriction. There is no innervation of the vessels of the retina and the optic nerve head, although receptors for α and β–adrenergic and cholinergic agents as well as for angiotensin are present (Anderson and Braverman, 1976; Hayreh, 1978). Autoregulation keeps local perfusion pressure constant and adapts to local metabolic needs.

Serotonin is an autocoid that is stored in platelets and can be released to potentiate local vascular contraction. Serotonin has been shown of effectively constrict the long posterior ciliary artery in a dose dependent manner. A characterization of the cellular mechanisms mediating the

serotonin-induced contraction could be useful in understanding the function and role of the serotonin in this vascular bed.

Aqueous Humor Dynamics

The aqueous humor is a clear, watery, cell-free, protein-free fluid that fills the anterior chamber and part of the posterior chamber of the eye. It has a pH varying from 7.1 to 7.3 and total volume of 125 μl (Vaughn and Asbury, 1986). With the exception of ascorbate (vitamin C) which is present in higher concentrations, the composition of aqueous humor is very similar to protein-free plasma. Aqueous humor brings nutrients such as glucose and oxygen to the avascular tissues of the eye such as the cornea, the lens and the trabecular meshwork. Aqueous humor also removes metabolites such as lactic acid and CO_2 from these same tissues.

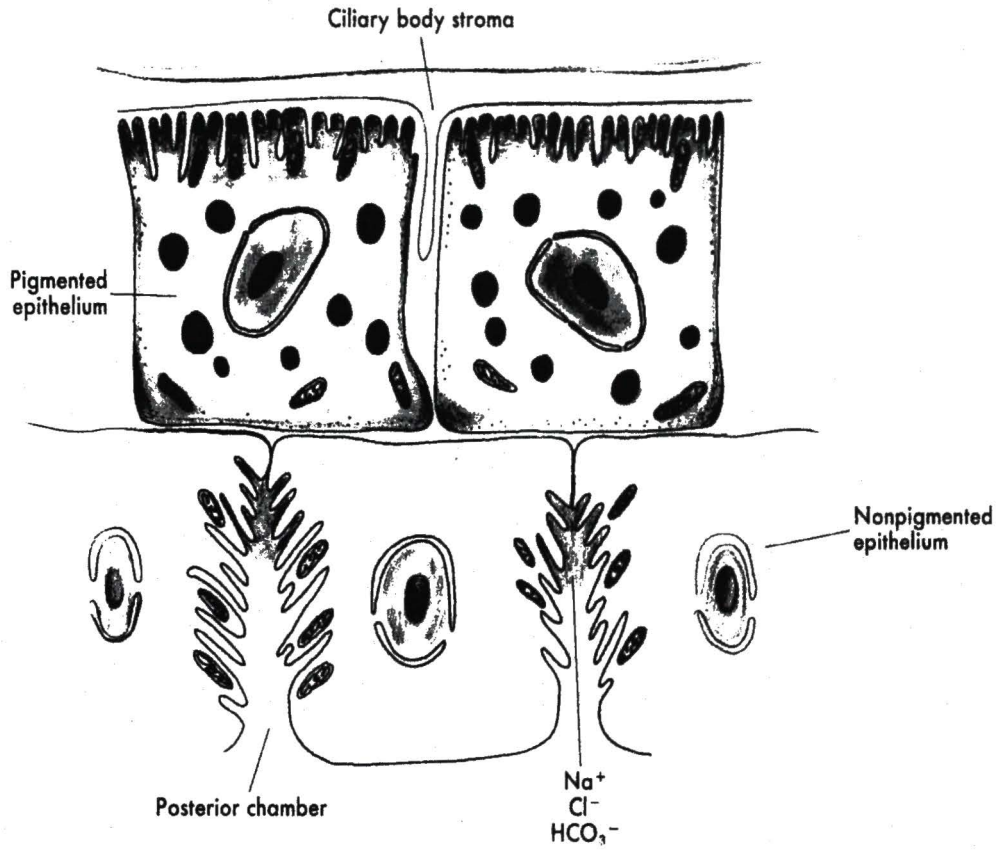
Aqueous humor provides a continuous supply of nutrients to all of the tissues it perfuses and is necessary to maintain normal tissue function. The formation of aqueous humor involves three processes that occur in series: blood flow to the ciliary process, ultrafiltration, and active secretion by ciliary epithelial cells.

The ciliary body is composed of the ciliary muscle, vascular tissue and the ciliary epithelium. Ciliary epithelial cells line the linear folds that project from the ciliary body into the space behind the iris. A bilayer of these cells comprise the ciliary processes and they actively secrete aqueous humor. The ciliary processes are covered by a double layer of epithelial cells. The nonpigmented epithelium (NPE) lies adjacent to the posterior chamber and the pigmented layer rest on the stroma. It has been estimated that each cell must secrete a volume of aqueous humor per minute equal to one third of its own intracellular volume (Brubaker, 1991).

The aqueous humor formed by the ciliary processes circulates around the lens, from the posterior chamber through the pupil and into the angle formed by the iris and the anterior chamber. The fluid exits the eye by flowing through the trabecular meshwork, juxtacanalicular

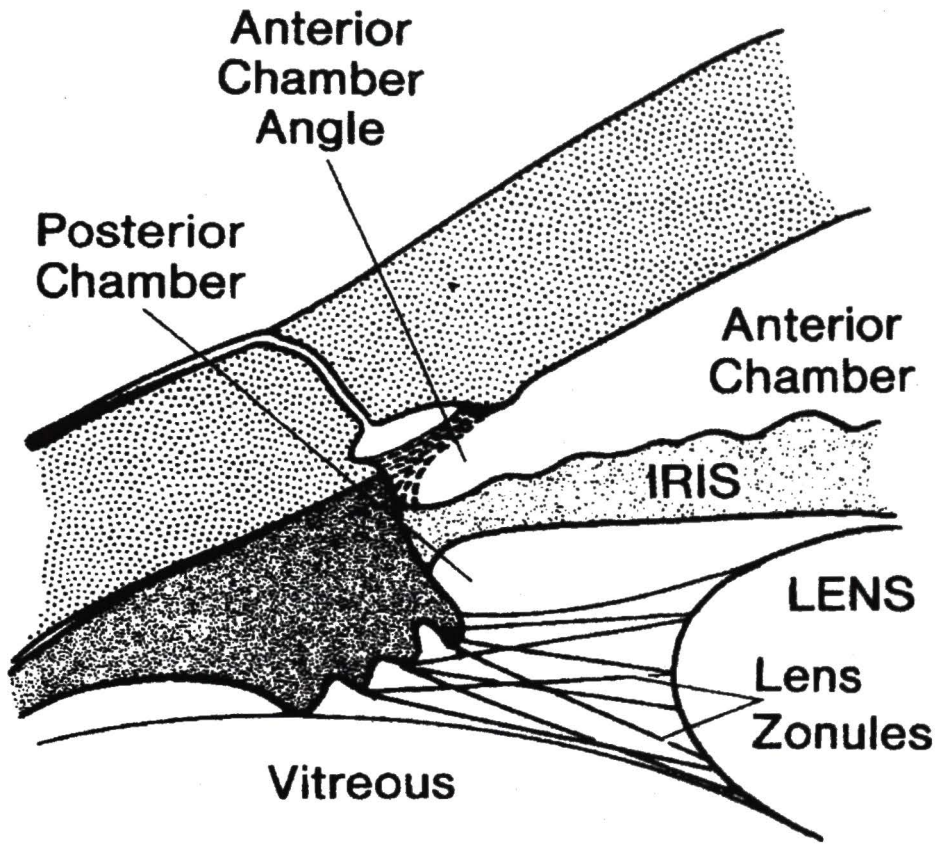
tissue, Schlemmm's canal, collector channels and into the venous system. A smaller fraction of the total aqueous humor leaves in a pressure independent fashion through the uveoscleral outflow. Current estimates are that in the normal eye, uveoscleral flow accounts for less than 10% of the total.

Figure 4
Aqueous Humor Formation



Schematic diagram of nonpigmented and pigmented epithelial cells facing each other apex-to-apex. Ions are secreted into the intercellular clefts of the nonpigmented epithelial cells. The ions create sufficient osmotic force to attract water. Aqueous humor flows from the nonpigmented epithelium into the posterior chamber (Reprinted from Drance's *Glaucoma: Applied Pharmacology in Medical Treatment*, 1984).

Figure 5
Structures Involved in Aqueous Humor Dynamics



Aqueous humor is formed by the ciliary processes. It exits the posterior chamber by flowing between the lens and the iris and through the pupil to the anterior chamber. There it flows into the anterior chamber angle and exits the eye through the trabecular meshwork and into the canal of Schlemm (Reprinted from Shields's *Textbook of Glaucoma*, 1992).

It has been postulated that most of the aqueous humor results from active secretion of ions by the NPE (Cole, 1977).

Part of the plasma exiting through the fenestrated capillaries in the interstitial spaces between the vessels and the ciliary epithelia will move through the uvea to leave the eye by the uvealscleral outflow pathway. The major portion of the filtrate is available to the ciliary epithelia for formation of aqueous humor. The third component of aqueous humor formation involves the steady osmotic gradient maintained in the intercellular channels between the NPE cells. A concentration gradient is created by active secretion of ions into the intercellular channel that forces water to flow into the posterior chamber.

The IOP is determined by the relative rates of production of aqueous humor and its outflow through the trabecular meshwork and the canal of Schlemm. When the rates of inflow and outflow are in equilibrium, the IOP stays relatively constant. Inflow is a consequence of arterial blood pressure and production of aqueous humor, whereas, outflow is dependent on the resistance to outflow and the episcleral venous pressure (Eid and Spaeth, 1999).

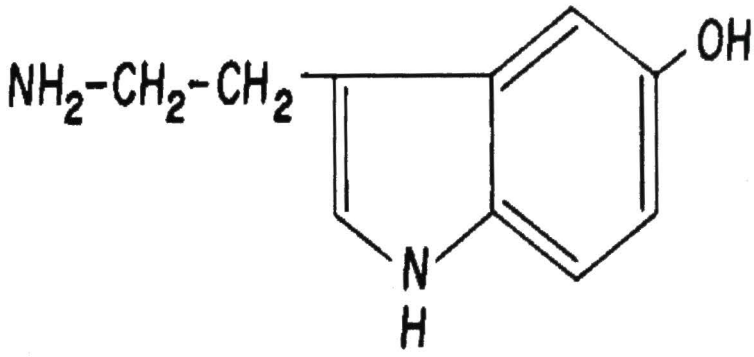
The long posterior ciliary artery, the anterior ciliary artery and the major arterial circle provide the vascular supply to the anterior segment of the eye that includes the ciliary body. The precapillary arterioles divide into tortuous vessels within each ciliary process. The process of ultrafiltration helps to move fluid out of the capillaries into the stroma but is insufficient by itself to account for the amount of fluid moved into the posterior chamber. This latter step requires an active metabolic process that occurs in tandem with the ultrafiltration. Active transport is an energy dependent process that selectively moves a substance across a cell membrane against its electrochemical gradient.

History of Serotonin

Serotonin (5-HT) , 5-hydroxytryptamine, is a neurotransmitter that has received much attention in recent years. It was first identified by Rapport in 1948 as an agent present in serum

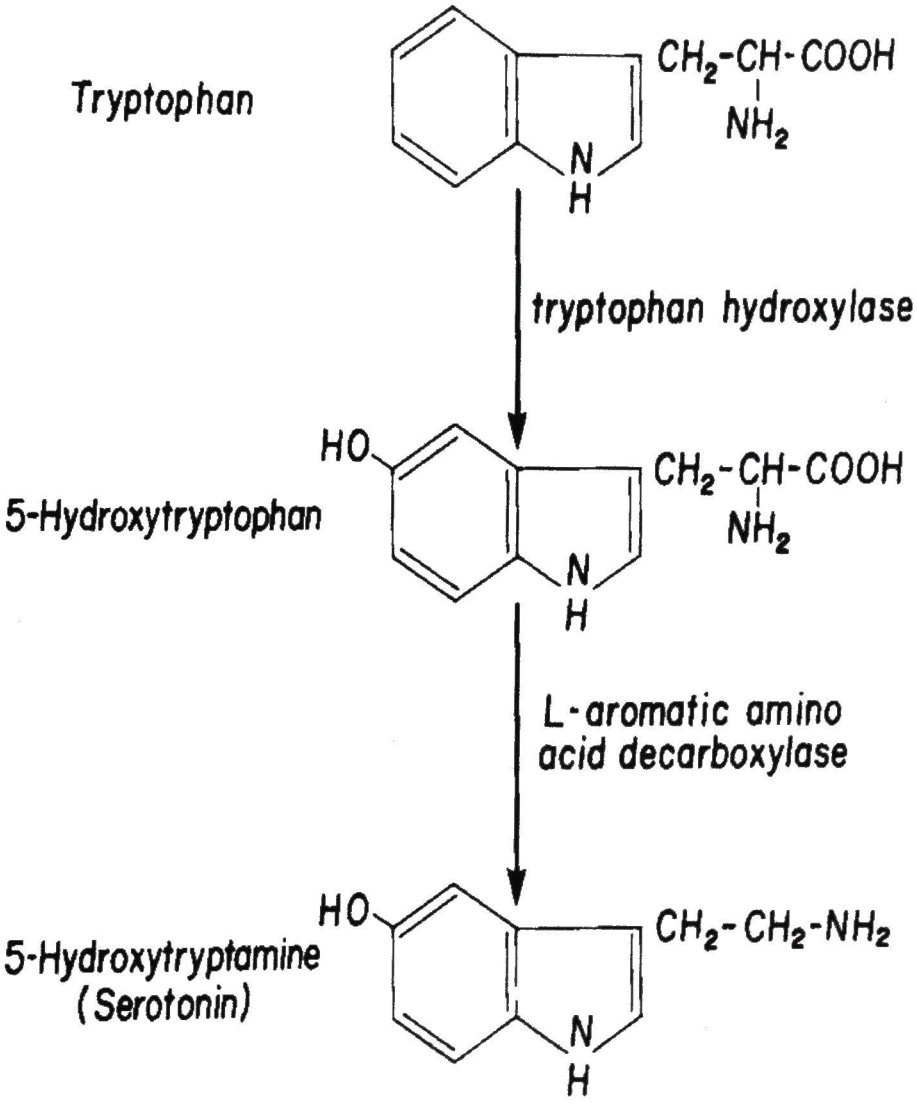
after blood is allowed to clot. Investigators at the Cleveland Clinic crystallized the substance and named it *serotonin* (Rapport, Green, and Page, 1948). Independently, in the 1930s Erspamer and colleagues characterized a substance that imparts peculiar histochemical properties to enterochromaffin cells of the gastrointestinal mucosa. Their experiments isolated a gut-stimulating factor that they named *enteramine*. In 1952, Erspamer and Asero identified enteramine as 5-HT (Erspamer and Testini, 1959).

Figure 6
Chemical Structure of Serotonin



Serotonin is found in high concentrations in enterochromaffin cells of the gastrointestinal tract, in brain tissue and in platelets. Serotonin serves as a chemical transmitter for tryptaminergic neurons in the brain and as a precursor for the pineal hormone melatonin (see Figure 7, page 21). In the periphery, serotonin plays a role in regulation of gastric motility and participates in hemostasis. While approximately 90% of the serotonin in the human body is located in the enterochromaffin cells of the gastrointestinal tract, platelets actively accumulate 5-HT during their passage through the intestinal blood vessels and are the greatest source of serotonin in the vascular periphery. During aggregation at sites of vascular injury, platelets release 5-HT through activation of 5-HT_2-sertonergic receptors on the platelets. The 5-HT accelerates the aggregation process thus exerting a positive feedback on its own release (De Clerck, David and Janssen, 1982). Since the vascular endothelial cells may be damaged at the site of injury, 5-HT can also act directly on the smooth muscle cells of the vessel wall, thereby causing contraction and aiding hemostasis (Houston and Vanhoutte, 1986; Hollenberg, 1988).

Figure 7
Biosynthesis of Serotonin from L-Tryptophan



Based on ligand-binding assays and pharmacological classification, at least seven major classes or families of serotonin receptors have been identified, each with several subtypes. With the exception of 5-HT_3 receptors which are ligand-gated cation channels, all the other 5-HT receptors are G-protein coupled (Chilmonczyk, 1995; Hoyer *et al.*, 1994). The families of receptors are defined according to their amino acid sequence homology and their coupling to secondary messengers: negatively coupled to adenylyl cyclase (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B}); positively coupled to adenylyl cyclase (5-HT_4, 5-HT_6, 5-HT_7); and phosphoinositol-coupled (5-HT_{2A} - previously 5-HT_2, 5-HT_{2B}, 5-HT_{2C}- previously 5-HT_{1C}).

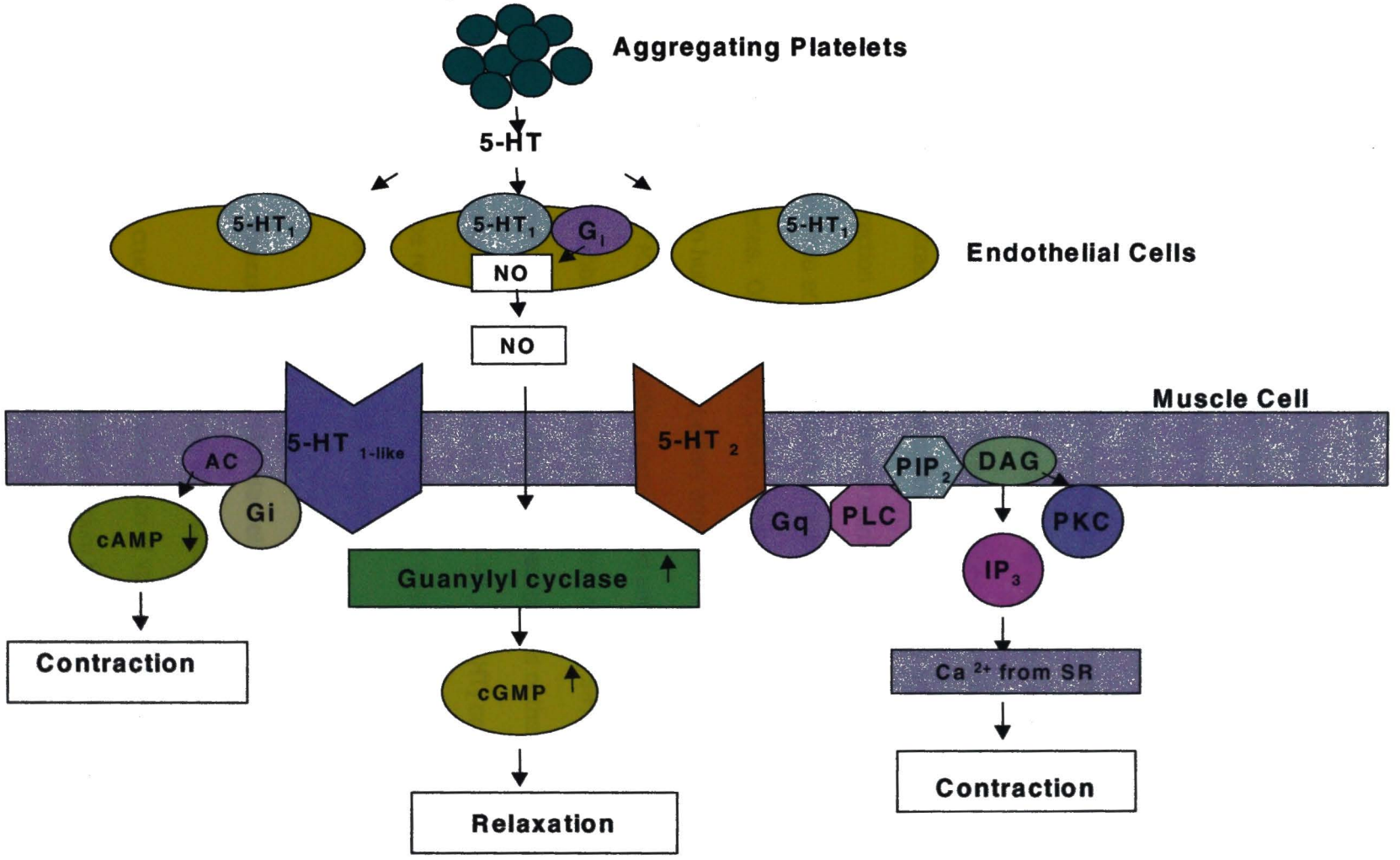
In the vascular periphery, aggregating platelets constitute the main source of serotonin. Serotonin receptors can contract or relax vascular smooth muscle cells, influence the release of norepinephrine from adrenergic nerves and stimulate endothelial cells to release nitric oxide and prostaglandins (Zschauer, Breeman and Uusitalo, 1991). In addition, serotonin can amplify the effects of other contractile agonists such as norepinephrine, histamine, and angiotensin II (Van Nueten *et al.*, 1982).

Until recent years, most serotonin-induced vascular constriction was identified as being mediated through the activation of a 5-HT_2 receptor. However, vasoconstriction in the basilary artery of bovine and the canine coronary artery is mediated through activation of 5-HT_1 receptors (Ebersole *et al.*, 1993). In peripheral blood vessels, the receptor responsible for this response has been further identified as a 5-$HT_{1\text{-like}}$ receptor (see Figure 8, page 24). 5-$HT_{1\text{-like}}$ receptors are a heterogeneous group of receptors that have been classified on the basis of a high affinity for the agonist, 5-carboxamidotryptamine (5-CT), a low affinity for ketanserin and susceptibility to antagonism by methiothepin (Bradley *et al.*, 1986). The 5-$HT_{1\text{-like}}$ response in cerebral arteries shows a similar but not identical pharmacological profile with the 5-HT_{1D} receptor. 5-$HT_{1\text{-like}}$ receptors in cerebral arteries have received particular attention in recent years as targets for the development of anti-migraine drugs (Saxena and Ferrari, 1989). Sumatriptan, a selective 5-$HT_{1\text{-like}}$ agonist, is prescribed to constrict cerebral arteries that are dilated during a migraine.

Stimulation of 5-HT_2 receptors in vascular smooth muscle cells can constrict most blood vessels including most arteries, veins, and venules (Garland, 1987). Vasoconstriction is the classical response to serotonin. The splanchnic, renal, pulmonary, and cerebral beds are particularly responsive to serotonin. All of the these effects are thought to aid in the hemostatic actions of platelets, especially in damaged vessels where the ability of endothelial cells to mediate vasodilation is compromised (Houston and Vanhoutte, 1986).

Figure 8
Cellular Effects of Serotonin on Vascular Smooth Muscle Cells

Schematic diagram illustrating the cellular effects of serotonin on vascular smooth muscle cells. Abbreviations used are as follows: 5-HT_1 = 5-HT_1 receptor, G_i = G-protein-inhibition, NO = nitric oxide, 5-$HT_{1\text{-like}}$= 5-$HT_{1\text{-like}}$ receptor, PLC = Phospholipase C, PIP_2 = Phosphatidylinositol 4,5, bisphosphate, PKC= protein kinase C, DAG=Diacylglycerol, G_q = G_q- protein, AC = adenylyl cyclase, SR = sarcoplasmic reticulum, cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophosphate, IP_3 = inositol trisphosphate.


Aggregating Platelets
5-HT
5-HT1
5-HT1
Gi
NO
5-HT1
Endothelial Cells
NO
Muscle Cell
5-HT 1-like
5-HT 2
AC
Gi
cAMP
Contraction
Guanylyl cyclase
cGMP
Relaxation
PIP2
DAG
Gq
PLC
PKC
IP3
Ca 2+ from SR
Contraction

The objective of this research is to characterize the serotonin receptors in the extraocular blood vessel that provides the blood supply for the structures responsible for the production of aqueous humor. While several others have looked at ocular vascular reactivity in a variety of species, there has been no information on the serotonin receptor subtype responsible for mediating a serotonin-induced vascular contraction in the long posterior ciliary artery.

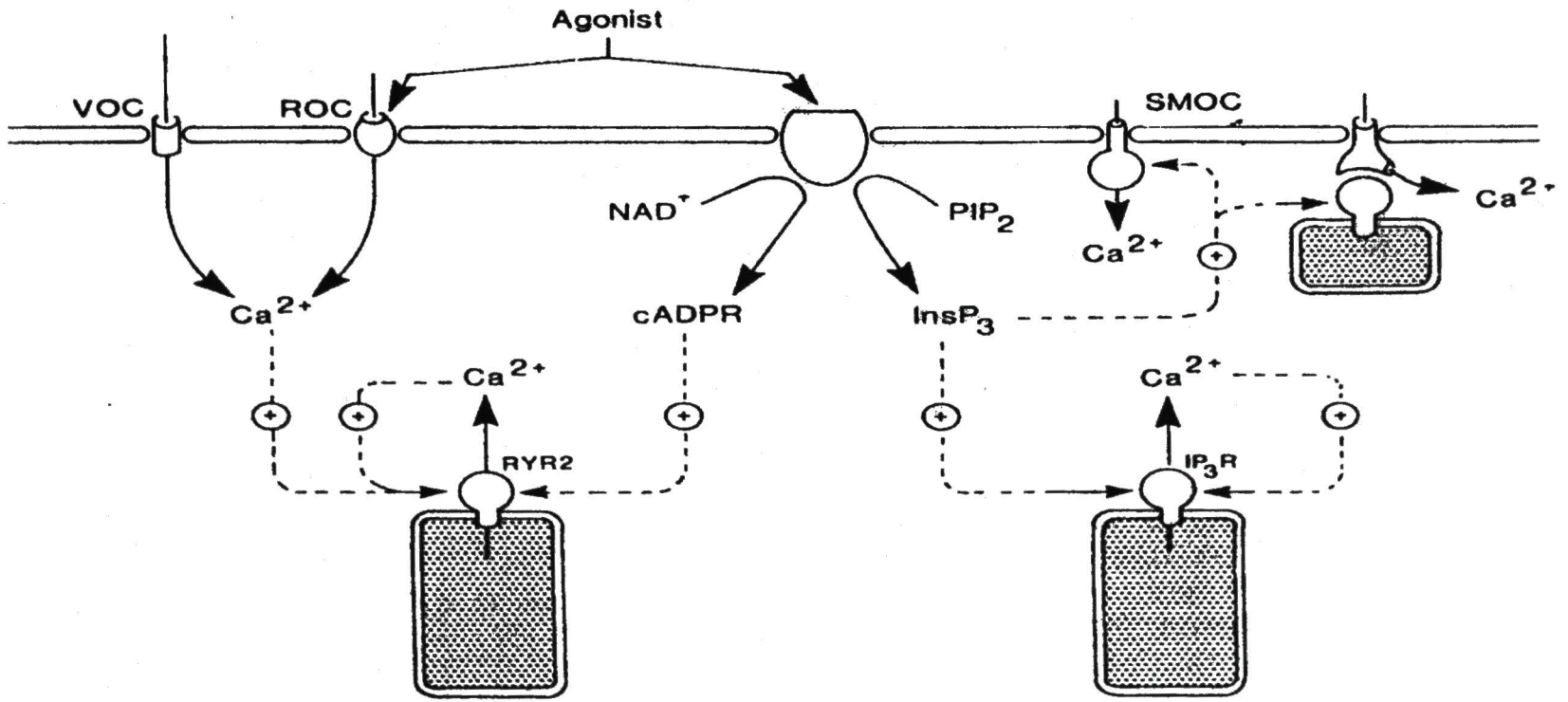
Dalske (1974) reported vascular contractions induced by histamine and serotonin in bovine ciliary arteries. More importantly, he reported the usefulness of the vascular contraction model in testing ocular vascular reactivity. When Haefliger, Flammer, and Luscher (1993) investigated vascular contractions in porcine ophthalmic and ciliary arteries, they found that the serotonin-induced contraction of both vessels was mediated by a 5-HT_2 receptor, which could be inhibited by ketanserin. Yao *et al.* (1991) demonstrated potent serotonin-induced contractions in porcine ophthalmic arteries. Ohkubo and Chiba (1988) demonstrated that serotonin produces a potent vasoconstriction in human posterior ciliary arteries. The threshold dose of 5-HT in these experiments was 1×10^{-9} M. Using selective agonists and antagonists, 5-HT responses in the ophthalmic artery of the rabbit were attributed to activation of both 5-HT_1 and 5-HT_2 receptors (Zschauer, Breeman, and Uusitalo, 1991). Although investigations of the 5-HT induced vascular reactivity in a variety of species have implicated either 5-HT_1 or 5-HT_2 or both, no one has fully detailed the 5-HT subtype responsible for these vascular responses or fully investigated the cellular mechanisms responsible for the vasoconstriction.

Vascular Smooth Muscle Cell Contraction and Calcium Mobilization

Calcium plays a crucial role in the contraction of vascular smooth muscle. It is the change in the concentration of free, intracellular calcium that is the principal mechanism that initiates the contraction (Somlyo and Somlyo, 1994; Hughes and Schachter, 1994). A rise in the intracellular free calcium concentration results from either an influx of extracellular calcium into

the cell or from the release of calcium into the cytosol from intracellular calcium stores. A contraction is the result of intracellular calcium binding to calmodulin, that in turn complexes with the catalytic subunit of myosin light-chain kinase (MLCK). Activated MLCK phosphorylates serine at position 19 on the regulatory light chain of myosin. Phosphorylated myosin ATPase then binds to actin. The crossbridging between the actin and myosin filaments results in contraction (Rembold, 1992)

Figure 9
Calcium Mobilization



Summary of calcium signalling. Calcium enters from the outside through voltage-operated channels (VOC), receptor-operated channels (ROC) or second messenger-operated channels (SMOC). Calcium can also be released from internal stores regulated by ryanodine receptors (RYR). Ryanodine receptors in non-muscle cells may be under the control of cyclic ADP ribose (cADPR). IP_3 receptors (IP_3R) regulate the other major pool of internal calcium. (Reprinted from Berridge, 1994)

Extracellular Calcium Entry

A number of mechanisms exist to mediate calcium influx into the cell: 1) receptor operated calcium channels that open as a result of an agonist binding to a membrane receptor, 2) voltage operated calcium channels that open in response to depolarization, 3) second messenger activated calcium channels, 4) G-protein coupled channels, 5) calcium activated calcium channels, and 6) channels driven by the depletion of calcium from the intracellular stores. The cross-talk between the various mechanisms is not clearly understood (Marin *et al.*, 1999).

Intracellular Calcium Storage and Release

The stores from which calcium is released are characterized by three components: pumps to sequester calcium, binding proteins to store calcium and the specific IP_3 or ryanodine channel to release the calcium back into the cytosol. For this signaling mechanism to work effectively, the concentration of calcium in the cytosol must be kept low (approximately 10^{-7} M) whereas extracellular calcium is approximately 10^{-3} M. Both the plasma and the sarcoplasmic reticulum contain ATPases that utilize ATP hydrolysis to transport calcium out of the cytosol against a steep concentration gradient. The calcium is stored in the sarcoplasmic reticulum, typically bound to the protein calsequestrin.

IP_3 and ryanodine receptors represent the two principle channels for the mobilization of stored intracellular calcium. Both the IP_3 and the ryanodine receptors are tetramers located on the sarcoplasmic reticulum with the binding site located on the amino terminus, which projects into the cytosol (Berridge 1993). Although they are similar in structural appearance, the two channel types are functionally distinct. (Ehrlich *et al.* 1994).

Calcium induced calcium release is thought to be the primary activator of the ryanodine receptor in skeletal muscle (Chandler 1976). Ryanodine is an inhibitor of the ryanodine receptor (Smith *et al.*, 1988) but not of the IP_3 receptor (Erlich and Watras 1988) thereby providing a

means of distinguishing between the two receptors. The distribution of the IP_3 or ryanodine sensitive stores varies considerably from cell to cell. Skeletal muscle typically contain ryanodine sensitive stores whereas other cells such as vascular smooth muscle cells or atrial cells may contain both types (Berridge, 1993). Most studies indicate that the two types of stores are distinctly operated by their respective receptors (Galione *et al.*, 1991).

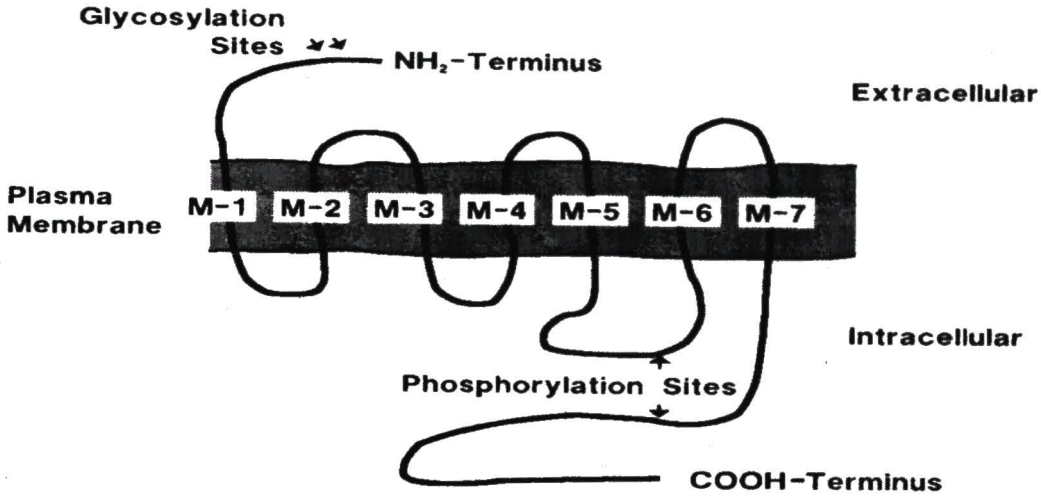
Stimulation of PLC through activation of a G-protein (probably a $G_{q\backslash 11}$) increases the intracellular level of inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). When IP_3 binds to its receptor, calcium contained within the intracellular store is released into the cytosol (Blatter, 1995).

The results of the proposed experiments will help to elicit the role that the mobilization of intracellular and extracellular calcium plays in the serotonin induced vascular contraction of the long posterior ciliary artery of bovine eyes.

Signal Transduction Pathway

All serotonin receptors identified to date, with the exception of 5-HT_3 receptors, which are ligand-gated cation channels, are G-protein coupled receptors. All G-protein linked receptors are characterized by having seven membrane spanning domains connected by extracellular and intracellular loops (Simon *et al.*, 1991). The transmembrane helices of the receptor form the binding site for the agonist. Once the agonist binds to the receptor, a conformational change takes place, which activates the G-protein. It is the second and third intracellular loops of the receptor that play an essential role in activating the heterotrimeric G-protein.

Figure 10
G-protein Linked Receptors Seven
Membrane Spanning Domains



The G-protein is composed of three subunits, alpha, beta, and gamma (Hepler and Gilman, 1992). Upon stimulation of the receptor, GTP displaces GDP on the alpha subunit causing dissociation of the GTP-alpha complex from the beta/gamma subunit (Johnson and Dhanasekaran, 1989). The Gq activates phospholipase C (PLC).

Once activated, PLC hydrolyses membrane associated phosphotidylinositol 4,5 biphosphate (PIP_2) to yield two second messengers, diacyglyerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG remains membrane associated and activates protein kinase C that is responsible for the phosphorylation of a number of intracellular proteins. IP_3, which is hydrophilic, is responsible for causing the release of calcium from intracellular stores by activation of an IP_3 receptor on the sarcoplasmic reticulum (Berridge, 1993). PLC is active until the inherent GTPase activity of GTP-alpha complex limits the reaction (Johnson and Dhanasekaran 1989, Katzung, 1989).

DAG with phosphotidylserine in the cytoplasmic half of the plasma membrane binds to PKC thereby increasing the affinity of the enzyme for calcium. When activated by DAG, PKC transfers the terminal phosphate group from ATP to specific serine or threonine residues on target proteins that vary depending on the cell

In a similar manner, Gs activates adenylyl cyclase to convert ATP to cAMP. In an inhibitory manner, Gi inhibits cAMP and results in vascular contraction.

Experiments with selective antagonists were performed to determine the signaling pathway involved in modulating the serotonin-induced vascular tension.

Experimental Rationale

Therapeutic agents prescribed for the treatment of glaucoma or ocular hypertension lower the pressure inside the eye by either decreasing the production of aqueous humor by the ciliary processes or by increasing the outflow of aqueous humor through the trabecular meshwork. The long, posterior ciliary artery is the major blood supply for the ciliary processes where aqueous humor is formed. Serotonin has been identified as an agent that effectively constricts this blood vessel. A classification of serotonin receptor subtypes, which are active in this ophthalmic artery, could provide valuable information about control of ocular blood flow to this tissue. The objective of this research is to determine which serotonin receptor subtype(s) is (are) responsible for the serotonin-induced contraction in the long posterior ciliary artery and to characterize the cellular mechanisms mediating that response.

In most vascular tissue, the vasoconstrictor effects of serotonin are the result of the activation of 5-HT_2 receptors. However, there are reports in the literature of a vascular contraction being mediated through activation of a 5-$HT_{1\text{-like}}$ receptor. Experiments will be performed to definitively identify the serotonin receptor subtypes and to identify the role of each subtype in mediating the response.

5-HT_2 receptors mediate their effects through activation of secondary messengers of the phosphatidylinositol pathway. Therefore, to characterize the cellular mechanisms responsible for the serotonin-induced vasoconstriction, experiments will be conducted to determine if the response is mediated through activation of protein kinase C or phospholipase C.

The long posterior ciliary artery of bovine eyes will be used to test the hypotheses that:

(1) the serotonin-induced vasoconstriction of the long posterior ciliary artery is modulated through activation of more than one serotonin subtype.

(2) the serotonin-induced contraction is dependent on calcium mobilization.

(3) the serotonin-induced response is mediated through activation of protein kinase C or phospholipase C.

CHAPTER II

MATERIALS AND METHODS

Bovine eyes are obtained from Aries Scientific in Richardson, Texas. The eyes are rapidly removed from the animals following slaughter and are placed in ice cold physiological saline solution to be transported on ice to Alcon Research, Inc. in Fort Worth, Texas. The eyes remain on ice until they are prepared for dissection.

Experimental Methods

Vascular Contraction Experiments

Within four (4) hours of collection, the eyes were received and medial or lateral segments of the long posterior ciliary artery (LPCA) were dissected. The eyes were dissected along the equator and the posterior portion was secured under a dissecting microscope. The long posterior ciliary arteries were located based upon diagrams (Fraudson, 1974 and Prince, 1960) and personal instruction from Byron Li, a technician in the toxicology department at Alcon. The vessels were visible on the exterior of the eye and run parallel along the optic nerve. A 2 to 3 cm segment was dissected free of surrounding tissue under a dissecting microscope and was placed in cold physiological buffer. Both ends of the segment were cut perpendicular to the long axis of the vessel and discarded so that each ring segment used in the experiments would be exposed to a minimum amount of handling and trauma. Each vascular ring was approximately 1.5 to 2 mm in width.

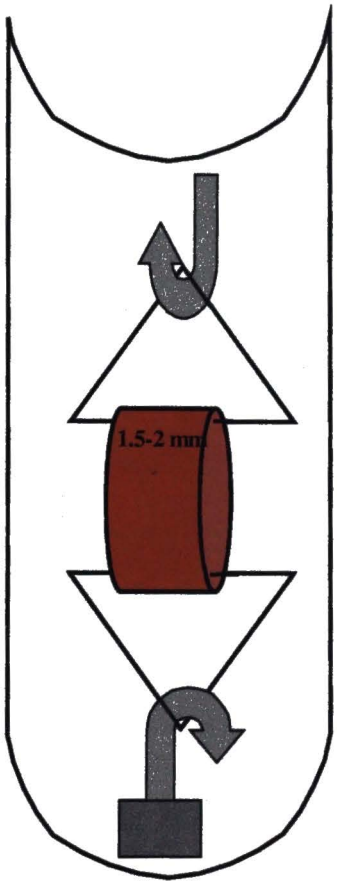
Tungsten triangles were made by cutting an 8 mm segment of tungsten wire ($1/5000^{th}$ in.) and shaping it to form a triangle. One end of the wire was bend upward to form a hook to allow closure of the triangle after the vessel was mounted. The other end of the triangle was

polished to a smooth surface to prevent damage to the vessel as the wire was passed through the lumen of the vessel. The vascular rings were mounted between two tungsten triangles (see Figure 11, page 36. The lower triangle was attached to a stainless steel rod mounted in a 18 ml tissue bath (Radnoti) filled with physiological buffer. Physiological buffer contained 1.5 mM $CaCl_2$, 10 mM dextrose, 10mM Hepes, 130 mM NaCl, 5.0 mM KCl, 0.3 mM KH_2PO_4, and 1.0 mM $MgCl_2$ at a pH of 7.4. The upper triangle was attached to a Grass force displacement transducer Model 79 (Grass Instruments, Quincy, Mass.) for measurements of isometric force which were recorded on a Narco physiograph, model DMP-4A (Narco Bio-systems, Inc., Houston, TX)(see Figure 12, page 37). Prior to mounting the ring segments on the transducer, the tension measured by the instrument was calibrated using standard weights. The solution in the tissue bath was aerated with 100% O_2 and maintained at 37°C for the duration of the experiments.

Each vessel segment was allowed to equilibrate for 30 minutes and then the tension was adjusted until a final resting tension of approximately 1.0 g was obtained on each vessel. During the conduction of the experiments, the vasoactive agents were added directly to the tissue bath. For dose-response experiments, additional doses were added to the tissue bath to provide a cumulative effect (Figure 13). For other experiments, the tissue bath was drained and rinsed with PB between experiments and the vessels were allowed to relax to baseline tension.

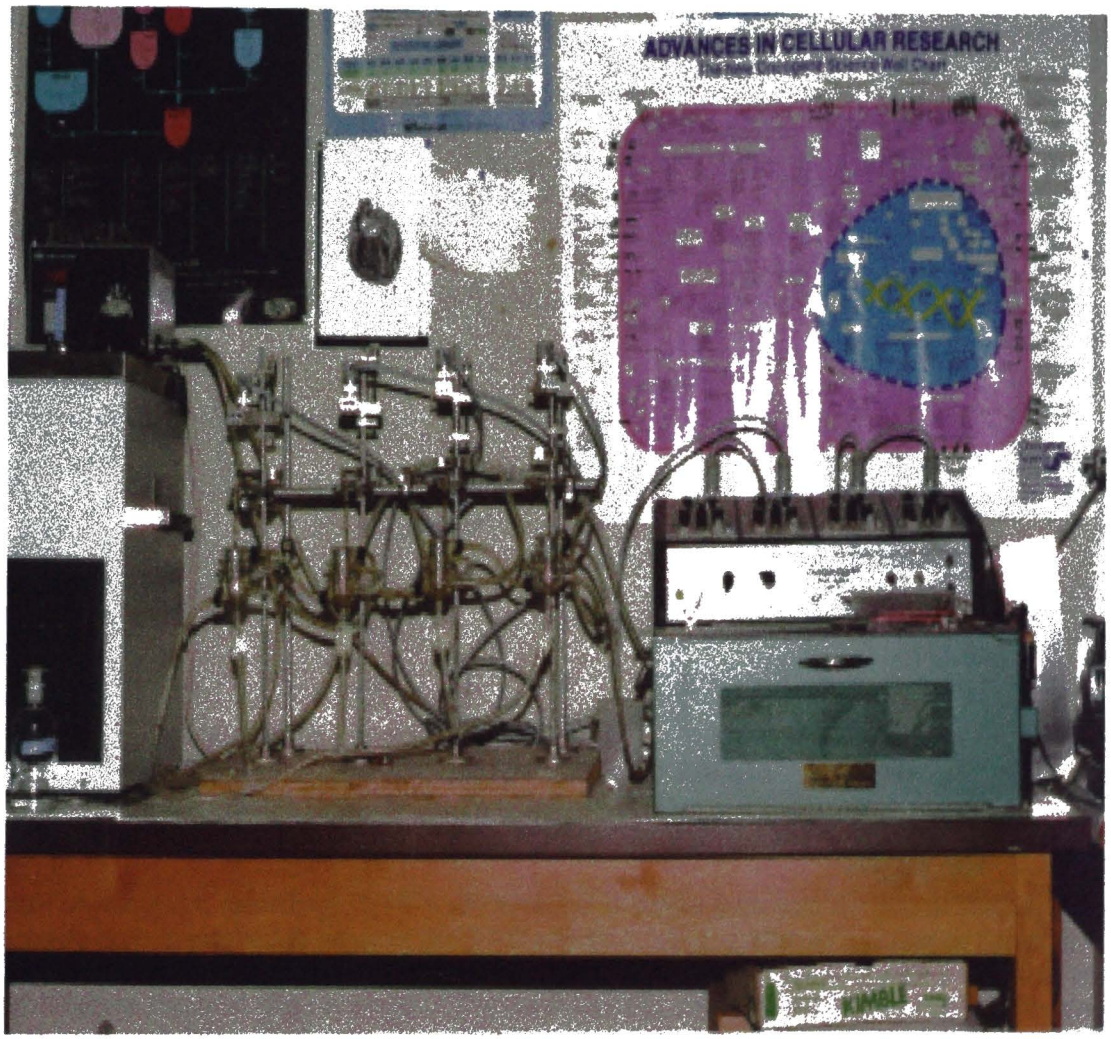
At the beginning of each set of contraction experiments, the response of each vessel to high KCl (KCl 40 mM) was determined. The tissue bath was rinsed and the vessel was allowed to return to baseline. Serotonin 1 μM was then added to each tissue bath. Only vessels that demonstrated potent contractions to both high KCl and 5-HT were considered to be viable and were used for further experiments. Dissected vessels were viable up to 48 hours following dissection if they were removed from the eye and maintained in aerated PB.

Figure 11
Vascular Ring Mounted on Tungsten Triangles



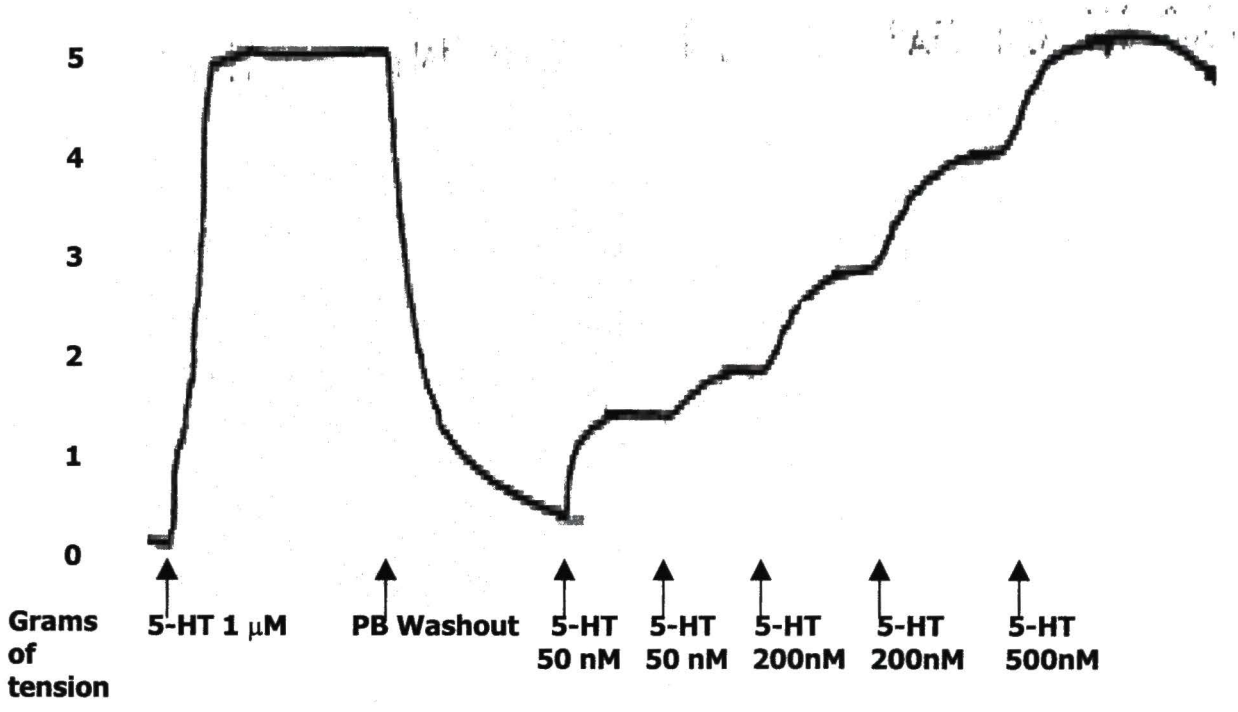
Graphical representation of ring segment of long posterior ciliary artery mounted on tungsten wires in 18 mL glass tissue bath.

Figure 12
Instrumentation for Measurement of Vascular Contraction



Copy of photograph of equipment used in vascular contraction experiments: four glass tissue baths and physiograph recorder.

Figure 13
Example Physiograph Recording



Example of typical physiograph recording. 5-HT 1 μM is added to tissue bath and the vessel contraction is recorded. The tissue bath is washed to remove the 5-HT and the vessel is allowed to relax to baseline. Subsequently, doses of 5-HT are added in a cumulative fashion. The vessel contracts with each dose and is allowed to plateau before the next dose is added. The results of this example experiment reflect the data that would constitute a cumulative dose response curve.

Primary Cell Culture Technique

Primary cell cultures of the vascular smooth muscle cells were grown using the explant method previously described by Diglio *et al.* 1989. Segments of the long posterior ciliary artery located approximately 2 cm from the posterior portion of the globe were aseptically dissected from the bovine eye and placed in cold Hank's balanced salt solution (HBSS) in a tissue culture dish. Sterilized forceps and iris scissors were used to remove all connective tissue. The tissue was rinsed twice with HBSS to remove all blood elements. The vascular segment was then cut into ring segments (1 to 1.5 mm) using a sharp scalpel. These vascular rings are place into multi-well tissue culture plates (24 well plates/2.1 cm^2/well) containing just enough complete medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) to keep the tissue moist. The ring segments were incubated overnight at 37° in 95% air/5% CO_2 atmosphere. On day 2 additional media was carefully added to each well (0.25 ml) as to not disturb explant attachment. Cultures were monitored daily and fresh media was added every two (2) days. Transfer of the same rings into new culture wells at four (4) day intervals resulted in obtaining a pure population of smooth muscle cells.

Calcium Imaging

The vascular smooth muscle cells were seeded onto six well plates (Costar, Cambridge, MA) containing #0 sterile glass coverslips and grown to 40-50% confluency. On the day of assay, the cell coated coverslip was placed into a specially designed mounting chamber (Medical Systems Corp., Greenvale, NY) for holding the coverslip and buffer. After the coverslip was placed into the chamber and rinsed twice with a loading buffer (125 mM NaCl, 5 mM KCL, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2P0_4, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose, 0.1 % bovine serum albumin, pH 7.2), it was incubated at room temperature in the dark for 60 minutes with 5 μM of the calcium fluorescent dye Fura 2 acetoxymethyl ester (Fura 2 AM, Molecular

Probes, Inc., Eugene, OR). After the incubation, the coverslip was rinsed three times with the loading buffer (minus bovine serum albumin) and then mounted onto the stage of the microscope (Nikon Diaphot, Nikon, Garden City, NY) with 2 ml of buffer in the chamber. Intercellular calcium concentration ($[Ca^{2+}]$) was measured on a real time basis by ratio fluorometry. The ratio fluorometer (Delta Scan 4000, Photon Technology International, South Brunswick, NJ) was either fitted with a SIT microscope video camera (Hamamatsu model C2400, Hamamatsu City, Japan) for images or a microscope photometer with photon counting photomultiplier detector for ratio fluorometry tracings of single cells. The fluorescent indicator was excited by UV light at alternating wavelengths of 340 and 380 nm and the fluorescent emission measured at 510 nm every 0.2 seconds (photomultiplier) or 5 seconds (camera). The emission intensity ratio was calculated for each time point. After obtaining a baseline measurement, 20 μl of the test drug was added to the chamber at the indicated time. To bring the calcium levels back to baseline, between drug additions, the chamber was rinsed three times with 2 ml of buffer. At the conclusion of each experiment, on the same cell, 5 μM ionomycin was added and the minimum and maximum 340/380 ratios obtained in the presence of 2 mM EGTA or 10 mM $CaCl_2$, respectively. Calcium concentration was calculated according to the Grynkiewicz et al. (Grynkiewicz, Poenie and Tsien, 1985) with 135 nM as the Kd of Fura 2 for calcium.

Experimental Agents

Experimental agents used in the conduction of this research are listed in the following table:

Table 2
List of Experimental Agents

Drug class	Drug	Source
5-HT agonist	Serotonin (5-HT)	Research Biochemicals, Inc. Natick, MA
5-HT_1 agonist	5-Carboxamidotryptamine maleate (5-CT)	Research Biochemicals, Inc. Natick, MA
5-HT_2 agonist	α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT)	Research Biochemicals, Inc. Natick, MA
5-HT_1 antagonist	Methiothepin mesylate	Research Biochemicals, Inc. Natick, MA
5-HT_2 antagonist	Ketanserin tartrate	Research Biochemicals, Inc. Natick, MA
Calcium channel blocker	Diltiazem	Marion Laboratories, Kansas City, MO
Calcium channel blocker	Nifedipine	Pfizer Company, Brooklyn, NY
Ryanodine receptor inhibitor	Ryanodine	Research Biochemicals, Inc. Natick, MA
IP3 receptor inhibitor	Caffeine	Sigma Chemical Co., St. Louis, MO
Phospholipase C inhibitor	2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (2-NCDC)	Sigma Chemical Co., St. Louis, MO
Phospholipase C inhibitor	U73122	Research Biochemicals, Inc. Natick, MA.
Phospholipase C inhibitor	Neomycin	Sigma Chemical Co., St. Louis, MO
Protein kinase C inhibitor	Chelerythrine	LC Services, Woburn, MA
Protein kinase C inhibitor	Bisindoylmalemide II	Calbiochem, La Jolla, CA
Protein kinase C inhibitor	Bisindoylmalemide V	Calbiochem, La Jolla, CA
Protein kinase C inhibitor	1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H-7)	Research Biochemicals, Inc. Natick, MA
Histamine agonist	Histamine	Sigma Chemical Co., St. Louis, MO
Calcium fluorescent dye	FURA-2	Molecular Probes, Inc., Eugene, OR
Muscarinic agonist	Carbachol	Research Biochemicals, Inc. Natick, MA

Experimental Protocols

Protocol #1

Serotonin Receptor Subtype Identification

Vessels were mounted as described in the Experimental Methods section to measure the vascular response of ring segments of the long posterior ciliary artery to various vasoactive agents.

Study 1: Selective serotonin agonists were added and the response was recorded on physiograph paper. Each experiment was performed with a high KCl (40 mM KCl) control.

Agonists: 5-HT, 5-$HT_{1\text{-like}}$(5-CT), 5-$HT_{2A\text{-}C}$ (α-methyl-5-HT)

Experiments were conducted with each of the agents listed above. The agonists were added in a cumulative manner to establish dose-response curves and to determine the EC_{50} for each agent.

Study 2: Experiments were performed using selective antagonists to further substantiate the receptor subtype(s) identified in Study 1. Shield regression analyses were performed by incubating the vascular rings with an antagonist for 20 minutes prior to the addition of serotonin. The vessels were incubated with a specific concentration of antagonist and then the same concentrations of serotonin used previously to establish a dose-response curve were added in a cumulative fashion. The tissue bath was washed out with PBS and the vessel was allowed to return to baseline. Then the vessel was incubated with a higher concentration of antagonist and the dose response curve was repeated. Similar experiments were repeated using a higher concentration of antagonist in each series.

Antagonists: 5-$HT_{1\text{-like}}$(methiothepin) and 5-$HT_{2A\text{-}C}$ (ketanserin).

Serotonin dose-response curves generated in the presence of various concentrations of the antagonists listed above were compared to estimate the IC_{50} for each agent. In each set of

experiments, a dose-response curve for serotonin was generated. Then the vessels were incubated for 20 minutes with the lowest concentration of antagonist to be tested and the serotonin dose-response curve was repeated using the same concentrations of serotonin as the original curve. Experiments continued in this fashion with each increasing concentration of antagonist and the curves were compared.

Protocol #2

Determination of the role of calcium mobilization in the serotonin induced vascular contraction

Study 1: To determine if the serotonin-induced vascular contraction was dependent on extracellular calcium mobilization, experiments were conducted by allowing the vascular rings to incubate in calcium free PB with 0.5 mM EGTA for 20 minutes prior to the addition of serotonin (1 μM). The vascular response of the vessels incubated in 0 Ca^{2+} PB with 0.5 mM EGTA was compared for both duration and magnitude to the serotonin-induced response of vessels incubated in PB containing 1.5 mM calcium.

Study 2: To determine if the contraction was dependent on extracellular calcium entering the cell through voltage gated L-type calcium channels, experiments were performed by incubating the vascular rings in PB with calcium channel blockers, diltiazem (10 μM) or nifedipine (10 μM), prior to the addition of serotonin (1 μM). As a control, the experiments were repeated with the ring segments equilibrating in PB with the calcium channel blocker prior to the addition of high KCl (40 mM) buffer instead of serotonin.

Study 3: To determine if the release of calcium from intracellular calcium stores in the sarcoplasmic reticulum (SR) plays a role in the serotonin-induced vascular contraction, vessels were incubated with agents that deplete calcium or block the release of calcium from the intracellular calcium stores in the sarcoplasmic reticulum by activation of either the ryanodine

receptor or the IP_3 receptor. Vascular contraction was compared in seven sets of vessels: 1) vessels incubated in PB treated with serotonin (1 μM), 2) vessels incubated for 20 minutes in 0 Ca^{2+} PB plus 0.5 mM EGTA prior to the addition of serotonin (1 μM), 3) vessels incubated for 20 minutes with ryanodine (10 μM) prior to serotonin (1 μM), 4) vessels incubated for 20 minutes in 0 Ca^{2+} PB with 0.5 mM EGTA and ryanodine (10 μM) prior to serotonin (1 μM), 5) vessels incubated for 20 minutes in 0 Ca^{2+} PB with 0.5 mM EGTA, diltiazem (10 μM), and ryanodine (10 μM) prior to the addition of serotonin (1 μM), 6) vessels incubated for 20 minutes in PB with caffeine (20 mM) prior to the addition of serotonin (1 μM), and 7) vessels incubated for 20 minutes in 0 Ca^{2+} PB with 0.5 mM EGTA and caffeine (20 mM) prior to serotonin (1 μM).

Study 4: To measure the change in intracellular calcium associated with the serotonin induced contraction, experiments were performed using ratio fluorometry. Primary cell cultures were grown to semiconfluency in 6-well polystyrene plates and prepared as described in Calcium Imaging, page 39. A baseline measurement was recorded for 3-5 minutes or until a steady baseline was reached. Several agents known to contract vascular smooth muscle cells, such as carbachol, serotonin, and histamine, were used in this set of experiments. The agonists were added directly into the chamber and the calcium response was recorded. The chamber was washed with HEPES buffer between the addition of each drug. At end of the experiment, the minimum and maximum fluorescence ratios were obtained using the calcium ionophore with 2 mM EGTA, followed by 10 mM $CaCl_2$, respectively.

Protocol #3

Determination of the role of phosphoinositide specific protein kinase C or phospholipase C in the serotonin induced contraction

Study 1: To determine the role of PLC in mediating the serotonin induced vascular response, experiments were performed by incubating vascular rings for 20 minutes in the PLC inhibitors, U73122 (0.5 μM) (Osol, Laher and Kelly, 1993), 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (70 μM, NCDC) (Clarke and Garland, 1991), and neomycin (5 mM) prior to the addition of serotonin. Results were compared to control vessels that had been contracted with serotonin and with high KCl.

Study 2: To determine the role of PKC in mediating the serotonin induced vasoconstriction, experiments were performed by incubating vessel rings for 20 minutes with PKC inhibitors, bisindolylmaleimide II (1 μM), bisindolylmaleimide II (10 μM), H-7 (5 μM), and chelerythrine (25 μM) (Hebert et al., 1990; Eckly-Michel, Le Bec and Lugnier, 1997) prior to the addition of serotonin (1 μM) (Kaye *et al.*, 1995). As a control, vessels were incubated 1) with serotonin without the PKC inhibitor, 2) with the PKC inhibitor and KCl, and 3) with bisindolylmaleimide V (10 μM) (an inactive analog) and serotonin.

Data Analysis

All data are presented as mean ± standard error of the mean (SEM); N refers to the number of individual vascular ring segments tested. Statistical analyses were performed using Student's t-test supplied by Microsoft Excel 97. Appropriate statistical analyses were applied to each set of data with $p < 0.05$ considered statistically significant. When comparing vascular contractions to serotonin responses, 1-tailed tests were used because the expected response to the agent being tested would be a decrease. In other comparisons when the response is compared between different agents, a 2-tailed test is performed. In experiments in which the %

of maximum contraction is reported, the response was compared to either the maximum response to serotonin or to high KCl (40 mM KCl), which ever was appropriate.

Prior to each set of experiments, the force transducer and the physiograph were standardized so that one gram of tension weight would be recorded as one centimeter displacement on the recorder. For all contraction experiments, vascular tension was recorded in centimeters by measuring the displacement of the recording pen of the physiograph and later converted to grams of tension. Graphical representation of the data was prepared using Microsoft Excel 97, TableCurve and Sigma Plot.

CHAPTER III

RESULTS

Specific Aim #1

Serotonin receptor subtype identification

In preliminary experiments, it was determined that serotonin produces potent, concentration dependent contractions of the long posterior ciliary artery. Prior to each set of experiments, approximately 1 gram of preload tension was placed on the vessels and each ring segment was challenged with 40 mM KCl and 1 μM serotonin. Vessels that demonstrated reproducible, sustained contractions were considered to be viable segments and were used in the experiments. The equipment allowed for 8 ring segments to be mounted at a time. Out of each set of 8 rings mounted on one day, it was typical for 7 to 8 segments to be viable.

Dose response experiments were conducted by adding incremental doses of serotonin to the tissue baths in a cumulative fashion. The response to each concentration was initially recorded by a physiograph on chart paper. The displacement of the physiograph pen was measured in cm and later converted to grams of tension based upon the instrument calibration. The response of each individual vessel recorded in grams of tension was then compared to the maximum response of that vessel to 40 mM KCl and the response was converted to a percentage.

A dose response curve with eight concentrations of serotonin was generated. The data were analyzed using TableCurve. The mean vascular response (±SEM) to serotonin is reported as a percent of the maximum contraction with 40 mM KCl for fifteen (n=15) sets of experiments and

is presented in Figure 14, page 50. In preliminary experiments, it was determined that selective 5-HT_1 and 5-HT_2 agonists both produced reproducible contractions in this vessel.

For this series of experiments, it was determined that 1.2 X 10^{-8} M was the minimum concentration (threshold) of serotonin that produced a measurable response and that 3.17 X 10^{-5} M produced the maximum response. The EC_{50}, the concentration at which the response was one half of its maximum, was calculated after normalizing the data so that the maximum response of each individual vessel was considered to be 100%. The dose-response curve was again generated with the normalized data and the EC_{50} was determined to be 2.89 X 10^{-7} M. The normalized dose response curve is presented in Figure 15 on page 52.

Additional experiments were conducted using selective 5-$HT_{1\text{-like}}$, agonist 5-carboxamidotryptamine maleate (5-CT) and the 5-HT_2 agonist, α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT). Dose-response curves for each of these agonists were constructed in a similar manner to the serotonin dose-response curve. The threshold concentration, EC_{50} and maximum concentration for each agonist can be found in Table 3, page 49.

The dose response curve for the 5-HT_1 agonist, 5-CT, was generated by adding seven (7) concentrations of the agonist in a cumulative fashion. The means graphed in Figure 14 and 15, represent the average of 14 (n=14) sets of experiments. The mean maximum effect of 5-CT compared to the mean maximum response to 40 mM KCl was 25.76%. The threshold concentration was 4.6 X 10^{-9} M and 6.0 X 10^{-6} M produced the maximum response. The EC_{50}, was determined from normalized data to be 3.63 X 10^{-7}M.

The mean responses presented for the 5-HT_2 agonist, α-methyl-5-HT, represent the average of seven (n=7) sets of experiments with seven concentrations of the agonist. The mean maximum effect was 104.91% of the maximum response to 40 mM KCl. For this series of experiments, it was determined that 7.3 X 10^{-8} M was the threshold concentration and that 3.17 X 10^{-5} M produced the maximum response. The EC_{50} was determined to be 1.77 X 10^{-6} M from

the normalized data. Figure 16 is a graph of the raw data collected in the generation of these dose-response curves. Similar graphs were prepared for both 5-HT and 5-CT. TableCurve was used to perform the analysis with each set of data to determine the threshold, the maximum response and the EC_{50}. The goodness of fit coefficients (r^2) for the graphs prepared with TableCurve were 0.989803, 0.966748, and 0.976407 for 5-HT, 5-CT, and α-methyl-5-HT, respectively.

These data indicate that the activation of the $5\text{-HT}_{1\text{-like}}$ receptor contributed about 15.13% to the contraction and the activation of the 5-HT_2 receptor contributed 61.61% to the contraction. The agonists appear to contribute to the contraction in a synergistic manner since the combined response of the individual selective agonists is less the vascular response to 5-HT. As shown in Figure 14, the efficacy of the 5-HT_2 agonist is 4 times the efficacy of the $5\text{-HT}_{1\text{-like}}$ agonist. The rank order of potency for these three agonists were 5-HT > 5-CT > α- methyl-5-HT. The EC_{50} of the 5-CT and 5-HT were very close, 336 nM (5-CT) compared to 283 nM (5-HT). In contrast, the EC_{50} of α-methyl-5-HT was 1.7 μM.

Table 3
List of Threshold Dose, E_{max} and EC_{50} for Serotonin Agonists

Agonists	Threshold (M)	E_{max}%*	EC_{50} (M)
5-HT	1.2×10^{-8}	170.27 ± 13.6	2.83×10^{-7}
5-CT ($5\text{-HT}_{1\text{-like}}$)	4.6×10^{-9}	25.76 ± 4.9	3.63×10^{-7}
α-methyl-5-HT (5-HT_2)	7.3×10^{-8}	104.91 ± 19.6	1.77×10^{-6}

* E_{max}% - average maximum response compared to maximum KCl response ± SEM.

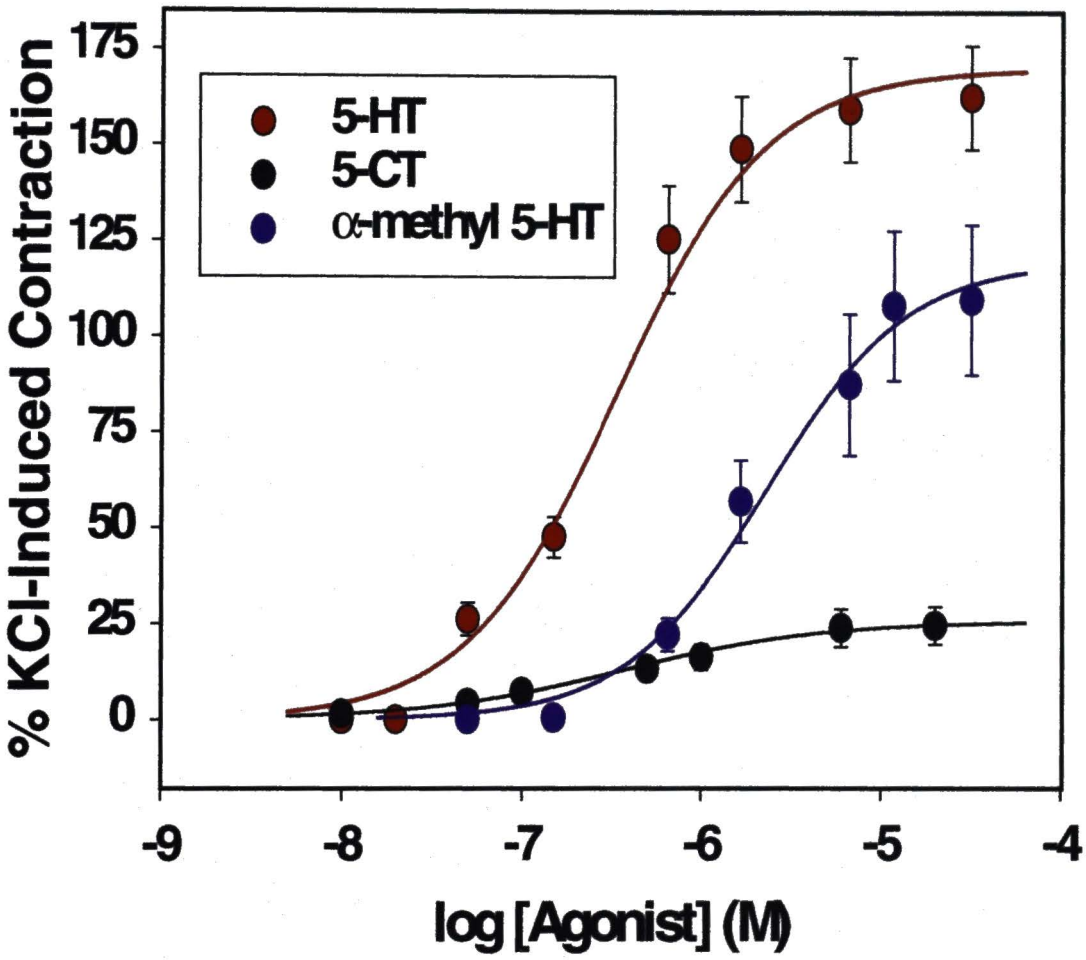


Figure: 14 Dose-response curves for raw data for 5-HT (n=15), 5-$HT_{1\text{-like}}$ agonist, 5-CT (n=14) and 5-HT_2 agonist, α-methyl-5-HT (n=7). Maximum response compared to maximum response to 40 mM KCl: 5-HT, 170.3%: 5-CT=25.8%: α-methyl-5-HT=104.9%. Data represent mean response ± SEM.

Figure 15
Normalized Dose-Response Curves for 5-HT, 5-CT and α-methyl 5-HT

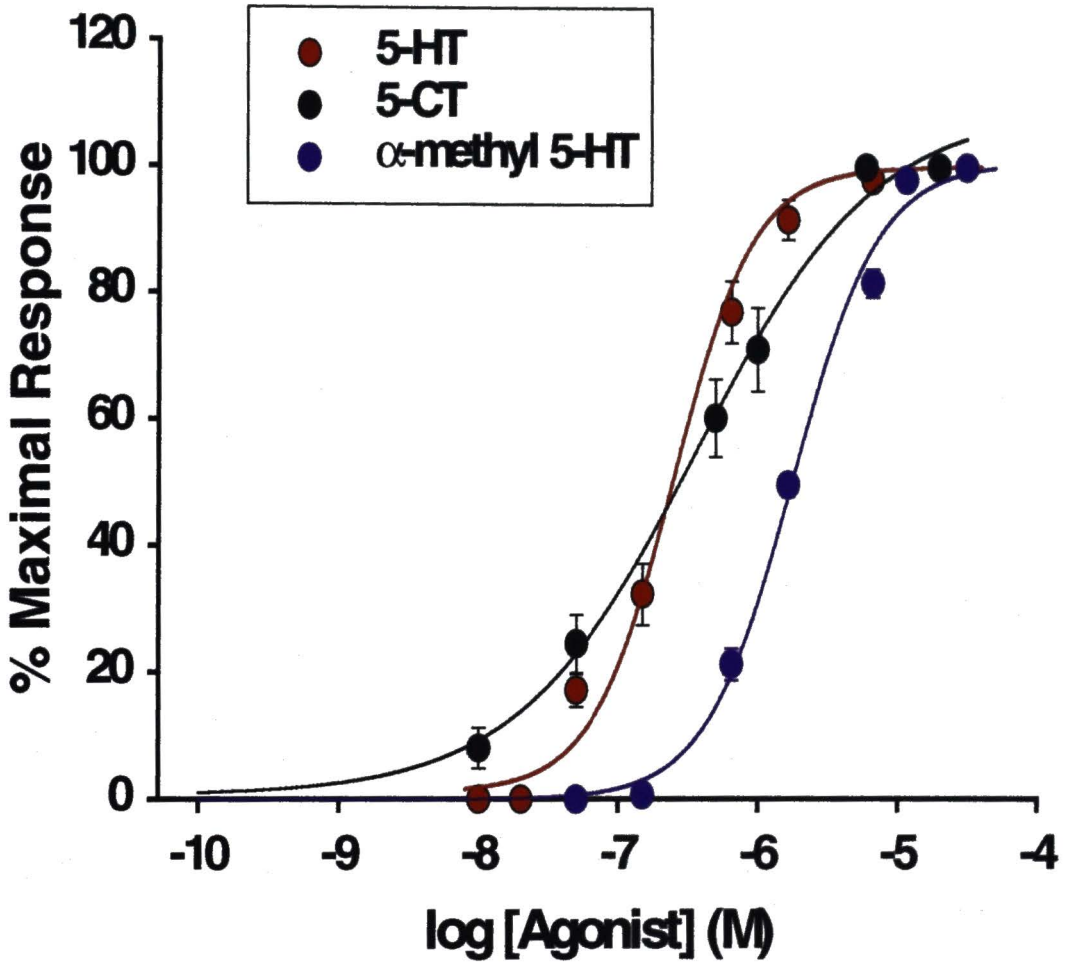
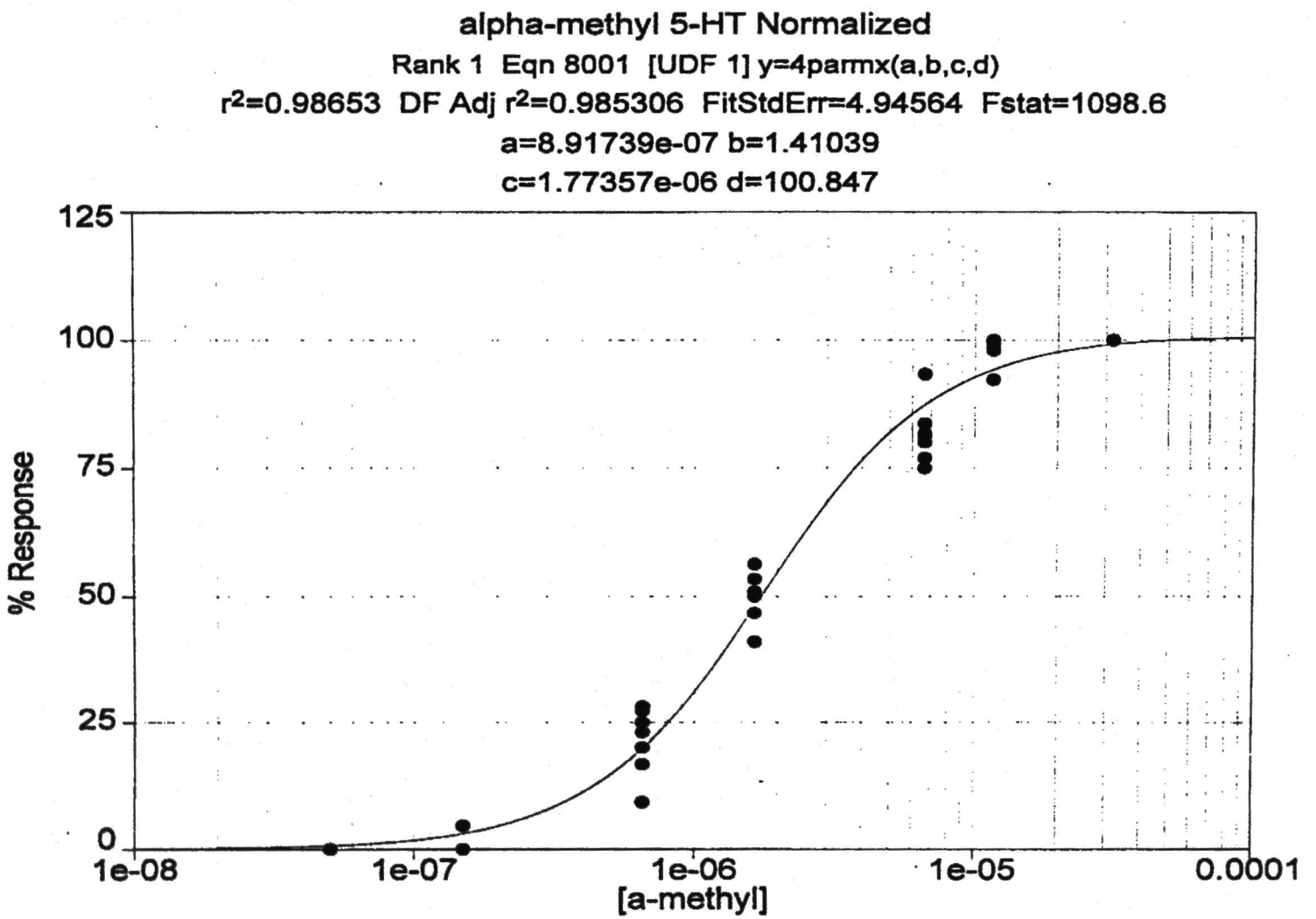


Figure: 15 Dose-response curves with normalized data for 5-HT (n=15), 5-$HT_{1\text{-like}}$ agonist, 5-CT (n=14) and 5-HT_2 agonist, α-methyl-5-HT (n=7). EC_{50} : 5-HT=283 nM, 5-CT = 363 nM, α-methyl 5-HT= 1.77 μM. Data represent mean response ± SEM.

Figure 16
Graph of Raw Data of Seven Dose-Response Curves Generated with α-Methyl-5-HT



TableCurve plot of normalized dose-response data with α-methyl-5-HT (n=7). a = minimum response concentration, b = Hill coefficient, c = EC_{50}, d = % maximum response, r2 = Goodness of Fit coefficient.

There have been reports of 5-$HT_{1\text{-like}}$ receptors acting like "silent receptors" that demonstrate greater efficacy if they are stimulated following the activation of another receptor (Yildiz, Smith and Purney, 1998) or depolarization. Vessels (n=5) were challenged with 40 mM KCl, 1 μM 5-HT and 1 μM 5-CT as in previous experiments. The initial 1 μM 5-CT induced contraction was 17.08% of the maximum. After the appropriate washout, a solution of 20 mM KCl was added to each tissue bath. The KCl contraction was allowed to reached a plateau and contraction was determined to be 48% of the maximum response to 5-HT. While the vessels were precontracted with the 20 mM KCl, 1 μM 5-CT was added to the tissue baths still containing KCl. The vessels treated with 20mM KCl and 1 μM 5-CT contracted an average of 124.26%. The addition of the 5-$HT_{1\text{-like}}$ agonist to a vessel already precontracted with KCl resulted in a contraction that was 4.46 times the contraction induced by 5-CT alone. Although I could not find a report in the literature of the 5-$HT_{1\text{-like}}$ receptor acting like a silent receptor in this tissue, the results of these experiments suggest that in fact the 5-$HT_{1\text{-like}}$ receptor does demonstrate greater efficacy following depolarization with KCl.

To determine if the agonists would constrict the vessel in a synergistic manner, vessels were constricted with each agonist individually and then with the agonists combined. The mean maximum response of vessels (n=4) constricted with 1 μM 5-CT was 13.8% and 10 μM α-methyl-5-HT was 71.3% (Figure 18, page 59). When the agonists were combined using the same concentrations, the response was 120% of the maximum response to 5-HT, indicating that the 5-$HT_{1\text{-like}}$ and 5-HT_2 agonists constrict the long posterior ciliary artery of the bovine eye in a synergistic manner.

Figure 17
Comparison of Vascular Response to 5-CT in Vessels
Pre-contracted with KCl

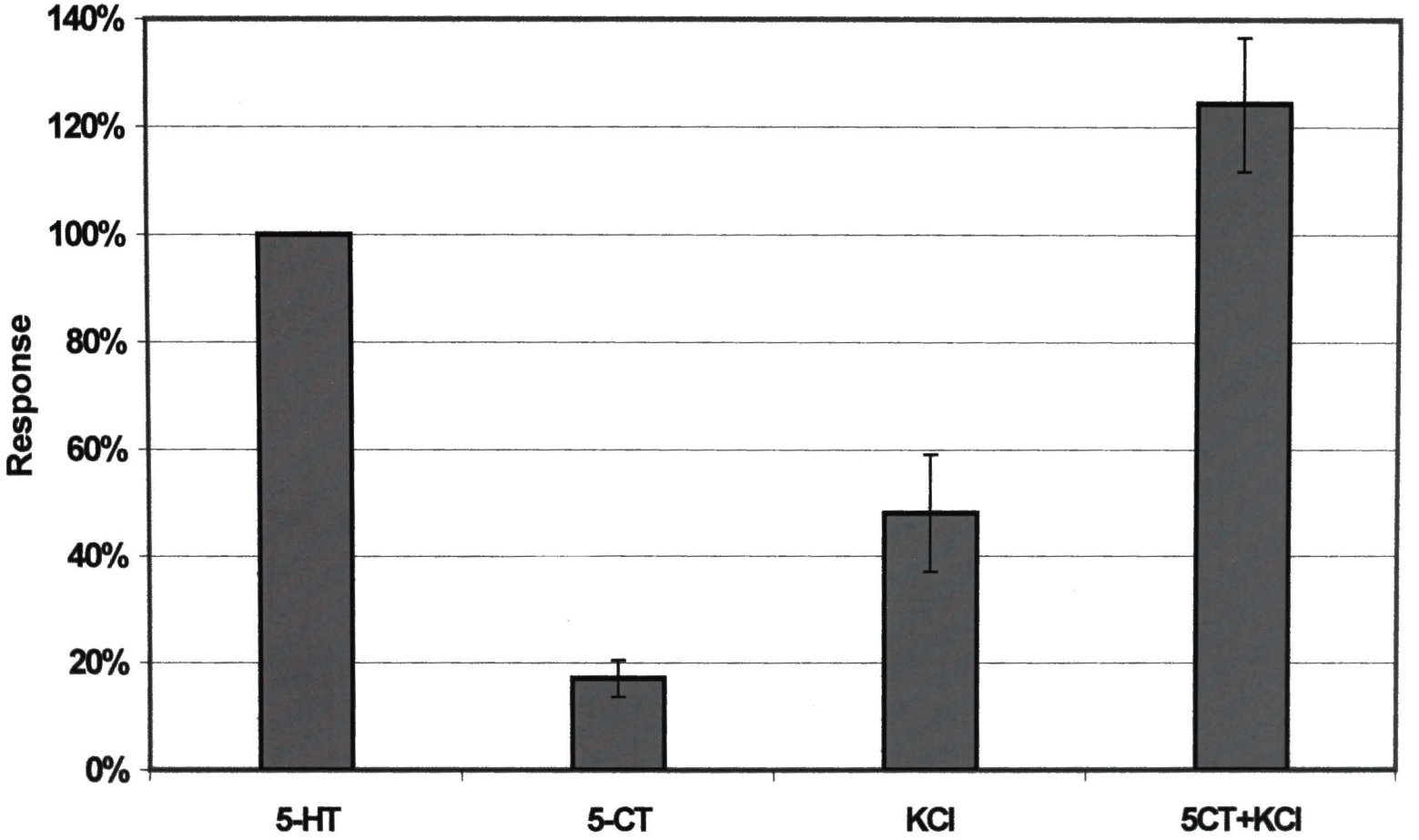


Figure 17: Comparison of mean vessel response to 5-HT $_{1\text{-like}}$ agonist (1 μM 5-CT)(n = 5) alone and 1 μM 5-CT plus 20 mM KCl. Data represent mean maximum response with mean value ± SEM shown by vertical bars.

Figure 18
Synergistic Affect of 5-CT and α-methyl-5-HT

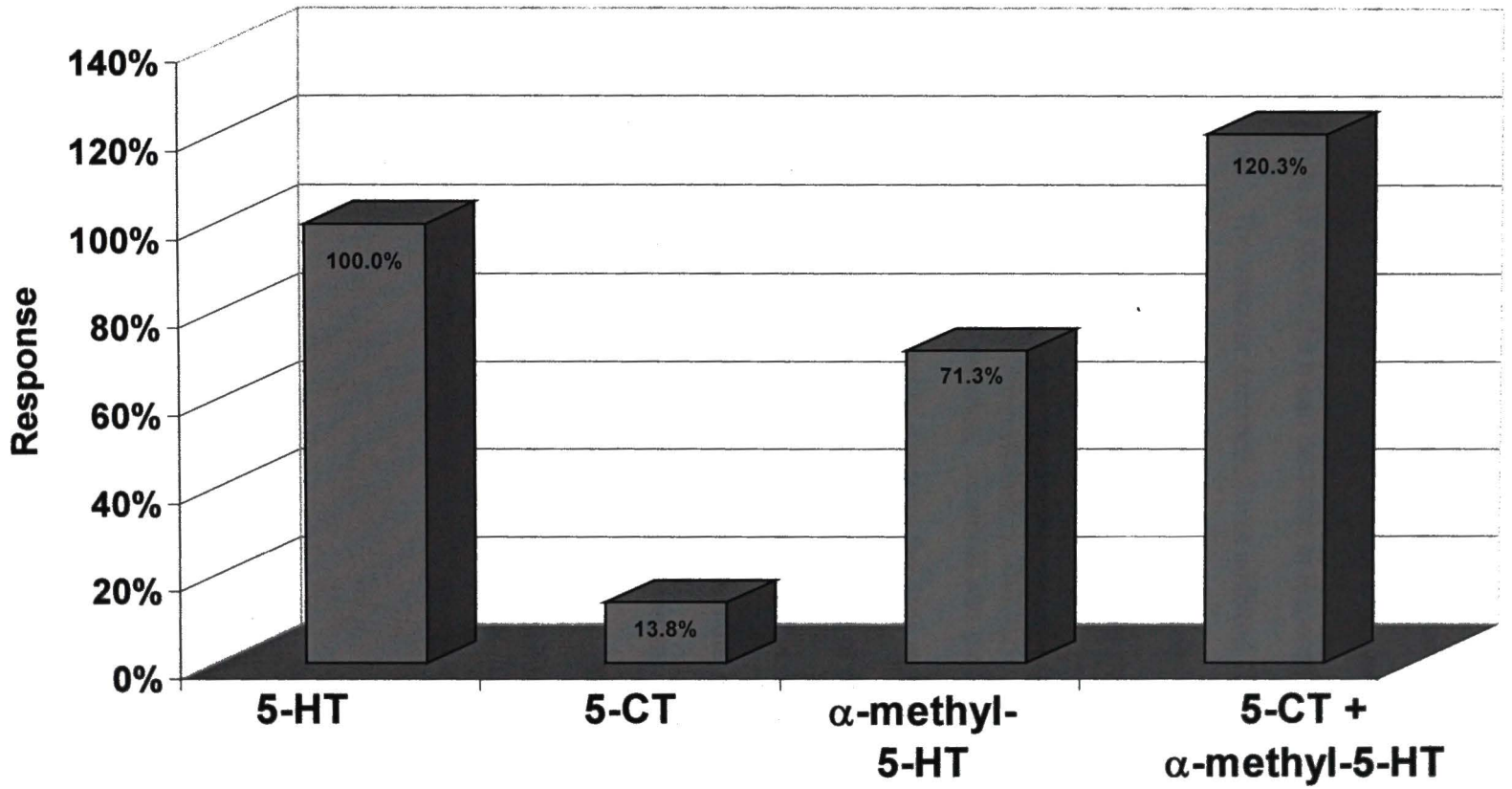


Figure 18: Comparison of vascular response (n=4) to 1 μM 5-$HT_{1\text{-like}}$ receptor agonist, 5-CT, 10 μM 5-HT_2 receptor agonist, α-methyl-5-HT, and 1 μM 5-CT + 10 μM α-methyl-5-HT.

In studies performed with selective antagonists, both the 5-HT $_{1\text{-like}}$ receptor antagonist, methiothepin, and the 5-HT_2 antagonist, ketanserin, effectively reduced the 5-HT induced contraction. Figure 19 shows the mean decrease in response to serotonin following incubation with methiothepin. The vascular contraction was inhibited 76.4% in vessels incubated with 1 nM methiothepin prior to the addition of 1 μM 5-HT. Incubation of the vessels with doses of methiothepin up to 100 nM resulted in decreases in contraction up to 85.5%.

For the dose inhibition curves, vessels were incubated with the lowest concentration of antagonist for approximately 20 minutes. Serotonin was then added in a cumulative fashion using the same concentrations used to generate the dose response curves. After each vessel had achieved the maximum contraction with the highest dose of serotonin, each vessel would undergo a washout period and then the next dose of antagonist would be added and the doses of serotonin would be repeated. Experiments continued in this manner until the vessels (n=12) had been incubated with 1 nM, 10 nM and 100 nM methiothepin.

The results of dose inhibition studies are shown in Figure 20. These data demonstrate that methiothepin inhibits the serotonin induced contraction in a noncompetitive manner.

Figure 19
Vascular Response to Serotonin Following Incubation with 5-HT_1 Antagonist Methiothepin

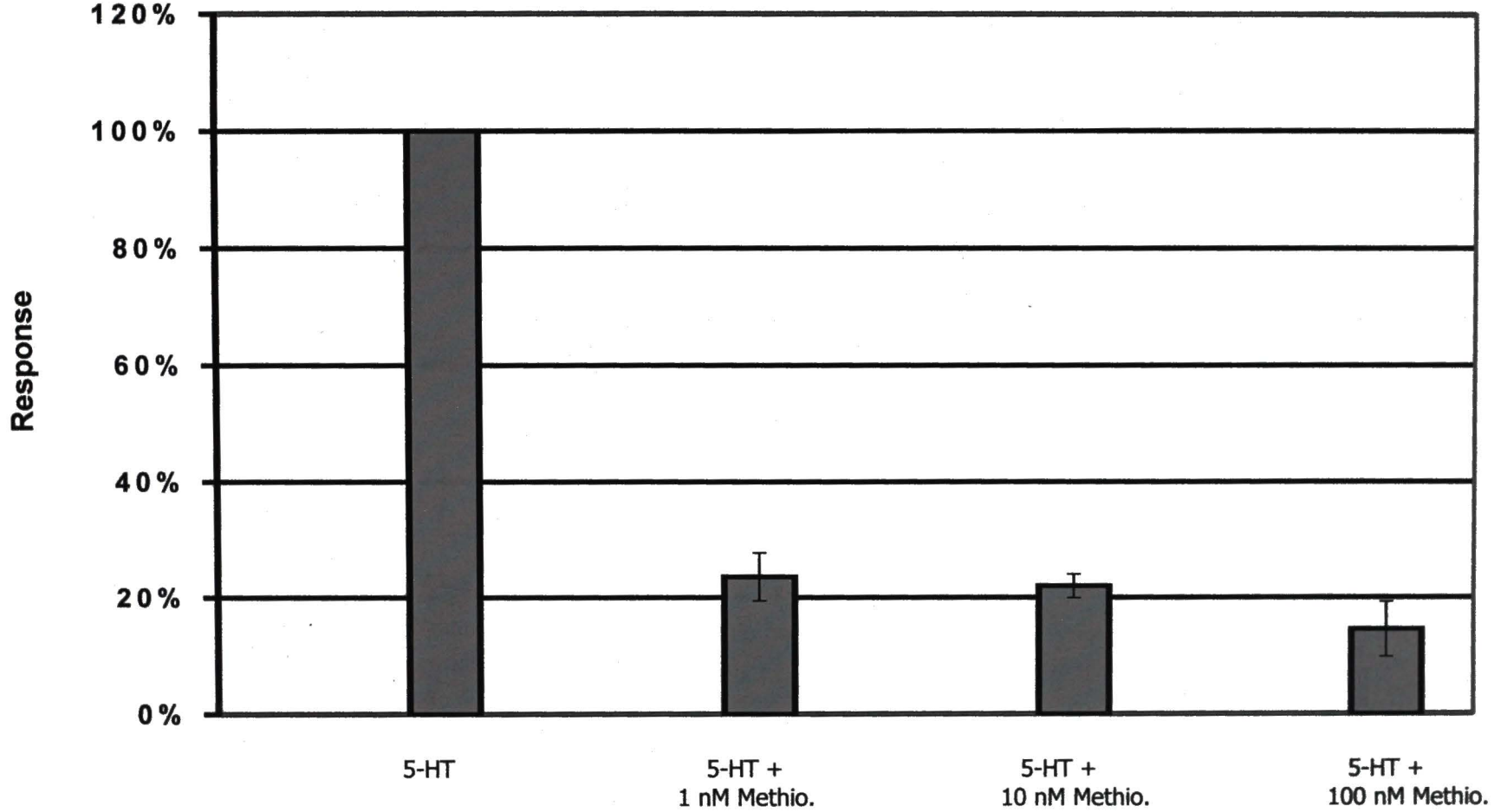


Figure 19: Comparison of response to 1 μM 5-HT in vessels (n=12) incubated with 5-HT $_{1\text{-like}}$ antagonist, methiothepin. Data respresent mean maximum response with mean value ± SEM shown by vertical bars.

Figure 20
Comparison of Vascular Response to Serotonin Following Incubation with 5-HT_1 Antagonist Methiothepin

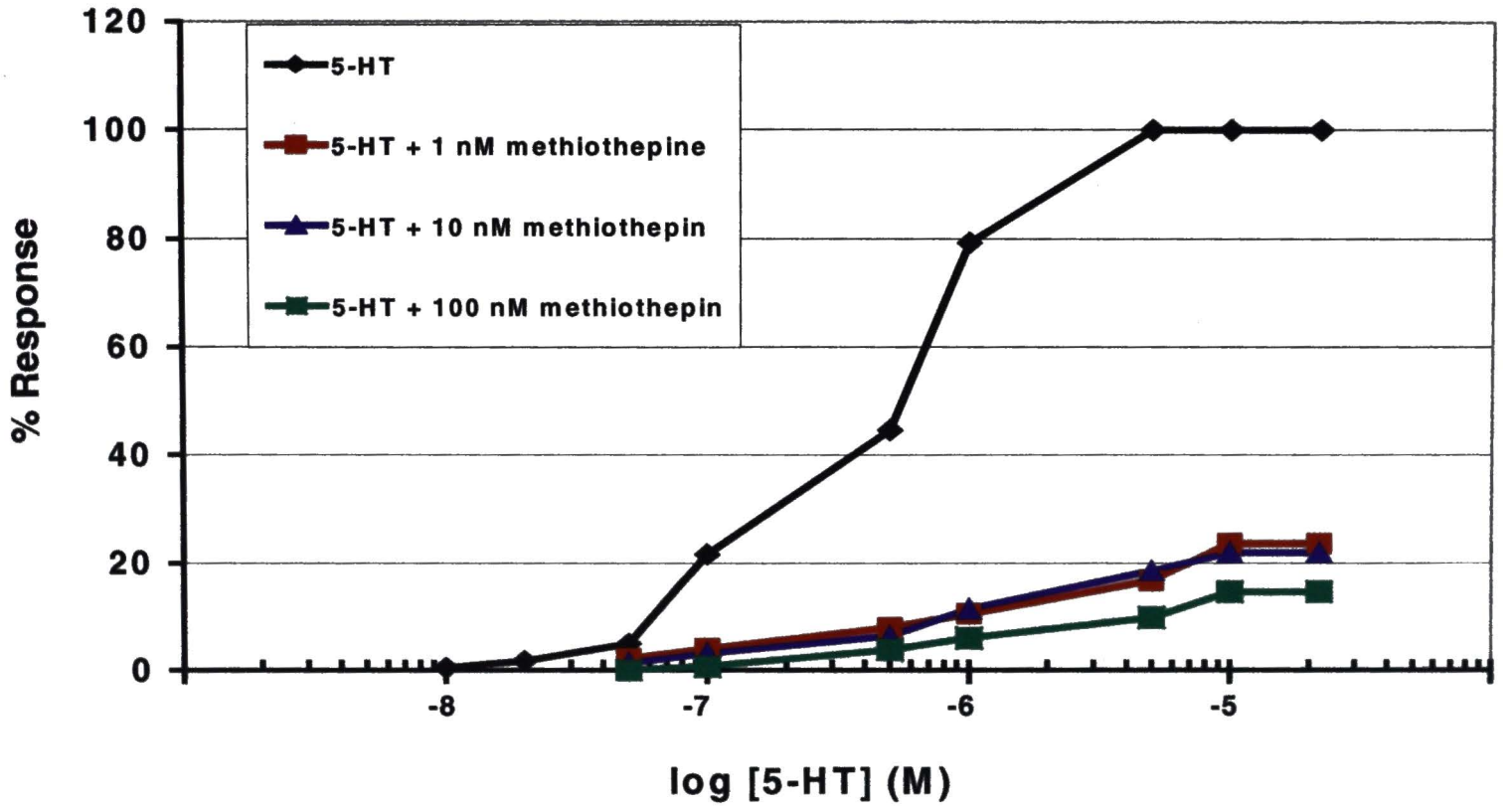


Figure 20: Comparison of 5-HT dose response curves (n=12) following incubation with increasing concentrations of the 5-$HT_{1\text{-like}}$ antagonist, methiothepin.

Similar experiments were conducted with the 5-HT_2 antagonist, ketanserin. Figure 21 shows the mean inhibition of the maximum serotonin response following incubation with each concentration of ketanserin. Vessels (n=14) were incubated with 1 nM, 10 nM, 50 nM, and 100 nM ketanserin prior to the cumulative doses of serotonin. These studies demonstrate that 1 nM ketanserin inhibits the serotonin induced contraction 56.2% in vessels incubated prior to the addition of 1 μM 5-HT. Incubation of the vessels with doses up to 100 nM resulted in decreases up to 75%.

The fact that neither selective antagonist at doses 100 times the IC_{50} totally blocked the serotonin induced contraction is evidence that the contraction is the result of activation of more than one serotonin receptor subtype. Additionally, when vessels are incubated with the antagonists together, the serotonin induced contraction was inhibited 100%. Vessels (n=8) incubated in 1 nM methiothepin and 1 nM ketanserin together failed to respond to 100 μM serotonin.

Figure 21
Vascular Response to Serotonin Following Incubation with 5-HT_2 Antagonist Ketanserin

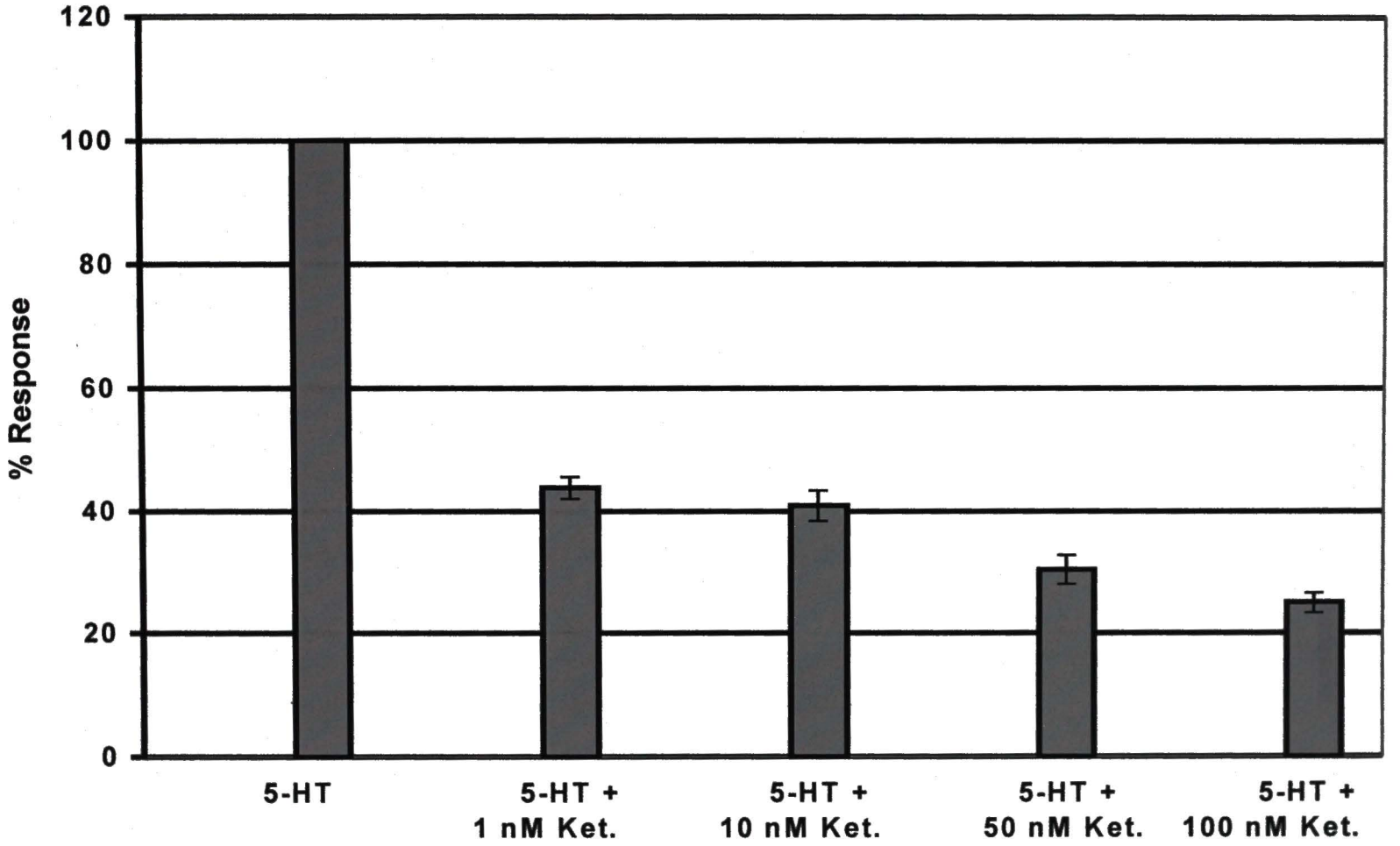


Figure 21: Comparison of response to 1 μM 5-HT in vessels (n=14) incubated with the 5-HT_2 antagonist, ketanserin. Data represent mean maximum response with mean value ± SEM shown by vertical bars.

Figure 22
Comparison of Vascular Response to Serotonin Following Incubation with 5-HT_2 Antagonist Ketanserin

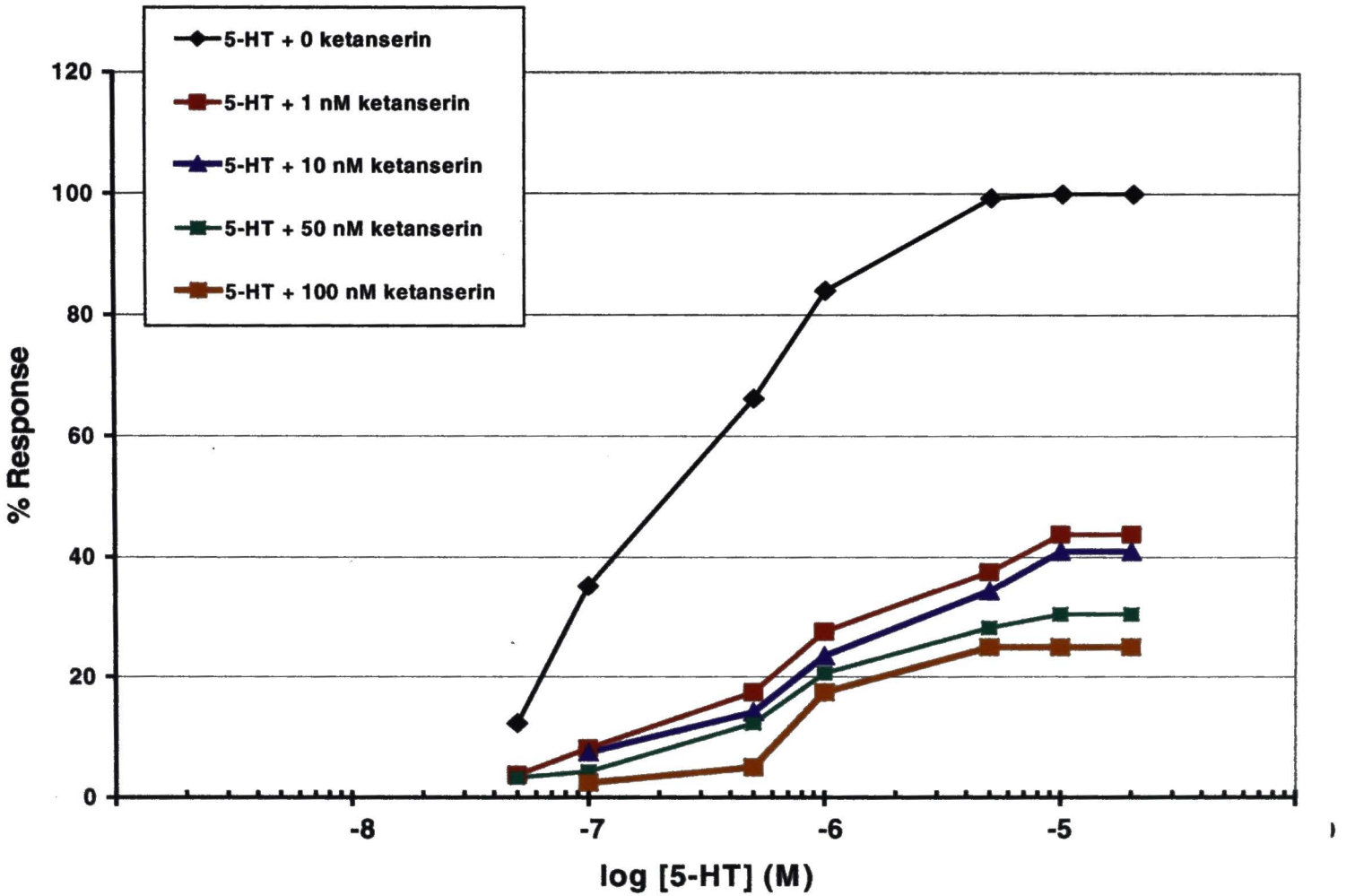


Figure 22: Comparison of 5-HT dose response curves (n=14) following incubation with increasing concentrations of the 5-HT_2 antagonist, ketanserin.

Specific Aim #2

Determination of the role of calcium mobilization

Vascular Contraction Experiments

Extracellular Calcium

The importance of extracellular calcium in the serotonin induced vascular contraction was examined by suspending the vascular ring segments in calcium free physiological buffer (PB) and by incubating the rings in PB with the calcium channel blockers diltiazem or nifedipine prior to the addition of serotonin. For each set of experiments, individual vessels were challenged initially with 40 mM KCl and 1 μM 5-HT to determine their maximum response.

Incubation of the vessels in calcium free PB (Figure 23, page 73) resulted in a significant reduction ($p<0.0001$) in maximum serotonin induced contraction. In the absence of extracellular calcium, the maximum response of the vessels ($n=18$) was reduced to 39.6%. Without extracellular calcium, the vessels were not able to maintain the contraction. Within 30 minutes, the vessels incubated in calcium free PB relaxed to baseline (Figure 24).

Similar results were found in experiments with the calcium channel blockers. Pretreatment of the vascular rings with diltiazem ($n=8$) and nifedipine ($n=4$) reduced the response to serotonin by 65.8% and 61.7%, respectively. Both methods used to investigate the role of extracellular calcium demonstrated significant reductions in the vascular contraction ($p<0.0001$) but there was no significant difference in the reduction produced by the two methods ($p=0.693$). In control vessels, the addition of either calcium channel blocker completely blocked the KCl response.

The results of these experiments indicate the influx of extracellular calcium plays a part in mediating the vascular contraction. However, the fact that a partial contraction was possible in

the absence of extracellular calcium would indicate that activation of the serotonin receptor(s) may stimulate the release of intracellular calcium or may increase the sensitivity of the contractile elements to calcium.

Figure 23
Response of Serotonin Induced Vascular Contraction
Following Incubation in Calcium Free PB
or with Calcium Channel Blockers

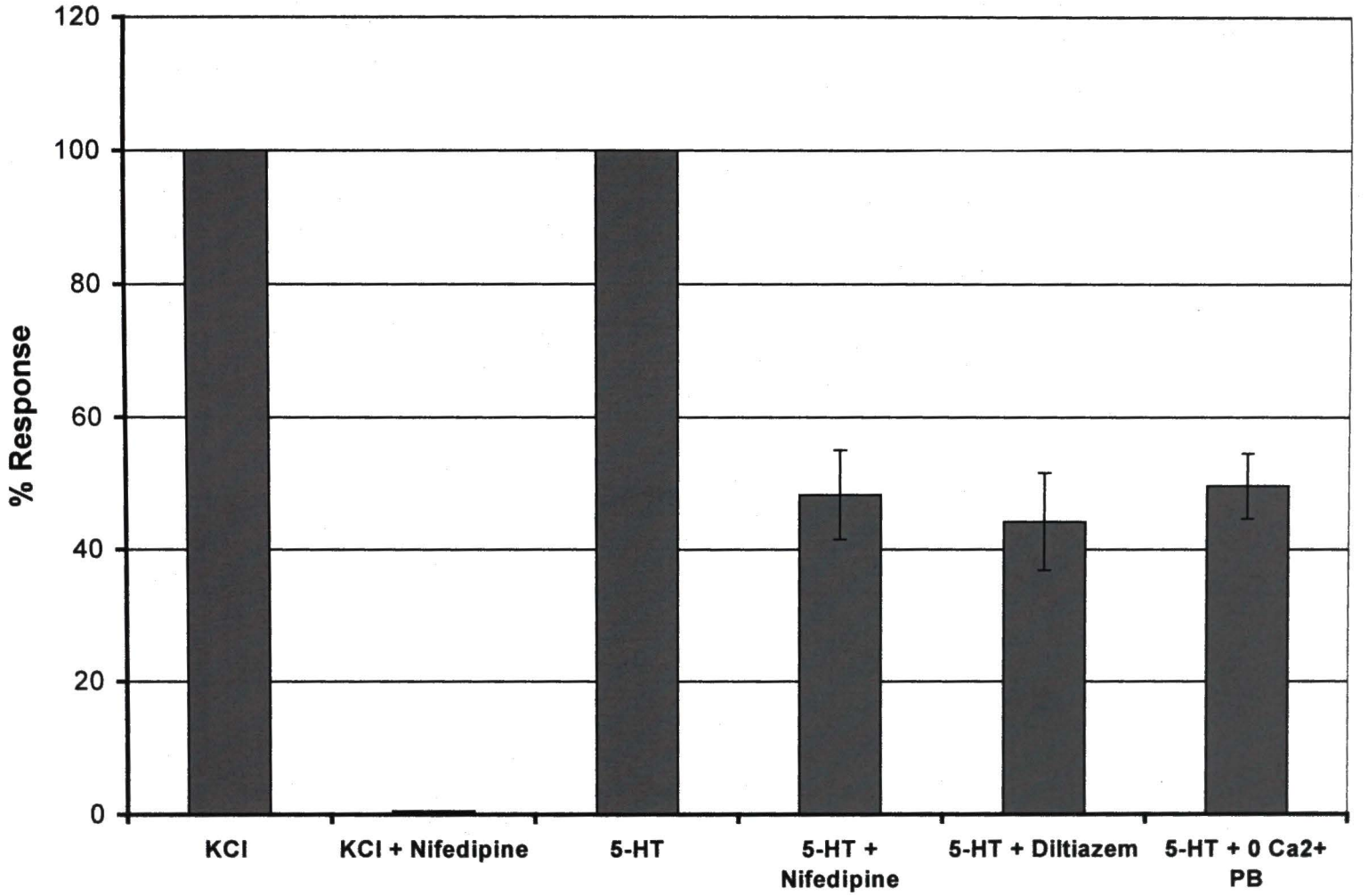
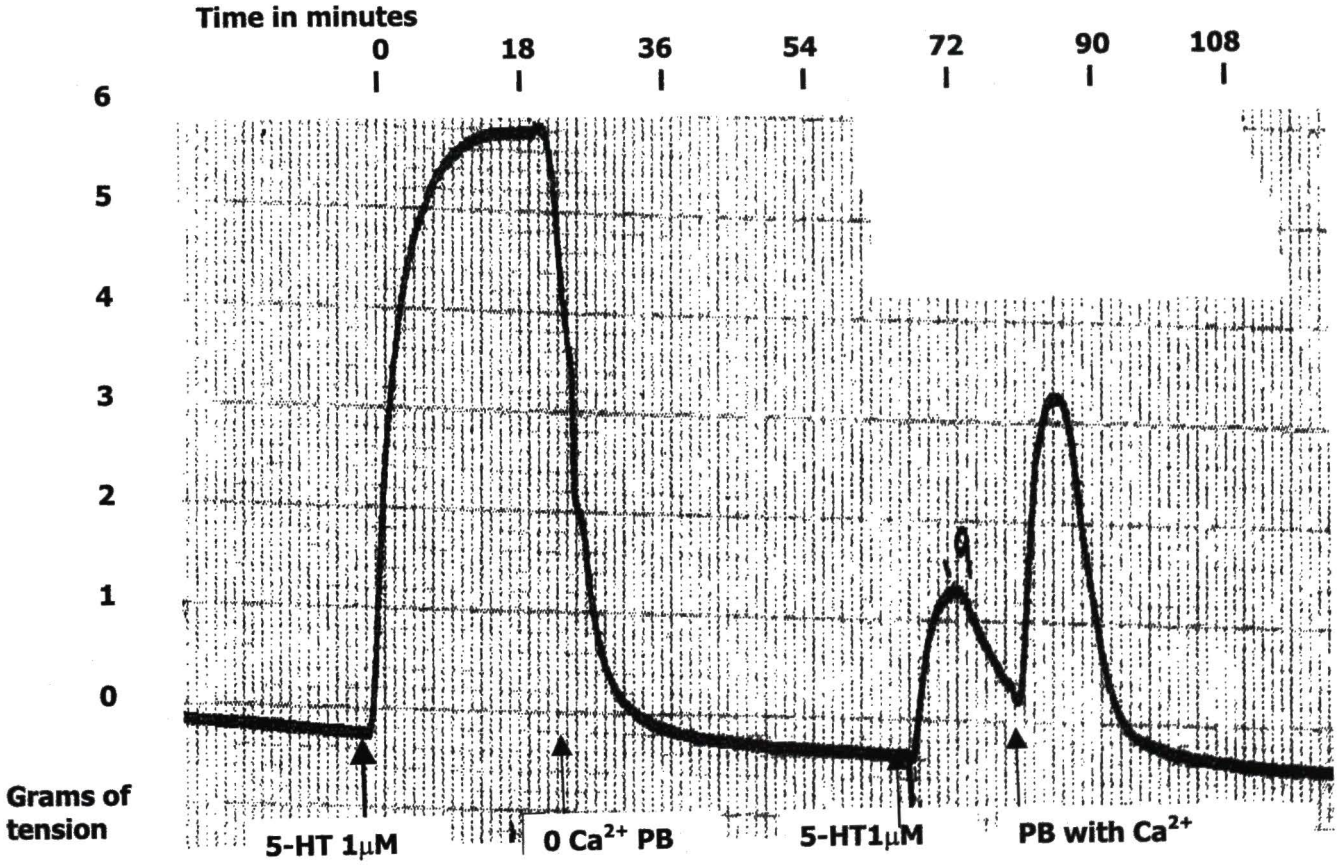


Figure 23. The mean vascular response to 1 μM serotonin following incubation of vascular rings in calcium free PB (n=18) or with calcium channel blockers, 10 μM Diltiazem (n=8) or 10 μM Nifedipine (n=4). Calcium free PB or calcium channel blockers significantly reduced the maximum response ($p < 0.0001$). There was no significant difference between responses with Diltiazem or Nifedipine ($p=0.986$) or between responses with calcium free PB and Diltiazem ($p=0.693$). Data represent mean maximum response with mean value ± SEM shown by vertical bars.

Figure 24
Physiograph Recording of Vessel Incubated in Calcium Free PB



Following incubation in O Ca^{2+} PB, 5-HT induced contractions are reduced and decline to baseline. Washout with PB (with Ca^{2+}) results in an immediate contraction.

Intracellular Calcium

The importance of the release of intracellular calcium to the contraction was determined by conducting experiments with agents that would either inhibit the release of intracellular calcium or would deplete the intracellular calcium stores. Ryanodine and IP_3 receptors are Ca^{2+} permeable channels that are associated with intracellular organelles. They are activated by second messengers to release calcium from intracellular stores. Although both receptors play a part in the storage and release of intracellular calcium, the two channel types are structurally and functionally distinct (Erlich *et al.*, 1994).

Experiments were performed with the ryanodine inhibitor, ryanodine. Vessels in this set of experiments were incubated with 1) 10 μM ryanodine (n=8), 2) 10 μM ryanodine plus the calcium channel blocker, 10 μM diltiazem (n=4), 3) 10 μM ryanodine in calcium free PB (n=9) and 4) 10 μM ryanodine plus 10 μM diltiazem in calcium free PB (n=3). The results of these experiments are shown in Figure 25. Ryanodine inhibited the serotonin induced contraction by 58.1%. When the vessels are incubated with ryanodine and a calcium channel blocker, the response was decreased to 25.9%. If both the ryanodine and diltiazem are added to calcium free PB, the response was reduced to 2.5% (Figure 25).

Additionally, experiments were conducted by incubating vascular ring segments in 20 mM caffeine that has been shown to deplete calcium from IP_3 sensitive stores in the sarcoplasmic reticulum (Endo, 1985). Vascular ring preparations were incubated in either physiological buffer with 20 mM caffeine or calcium free PB with 20 mM caffeine. In all vessels, the incubation with 20 mM caffeine (n=8) totally inhibited the vascular response to 5-HT (Figure 26).

These experiments were repeated and the concentration of caffeine was reduced to 10 mM. The results with the reduced concentration of caffeine were the same. These results would indicate that the serotonin induced vasoconstriction is 100% dependent on the release of intracellular calcium from IP_3 sensitive calcium stores.

Figure 25
Serotonin Response Following
Incubation with Ryanodine

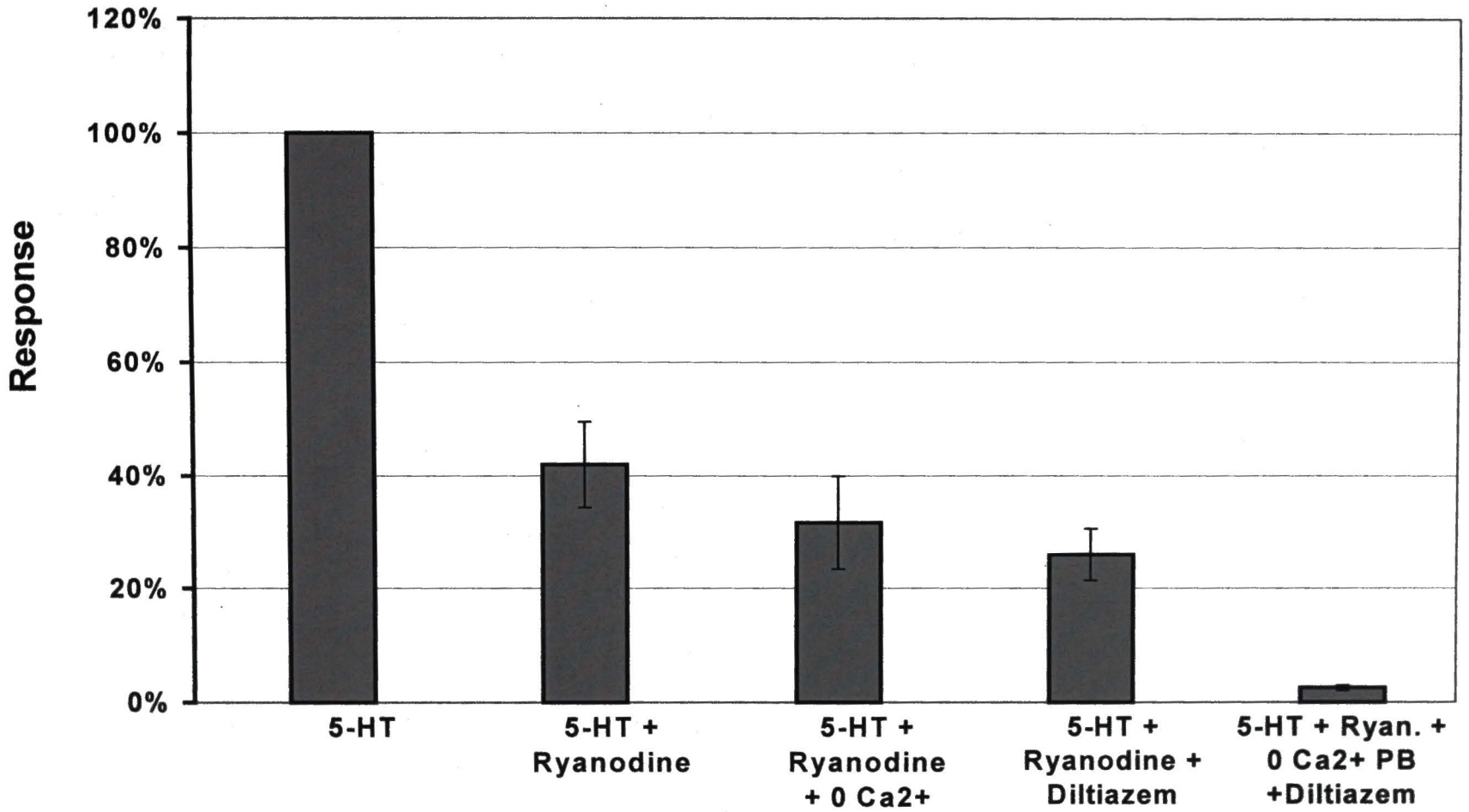


Figure 25: The mean serotonin induced vascular contraction was significantly inhibited following incubation with 10 μM ryanodine (n=8), 10 μM ryanodine with calcium free PB (n=9), 10 μM ryanodine and 10 μM diltiazem (n=4), and 10 μM ryanodine with calcium free PB and 10 μM diltiazem (n=4) ($p<0.0001$). Data represent mean maximum response with mean value ± SEM shown by vertical bars.

Figure 26
Inhibition of Serotonin Induced Vascular Contraction
by Ryanodine or Caffeine

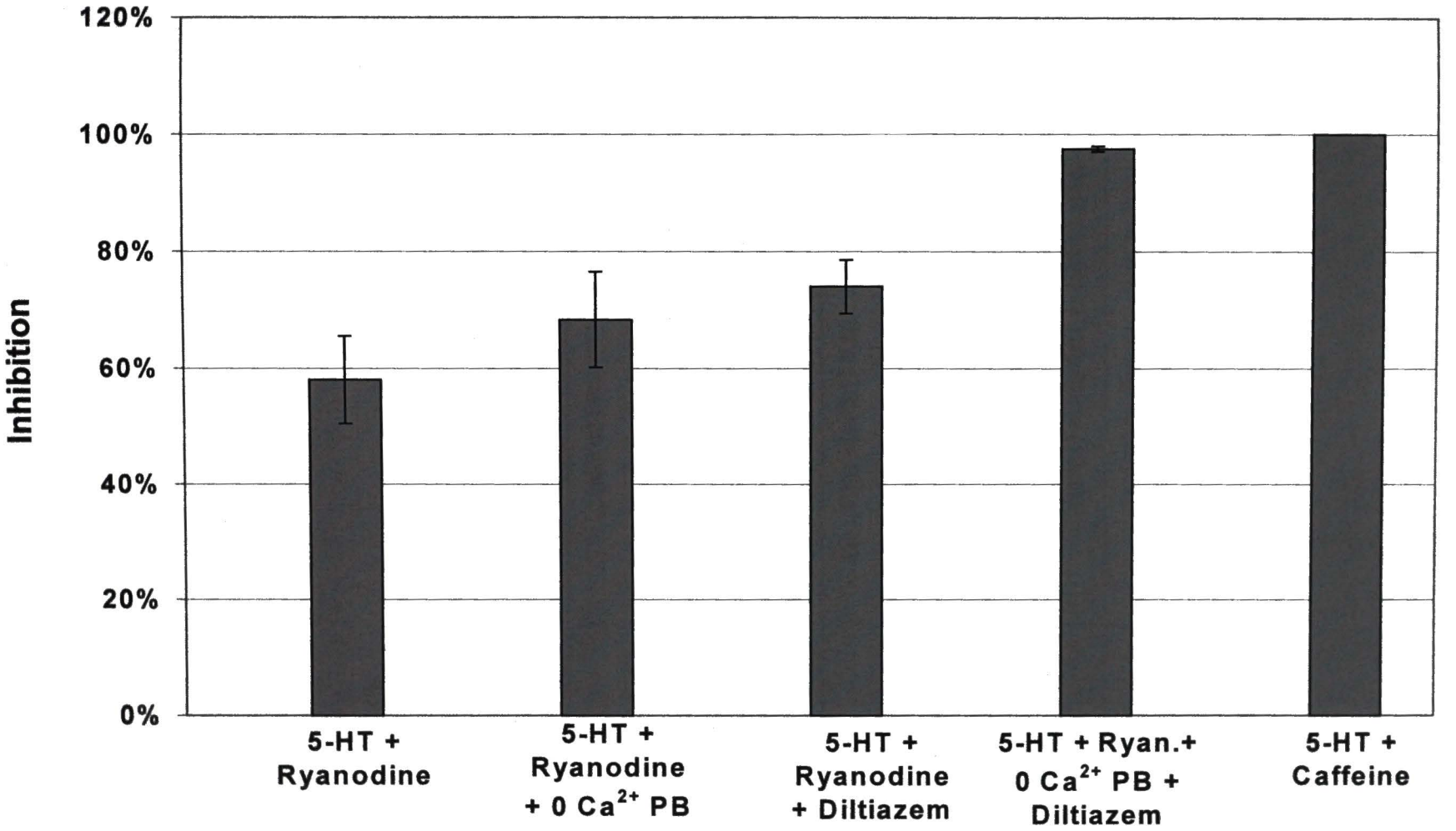


Figure 26. The mean inhibition of serotonin induced vascular contraction following incubation with 10 μM ryanodine (n=8), 10 μM ryanodine and calcium free PB (n=9), 10 μM ryanodine and 10 μM diltiazem (n=4), 10 μM ryanodine with calcium free PB and 10 μM diltiazem (n=4), and 10 mM caffeine (n=8)..

Table 4
Percent Inhibition by Agents that Affect Mobilization of Either Extracellular or Intracellular Calcium

Treatments	Mean % Inhibition ± SEM	n
KCl (40 mM) + Nifedipine (10μM)	*100	4
5-HT (1 μM) + Nifedipine (10μM)	*61.7 ± 6.7	4
5-HT (1μM) + Diltiazem (10μM)	*65.8 ± 7.3	8
5-HT (1 μM) + 0 Ca^{2+} PB	*60.4 ± 4.9	18
5-HT (1μM) + Ryanodine (10μM)	*58.0 ± 7.5	8
5-HT (1μM) + Ryanodine (10μM)+ 0 Ca^{2+} PB	*68.3 ± 8.2	9
5-HT (1μM) + Ryanodine (10μM) + Diltiazem (10μM)	*74 ± 4.5	4
5-HT (1μM) + Ryanodine (10μM)+ 0 Ca^{2+} PB + Diltiazem (10μM)	*97.5 ± 4.7	3
5-HT (1μM) + Caffeine (10 mM)	*100	8
5-HT (1μM) + Caffeine (20 mM)	*100	8

*$p<0.0001$

Dynamic Video Imaging Experiments

Primary cell cultures were grown and calcium imaging experiments were planned to quantitate the serotonin-mediated change in intracellular calcium. Cell cultures that appeared morphologically to be pure cultures of vascular smooth cells were prepared as described in the Methods section. Unfortunately, repeated attempts failed to demonstrate contraction of these cells as evidenced by changes in intracellular calcium concentrations. A variety of other vasoactive agents such as 100 μM carbachol and 10 μM histamine were also investigated but the cells failed to response to those also. These cells did stain positive for α–actin and myosin, suggesting that they were vascular smooth muscle cells. The failure of these cells to respond to these agents would indicate that either the cells were not a pure culture of vascular smooth muscle cells or they lost receptors during the culturing process.

Specific Aim #3

Determination of the role of PKC or PLC

To investigate the role that PLC or PKC plays in the serotonin induced vascular contraction, experiments were conducted using pharmacological agents that would selectively inhibit either PKC or PLC. The vascular response to serotonin following incubation with these agents was measured. For each set of experiments, individual vessels were challenged initially with high KCl or serotonin to determine their maximum response.

All three PLC antagonists reduced the contraction of the long posterior ciliary artery to serotonin. Incubation of the vascular ring segments with one of three PLC antagonists, 70 μM 2-NCDC (n=11), 0.5 μM U73122 (n= 12), or 5 mM neomycin (n=6), prior to the addition of 1 μM serotonin, significantly reduced the contraction of each vessel, $p<0.0001$. Vessels incubated with 5 mM neomycin (n=6) immediately relaxed below baseline. The addition of serotonin in

concentrations as high as 100 μM failed to produce a contraction. The fact that the vessels relaxed prior to the addition of serotonin would indicate that antagonism produced by neomycin was of a nonspecific nature. Therefore, these results did not allow conclusions to be drawn concerning the affect of neomycin on the serotonin induced contraction. Neomycin was the first of the three PLC inhibitors that was investigated and based upon the findings described above, the decision was made to repeat the experiments with additional selective agents.

The contraction in the vessels incubated with 70 μM 2-NCDC prior to the addition of 1 μM serotonin was inhibited an average of 87.7%. In each set of experiments, a control vessel was incubated in 2-NCDC prior to the addition of high KCl. 2-NCDC failed to block the high KCl contraction indicating that 2-NCDC did not reduce the serotonin induced contraction in a nonspecific manner.

Incubation of the vessels with 0.5 μM U73122 also significantly reduced the contraction ($p<0.0001$). Vessels incubated with U73122 prior to the addition of serotonin demonstrated a 66.39% reduction as compared to prior contractions measured with serotonin alone. Although there is a difference in the mean % inhibition demonstrated by both of these agents, the difference is not significant ($p=0.1201$).

Figure 27
Serotonin Response Following Incubation with PLC Inhibitors

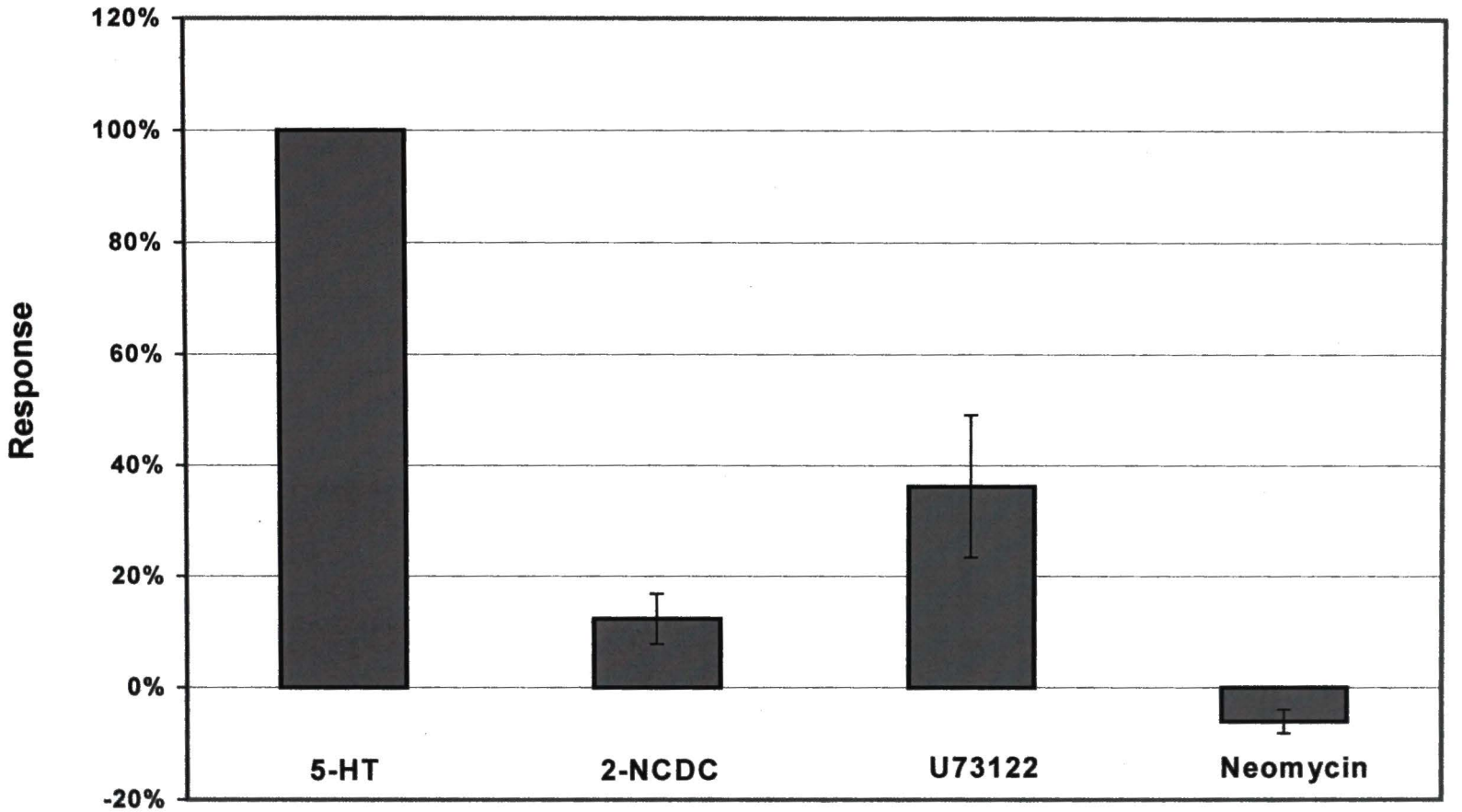


Figure 27: Vascular responses to 1 μM 5-HT (n=12) were inhibited following incubation of the vascular rings with selective PLC antagonists. A statistically significant reduction was demonstrated with 70 μM 2-NCDC (n=11), 0.5 μM U73122 (n=12) and 5 mM neomycin (n=6), $p<0.0001$. Data represent mean response with mean value ± SEM shown by vertical bars.

PKC inhibitors, 25 μM chelerythrine (n=11), 1 μM bisindolylmaleimide II (n=5), and 5 μM H-7, (n=8) significantly inhibited the serotonin induced vasoconstriction of the long posterior ciliary artery of bovine eyes ($p<0.0001$). Vessels were challenged with 40 mM KCl or 1 μM serotonin to determine their maximum response to these agents. The vessels were washed and allowed to return to baseline. Following incubation with either chelerythrine, bisindolylmaleimide II, or H-7, serotonin was again added to the tissue bath and the contraction was measured and compared to the initial contraction.

Bisindolylmaleimide II reduced the serotonin induced contraction in a dose-dependent manner. Bisindolylmaleimide II 1 μM and bisindolylmaleimide II 10 μM decreased the vascular response to serotonin by 19.8% and 55.7%, respectively. Bisindolylmaleimide V 10 μM (n=10), inactive analog, failed to significantly inhibit the vascular response ($p=0.2474$).

Chelerythrine and H-7 significantly reduced the vasoconstriction by 31.1% and 61.5%, respectively (Figure 28, page 87). Although all three agents tested in these experiments significantly reduced the vascular response to serotonin, the mean % reduction varied between agents. The only significant difference in reduction was between the reductions produced by chelerythrine and bisindolylmaleimide II 1 μM ($p=0.0120$). All other comparisons of inhibition failed to detect a significant difference between PKC inhibitors.

Figure 28

Serotonin Response Following Incubation with PKC Inhibitors

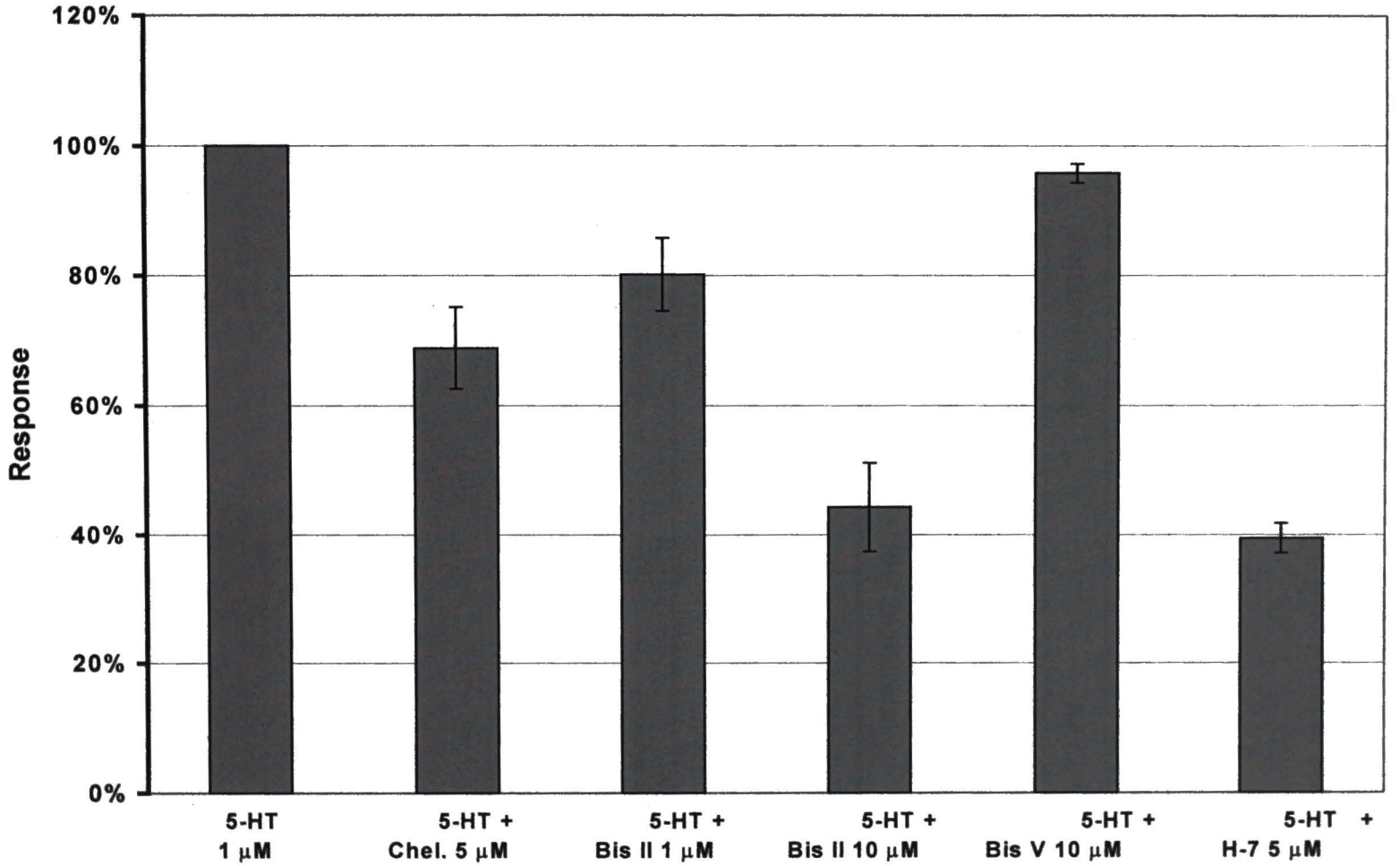


Figure 28: Vascular responses to 1 μM 5-HT (n=11) were inhibited following incubation of the vascular rings with selective PKC antagonists. Statistically significant inhibition of contraction 001was demonstrated with 25 μM Chelerythrine ** (n=11), 1 μM Bisindolylmaleimide II * (n=5), 10 μM Bisindolylmaleimide II ** (n=10), and 5 μM H-7 ** (n=8). Bisindolylmaleimide V 10 μM (n=10) did not significantly inhibited the contraction, p=0.2475. Data represent mean responses with mean value ± SEM shown by vertical bars. *p<0.05, **p<0.0001

Table 5
Percent Decrease in Serotonin Induced Contraction by Agents that Inhibit PLC or PKC

PLC Inhibitors		
Agents	**Mean % Inhibition ± SEM**	**n**
2-NDCD (70 μM)	87.7 ± 4.5**	11
U73122 (0.5 μM)	66.4 ± 12.8**	12
Neomycin (5 mM)	106.0 ± 2.1**	6
PKC Inhibitors		
Agents	**Mean % Inhibition ± SEM**	**n**
Chelerythrine (25 μM)	31.1 ± 6.2**	11
Bis II (1 μM)	19.8 ± 5.5*	5
Bis II (10 μM)	55.7 ± 6.9**	10
Bis V(10 μM)	4.3 ± 1.4	10
H-7 (5 μM)	61.5 ± 2.3**	8

*$p < 0.05$
**$p < 0.0001$

CHAPTER IV

DISCUSSION

Specific Aim #1

Serotonin receptor subtype identification

Glaucoma is a multifactorial disease and the pathogenesiof glaucomatous damage is poorly understood. It is clear that there are a number of risk factors that are involved in the disease process. Vascular changes such as systemic hypotension and vasospasm as well as increased intraocular pressure play a role in the disease. Furthermore, it is conceivable that an underlying disorder leading to vascular dysfunction might influence the intraocular pressure (Wiederholt *et al.*, 1994). Additionally, a disturbance of the autoregulation of blood flow in the optic nerve head, choroid, and other ocular tissues may contribute to the pathogenesis of the disease.

Serotonin has been shown to possess both vasoconstrictor and vasodilator properties in various vascular beds (Van Nueten *et al.*, 1984). Increasing evidence points to a multiplicity of peripheral vascular 5-HT receptors (McFadden *et al.*, 1991; Garland, 1987; Houston and Vanhouette, 1986). There has been a focus in migraine research to identify 5-HT binding sites in cerebral vascular tissue, but there is a need to identify functional peripheral 5-HT receptors. In many vascular beds, serotonin causes vasoconstriction by interacting with 5-HT_2 receptors. Vascular 5-HT_2 receptors have been identified in the rat caudal artery and in the gastrosplenic artery of the dog (Peroutka and Snyder, 1979; Leysen et al., 1981).

In other blood vessels, 5-HT induced contraction is mediated through activation of 5-$HT_{1\text{-like}}$ receptors that have been classified on the basis of a high affinity for the agonist 5-CT

and the susceptibiity to antagonism by methiothepin (Feniuk and Humphery, 1989; Bradley *et al.*, 1986; Ebersole *et al.*, 1993).

In canine basilar artery, the vasoconstrictor response to 5-HT has been shown to be mediated by both 5-HT_2 and 5-$HT_{1\text{-like}}$ receptors (Conner *et al.*, 1989; Frenken, 1989; Apperley et al, 1980) whereas in the rabbit and human basilar artery (Bradley *et al.*, 1986; Parson *et al*, 1989), the response to 5-HT appears to be mediated primarily by 5-$HT_{1\text{-like}}$ receptors.

The results of the vascular contraction experiments with ring segments of the long posterior ciliary artery of the bovine indicate that the serotonin induced vasoconstriction is mediated through the activation of more than one serotonin receptor subtype (see Figure 29). Dose-response curves constructed with selective 5-$HT_{1\text{-like}}$ and 5-HT_2 agonists demonstrate that both agents are able to effectively constrict the LPCA of the bovine eye.

The threshold doses of the three serotonin receptor agonists ranged from 10 nM to 73 nM. These concentrations are 10 times higher than those required in cat ophthalmociliary artery (Yu *et al.*, 1992), human ciliary artery (Okubo and Chiba 1988), monkey ophthalmic and ciliary arteries (Okubo and Chiba 1987), and in bovine long posterior ciliary artery as reported by Dalske (1974). The difference in potency is probably due not only to differences in species and ocular vascular tissues but also in experimental technique. The contraction experiments reported by Yu *et al.* (1992) were conducted under zero baseline tension, where experiments reported by Dalske in bovine LPCA were conducted under 5 grams of tension. The data reported by Okubo and Chiba in both the monkey and human were collected as changes in pressure utilizing a perfusion method instead of contraction.

In the results reported here, the potency demonstrated by these agonists are 5-HT >5-CT> α-methyl-5-HT. The potency order exhibited by these agonists strongly support the presence of the 5-$HT_{1\text{-like}}$ receptor. However, the 5-HT_2 agonist, α-methyl-5-HT, demonstrated greater efficacy than the 5-$HT_{1\text{-like}}$ agonist. The mean vascular response to α-methyl-5-HT was

4.1 times as great as the mean response to 5-CT. This further corroborates the supposition that there are two serotonin receptor subtypes responsible for the serotonin induced vasoconstriction of the LPCA.

The EC_{50} for 5-HT in this tissue was determined to be 283 nM. This is very similar to the 175 nM EC_{50} for 5-HT as reported by Dalske (1974) for bovine LPCA but it is 6 times the EC_{50} reported for human ciliary arteries (Ohkubo and Chiba, 1988).

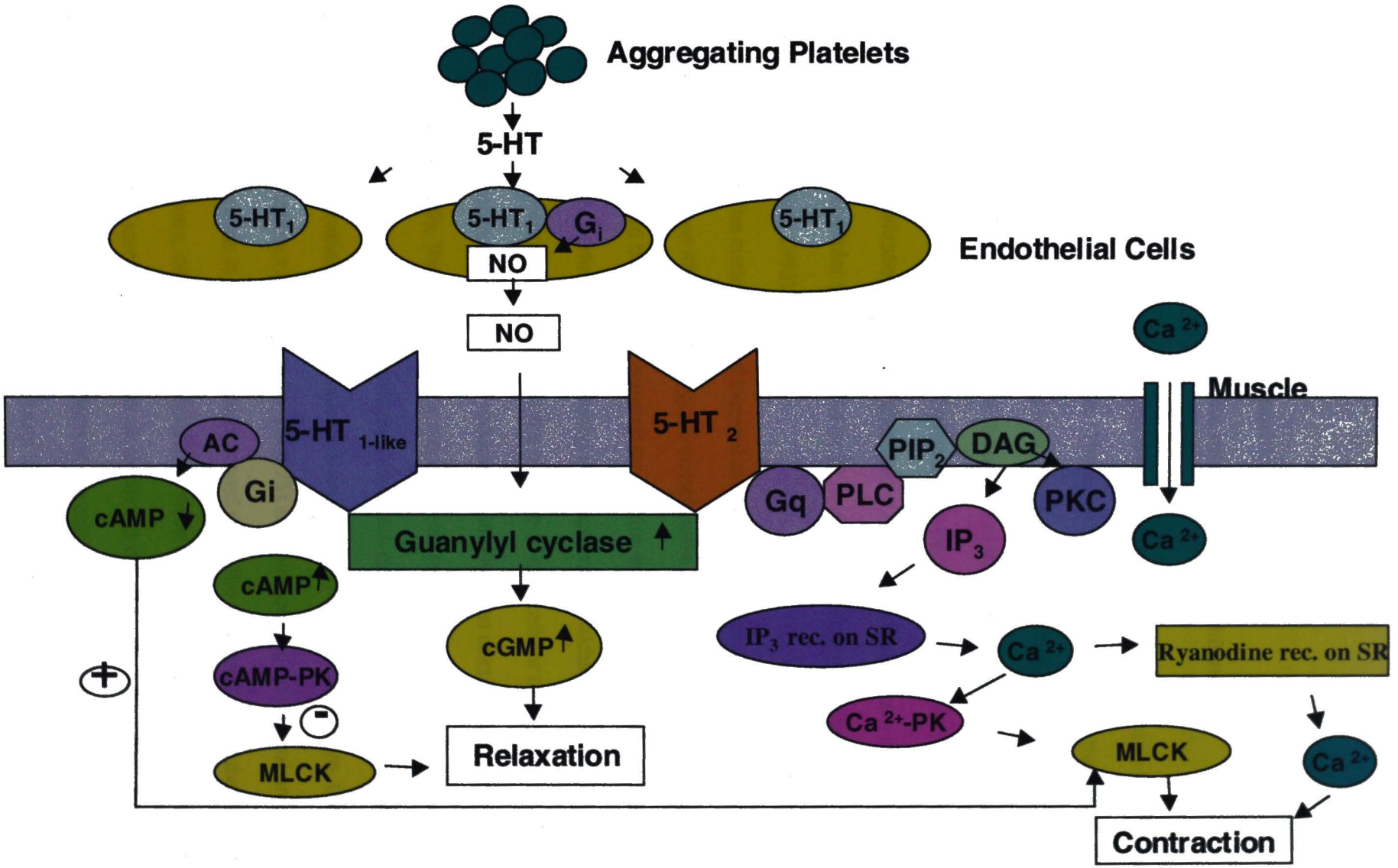
"Uncovered" or "silent" receptor characteristics for vasoconstrictor effects have been reported for 5-$HT_{1\text{-like}}$ receptors in guinea pig iliac artery (Sahin-Erdemli et al., 1991) and rabbit femoral arteries (MacLennan and Martin, 1992) by first inducing contractions with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or thromboxane A_2. In rabbit ear (Movahedi et al., 1995) and renal arteries (Choppin and O'Connor, 1993), 5-$HT_{1\text{-like}}$ receptors were shown to be uncovered following contractions induced with methoxamine and histamine, respectively. Current evidence suggest that only 5-$HT_{1\text{-like}}$ receptors display silent receptor characteristics in the 5-HT receptor families (Yildiz, Smith and Purdy, 1998). Results reported here with the 5-$HT_{1\text{-like}}$ agonist, 5-CT, indicate that the 5-$HT_{1\text{-like}}$ receptor is exhibiting the silent receptor characteristics in this tissue. In vessels precontracted with 20 mM KCl, the vascular response to the addition of 5-CT was 4.46 times the response of 5-CT in vessels not precontracted. Although the mechanisms that mediate this response are unclear, changes in cytosolic calcium may be important. Yildiz and Tuncer (1995) have proposed that the initial contraction with an agonist, elevates cytosolic calcium, and that the potassium opens voltage sensitive calcium channels. The 5-$HT_{1\text{-like}}$ receptor is inversely coupled to adenylyl cyclase, lowering cAMP levels. It is proposed that at this point the receptor is silent because cytosolic calcium levels are close to or below the concentration required for threshold contraction. After stimulation by another agonist or by depolarization, the concentration of cytosolic calcium is elevated and is maintained constant in a dynamic steady state by the calcium elevating actions of the agonist and the calcium lowering actions of the second messenger, cAMP. Upon addition of

5-CT or 5-HT, the resulting 5-$HT_{1\text{-like}}$ receptor activation inhibits adenylyl cyclase, lowering cAMP. The elevation of cytosolic calcium leads to further vasoconstricition. Under these conditions, the 5-$HT_{1\text{-like}}$ receptor is enabled and appears to be mediating a greater than expected contraction. As part of this model, the 5-$HT_{1\text{-like}}$ receptor is lowering cAMP levels that would otherwise inhibit the vasoconstriction. In support of the model, α_2-adrenergic (Minneman *et al.*, 1994) and neuropeptide Y_1 receptors (Wahlestedt, Regunathan and Reis, 1992) are coupled inversely to adenylyl cyclase and the have been shown to display silent receptor characteristics including vasoconstrictor synergism.

The results of experiments using selective 5-HT receptor antagonists added additional support for the presence of two serotonin receptor subtypes in the long posterior ciliary artery of the bovine eye. Both methiothepin and ketanserin noncompetitively inhibited the serotonin response in these vessels. Noncompetitive inhibition has been demonstrated by both of these compounds in other studies (Haefliger, Flammer and Luscher, 1993; Yildiz et al., 1996, Van Neuten 1981). The IC_{50} for methiothepin and ketanserin were less than 1 nM. However, increasing the concentration of the antagonist 100 fold did not totally inhibit the response. If only one receptor subtype were present, one would expect that incubation of the vessel with a selective antagonist in concentrations 100 times the IC_{50}, would totally block the contraction. That did not occur in any of the 26 vessels tested. Additionally, 100% inhibition was demonstrated when vessels were incubated in a solution of 1 nM ketanserin plus 1 nM methiothepin indicating that it is the activation of both receptor subtypes that participate in the contraction.

Figure 29
Vascular Effects of Activation of 5-$HT_{1\text{-like}}$ and 5-HT_2 Receptors

Schematic diagram illustrating the cellular effects of serotonin on vascular smooth muscle cells. Abbreviations used are as follows: 5-HT_1 = 5-HT_1 receptor, G_i = G-protein-inhibition, NO = nitric oxide, 5-$HT_{1\text{-like}}$= 5-$HT_{1\text{-like}}$ receptor, PLC = Phospholipase C, PIP_2 = Phosphatidylinositol 4,5, bisphosphate, PKC= protein kinase C, DAG=Diacylglycerol, G_q = G_q- protein, AC = adenylyl cyclase, SR = sarcoplasmic reticulum, cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophosphate, IP_3 = inositol trisphosphate, MLCK = myosin light chain kinase, cAMP-PK = cyclic AMP dependent protein kinase (protein kinase A), Ca^{2+} = calcium, Ca^{2+} - PK = calcium dependent protein kinase.


Aggregating Platelets
5-HT
5-HT1
5-HT1
Gi
NO
5-HT1
Endothelial Cells
NO
Ca 2+
Muscle
AC
5-HT 1-like
5-HT 2
PIP2
DAG
Gi
Gq
PLC
PKC
cAMP
Guanylyl cyclase
IP3
Ca 2+
cAMP
cGMP
IP3 rec. on SR
Ca 2+
Ryanodine rec. on SR
cAMP-PK
Ca 2+-PK
MLCK
Relaxation
MLCK
Ca 2+
Contraction

Specific Aim #2

Determination of the role of calcium mobilization

Increases in intracellular calcium concentration is the primary mechanism that initiates contraction in vascular smooth muscle cells. Therefore to understand the role that calcium plays in the modulation of the serotonin-induced vascular contraction in the LPCA of bovine eyes, it is important to investigate the contribution of the release of intracellular calcium from intracellular stores and the contribution of the influx of extracellular calcium to the contraction. Receptor-activated calcium mobilization via inositol phosphate-calcium signaling system involves calcium release from an intracelllular store mediated by IP_3 and a more prolonged phase of extracellular entry (Putney and Bird, 1993).

To examine the contribution of external calcium to the contraction, the tissue was incubated in calcium free PB. Additionally, the vessels were incubated with calcium channel blockers that antagonize the influx of extracellular calcium through L-type calcium channels. The results of experiments performed by incubating ring segments in calcium free PB revealed that the contraction is significantly decreased in the absence of extracellular calcium. The magnitude of the vascular response to serotonin was reduced by 60.4% when calcium was removed from the media. Additionally, the duration of the response was reduced. The contraction continued to decline over a 30 to 40 minute period until the vessel was close to baseline tension. When the calcium free PB was drained from the tissue bath and replaced by PB with calcium, the vessels would immediately contract (see Figure 24, page 75) even without the addition of an agonist.

Similar reductions in response were observed when the vessels were incubated with calcium channel blockers, diltiazem and nifedipine. While both agents significantly decreased the maximum response of serotonin, there was no significant difference in the inhibitory responses of the two calcium channel blockers. The effects of the calcium channel blockers were not significantly different from the effect of calcium free PB on the contractions.

While the removal of extracellular calcium or the blocking of calcium entry significantly reduced the serotonin response in the long posterior ciliary artery, it is interesting to note that the serotonin contraction is not reduced in the absence of extracellular calcium in some blood vessels, such as the rabbit aorta and bovine coronary artery (Ratz and Flaim, 1984) and the rabbit ear artery (McCalden and Bevan, 1981). However, serotonin-induced contraction of the rabbit saphenous (Towart, 1981) and basilar arteries (Towart, 1981; Cain and Nicholson, 1989), rat tail artery (Berta et al., 1986) and dog saphenous vein (Sumner *et al.*, 1992) were almost completely abolished by the removal of extracellular calcium or by calcium channel antagonists.

Calcium is stored inside the cell primarily in the sarcoplasmic reticulum. There are at least two types of calcium channels in the sarcoplasmic reticulum that release calcium when activated. These channels are classified as IP_3 sensitive or ryanodine sensitive.

There have been three separate isoforms of the IP_3 sensitive receptor identified and there are quantitative differences of expression of the various subtypes between different tissues (Nakagawa *et al.*, 1991). This receptor has binding domains for IP_3 and calcium and a central aqueous channel that permits the passage of calcium from the lumen of the sarcoplasmic reticulum towards the cytosol. The interaction of IP_3 with the binding site on the surface of the IP_3 receptor induces the opening of an IP_3-gated calcium channel and the subsequent calcium release through it. The IP_3 receptor appears to be of physiological importance in vascular smooth muscle cells since its activation produces a large calcium release to the cytosol (Berridge 1993).

IP_3 is the only physiological activator of the IP_3 receptor and heparin and caffeine are the pharmacological inhibitors of the receptor (Ehrlich *et al.*, 1994). IP_3 is formed by the activation of G-protein linked receptors such as the 5-HT_2, that initiate the breakdown of polyphosphoinositides. The mechanism of inhibition of caffeine on the IP_3 receptor is less clear. Caffeine does not appear to act via its inhibition of phosphodiesterase activity because caffeine-induced inhibition occurred in the absence of Mg^{2+}-ATP and was measured in the bilayer, where the phosphodiesterase is presumably lacking (Bezprozvanny *et al.*, 1994). It has been suggested

that caffeine acts on the rate of opening of the IP_3-occuped channel rather that the ability of IP_3 to bind to the receptor (Bezprozvanny *et al.*, 1994). It has been demonstrated that caffeine has additional effects on the polyphosphoinositide pathway. In pancreatic acinar cells, caffeine inhibited agonist evoked IP_3 production with a K_d of 2 mm and a maximal inhibition of 50% at 10 mM caffeine (Toescu *et al.*, 1992). Similar results were obtained in PC12 cells (Zacchetti et al., 1991). These experiments would suggest that caffeine has an inhibitory effect on phospholipase C activity or on some intermediate steps in the coupling between receptor stimulation and phospholipase C activation in some cells. Phospholipase C is calcium dependent and hence this inhibitory action of caffeine may result from lowered cytosolic calcium levels as a consequence of decreased calcium release via the IP_3 receptor (Ehrich *et al.*, 1994).

Ryanodine receptors are the second type of receptor in the sarcoplasmic reticulum that can release stored calcium. The ryanodine receptor resembles four spheres closely associated to form a four leaf clover shape. There is a central pore that permits the passage of calcium and other divalent cations when it is activated (Franzini-Armstrong and Protasi, 1997; Ashley, Mulligan and Lea, 1991). Three isoforms have been described by cDNA cloning and sequencing. It appears that each isoform is a product of a different gene. Ryanodine receptors can be activated by caffeine, heparin, ryanodine (<10 μM), doxorubicin, or calcium. Ryanodine is a neutral plant alkaloid and a naturally occurring insecticide that produces, at low concentrations, a small and maintained calcium efflux from the sarcoplasmic reticulum to finally exhaust the calcium stores (Dulhunty, 1992). Ryanodine at concentrations < 10 μM binds to the ryanodine sensitive channel to lock them in an open state. At higher concentrations, ryanodine inhibits the opening of the channel and at concentrations greater than 500 μM, the receptor is transferred into a permanently closed state (Marin *et al.*, 1999; Erlich *et al.*, 1994).

The results of the experiments reported here would indicate that the long posterior ciliary artery of the bovine contains both IP_3 sensitive and ryanodine receptors. Since one of the

serotonin receptor subtypes identified is the G-protein linked 5-HT_2, it is not surprising that the IP_3 receptor appears to play a critical role. Caffeine totally inhibited the vascular response in all vessels. Experiments were initially performed with 20 mM caffeine and when it was determined that the response was blocked at that concentration, the experiments were repeated with 10 mM. Whether caffeine acts by blocking either phospholipase C activity as has been proposed or blocks the IP_3 receptor directly, it is clear that in this vascular tissue, the activation of the IP_3 receptor and the subsequent release of calcium is critical to the contraction.

Experiments with 10 μM ryanodine also significantly decreased the contraction although to a lesser extent than did caffeine. At concentrations less than 10 μM, ryanodine can be an activator of the ryanodine receptor. In these vessels, incubation with 10 μM ryanodine did not initiate a contraction. These results would indicate that ryanodine is blocking the release of calcium through ryanodine sensitive channels and that the inhibition of that release significantly decreases the response to serotonin.

Although a change in the concentration of free calcium inside the cell is required for smooth muscle contraction, an additional mechanism to produce a contraction at lower levels of calcium is by increasing the sensitivity of the cell to calcium. If the sensitivity to calcium increases, a response is possible at a lower concentration of calcium. The affect of a change in sensitivity was not addressed in any of the experiments performed.

Specific Aim #3

Determination of the role of PKC or PLC

Serotonin has been shown to increase phosphoinositide turnover in a variety of tissues including rat aortic smooth muscle (Doyle et al., 1986; Walenga *et al.*, 1980; Roth et al, 1984; Nakaki et al;.1985) and rat jugular vein, aorta, and uterus (Cohen and Wittenauer, 1987). 5-HT_2

receptors are coupled to phosphoinositide hydrolysis (Conn and Sanders-Bush, 1984; Roth *et al.*, 1984) producing the second messengers IP_3 and DAG. DAG activates PKC and IP_3 releases calcium from intracellular stores. To further characterize the cellular mechanisms mediating the serotonin-induced contraction, the effects of selective PLC and PKC inhibitors on the serotonin response were examined.

Evidence supporting a role for PLC in the contractile response to 5-HT in the long posterior ciliary artery is provided by the experiments with 2-NCDC, neomycin and U73122. Vessels incubated with 5 mM neomycin, immediately relaxed below baseline. They failed to contract with subsequent addition of serotonin in concentrations up to 100 μM. Neomycin has been shown to decrease isometric tone in canine cerebral arteries by a mechanism that appears to interfere with intracellular calcium accumulation (Gergawy, Vollrath and Cook, 1998). Conflicting results in cultured cells were reported by Sipma *et al.* (1996). They reported that Neomycin did not interfere with IP_3 formation or the release of calcium from internal stores. They concluded that the inhibiton was most likely exerted at the level of plasma membrane calcium channels. The neomycin mediated immediate relaxation of the LPCA would indicate that neomycin blocks the constriction by inhibiting a cellular mechanism that is contributing to basal tone in this blood vessel.

The most significant reduction by the selective PLC inhibitors was demonstrated by 2-NCDC. 2-NCDC decreased the response to serotonin by 87.7% but failed to inhibit the KCl induced contraction. This is evidence to rule out a non-specific action of 2-NCDC on the contractile process. These results are similar to those reported in rat aorta by Nakaki et al. (1985). In this tissue, 2-NCNC was shown to inhibit the 5-HT_2 induced contraction and subsequent phosphoinositide hydrolysis.

The widely used selective PLC inhibitor, U73122, significantly inhibited the 5-HT induced contraction. The response to 5-HT was reduced 66 % by U73122. U73122 has been used in

several studies to investigate the affect of the inhibition of PLC on 5-HT stimulated platelet aggregation (Lockhart and McNicol, 1999; Shah *et al.*, 1999).

Although none of the PKC inhibitors are totally selective, H-7 and bisindolylmaleimide are more active against PKC than the Ca^{+2}–calmodulin-dependent kinases such as myosin light chain kinase (MLCK). Both compounds act to some extent on cyclic AMP and cyclic GMP-dependent kinases. Bisindolylmaleimide II is a selective PKC inhibitor synthesized for its potency and selectivity with an IC_{50} of 5-70 nM (Toullec et al., 1991). The structural analogue, bisindolylamaleimide V, lacks activity as a PKC inhibitor and is used as a control compound (Dabdoub and Payne, 1999). Additionally, the benzophenanthridine alkaloid, chelerythrine, a potent, selective PKC antagonist with an IC_{50} of 0.6 μM was included in these experiments.

All three antagonists tested demonstrated significant inhibition of the serotonin vasconstriction. Bisindolylmaleimide II was tested at 1 μM and 10 μM. This compound significantly inhibited the response in a dose-dependent manner. The greatest inhibition was demonstrated by 5 μM H-7 with 61.5% inhibition, followed by 10 μM Bisindolylmaleimide II, 25 μM Chelerythrine, and 1 μM Bisindolylmaleimide II with inhibitions of 55.7%, 31.1%, and 19.8%, respectively.

Clark and Garland (1991) demonstrated that in the rabbit basilar artery, H-7 inhibited the contractile response to serotonin in a dose-dependent fashion. These findings were similar to those reported in cerebral arteries by Young *et al.* (1986). Although there have been differences reported between 5-HT_2 receptors found in cerebral and peripheral vessels in their dependence on extracellular calcium, there are consistent reports of their ability to increase phosphoinositide turnover.

The results presented here support the conclusion that the serotonin-induced contraction is mediated through activation of phospholipase C and protein kinase C. No other conclusions

can be drawn concerning the exact mechanism, as the inositol phosphates were not quantitated in these experiments.

CHAPTER V

SUMMARY

The posterior ciliary arteries supply blood to the optic nerve head, the choroid, the iris, and the ciliary body. Any impairment of their ability to control blood flow therefore may affect many aspects of ocular function involving the outer retina, the optic nerve head, the iris and the ciliary body. Vascular disease and vasospasm are implicated in the etiology of glaucoma. Therefore, information about the pharmacological control of the vascular smooth muscle of the long posterior ciliary would be helpful in understanding its normal and pathologic function.

Vascular contraction experiments performed with ring segments of the long posterior ciliary artery of bovine eyes have demonstrated that there were two serotonin receptor subtypes responsible for the serotonin-induced vasoconstriction. Both 5-HT_2 and 5-$HT_{1\text{-like}}$ receptors contribute to the vascular response. The activation of the 5-HT_2 receptor subtype was responsible for more than 60% of the contraction. There is further evidence that the 5-$HT_{1\text{-like}}$ receptor may contribute to the response in a synergistic manner. When the vessel was precontracted with 20 mM KCl, the ability of the 5-$HT_{1\text{-like}}$ receptor to evolve a contraction was increased 4.46 times the initial response. It is possible that 5-HT stimulation of the 5-HT_2 receptor may increase intracellular calcium levels through activation of G-protein mediated PLC. The subsequent elevated intracellular calcium levels may potentiate the 5-$HT_{1\text{-like}}$ receptor response.

The response of the LPCA to 5-HT was dependent on the mobilization of extracellular and intracellular calcium. The 5-HT response was decreased and could not be maintained without extracellular calcium. Calcium release from IP_3 and ryanodine sensitive calcium stores was critical to the contractions.

In summary, the 5-HT-induced vasoconstriction of the long posterior ciliary artery of the bovine eye is mediated through activation of both 5-HT_2 and 5-$HT_{1\text{-like}}$ receptors. The contraction is dependent on the mobilization of calcium and is mediated in part through PLC activated intracellular calcium release from IP_3 sensitive stores.

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