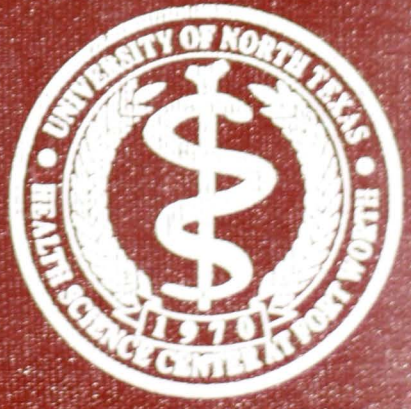
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
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Endothelins are a family of regulatory peptides which could have important implications in the regulation of aqueous humor outflow and intraocular pressure (IOP). The objectives of this dissertation were to investigate the cellular mechanism of endothelin (ET) receptor interactions in ocular tissues focusing on their effect on second messengers such as phospholipase C (PLC) and calcium, and their interactions with phospholipase A_2 (PLA_2) in ciliary muscle cells. The hypothesis was that in human ciliary muscle (HCM) cells, endothelin-1 (ET-1), via the ET_A receptor and a pertussis toxin sensitive G-protein, activates PLC, which in turn stimulates calcium mobilization. Independent of this pathway, ET-1 also activates PLA_2 and increases the release of prostaglandins. These two pathways provide a cellular second messenger balance that influences ciliary smooth muscle contraction.

The current study demonstrated that ET-1 and endothelin-2 (ET-2) stimulate calcium mobilization in HCM cells via an ET_A receptor subtype. It appears that the increase in intracellular calcium ($[Ca^{2+}]_i$) is the result of ET coupled to PLC via a pertussis toxin sensitive G-protein. A biphasic calcium response is elicited with ET stimulation consisting of a transient increase in

$[Ca^{2+}]_i$ which appears to be primarily due to release of intracellular stores, followed by a lower sustained phase which appears to be dependent on the influx of extracellular calcium. Endothelin-1 also appears to stimulate an increase in prostaglandin E_2 (PGE_2) formation through activation of PLA_2. Furthermore, it appears that the effects of ET-1 on PLC and calcium are independent of the ET-1 effects on PGE_2 production, such that the ET-1 induced increase in $[Ca^{2+}]_i$ are coupled to the PLC signaling pathway, whereas increase in PGE_2 production appears to be the result of an ET_A receptor coupled to PLA_2. Whether there are different subtypes of ET_A receptors or the receptor is coupled through different G-proteins is uncertain. Endothelin-1 and Big ET-1 immunoreactivity was also observed in both HCM and human nonpigmented ciliary epithelial (HNPE) cells. This is the first time that ET-1 and Big ET-1 immunoreactivity has been detected in the HCM cells, suggesting that these cells have the capability to synthesize both peptides. Furthermore, the increase in ET-1 and Big ET-1 immunoreactivity upon stimulation with TNF-α suggests that cytokines may be important regulators of ET synthesis and release.

The findings of this research aid in the understanding of the mechanism of action whereby ETs regulate aqueous humor dynamics and IOP. Through a better understanding of the cellular actions of ET, insight is gained into the development of new ocular selective agents acting at the ET receptor.

CELLULAR MECHANISMS IN THE OCULAR

ACTIONS OF ENDOTHELIN

Karen A. White, B.S.

APPROVED:

Major Professor

Minor Professor

Committee Member

Committee Member

Committee Member

Committee Member

Chair, Department of Pharmacology

Dean, Graduate School of Biomedical Sciences

CELLULAR MECHANISMS IN THE OCULAR ACTIONS OF ENDOTHELIN

DISSERTATION

Presented to the Graduate Council of the

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in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Karen A. White, B.S.

Fort Worth, Texas

December 1996

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Table

Table

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure

Page

CHAPTER I

INTRODUCTION

Statement of the Problem

Primary open-angle glaucoma affects millions of people and is the leading cause of blindness today. Glaucoma is characterized by cupping of the optic disc, changes in the visual field, and in most instances an elevation in intraocular pressure (IOP). The currently acceptable pharmacological treatments for glaucoma involve either lowering the production of aqueous humor or increasing its outflow facility. Several new types of compounds are currently being studied for their potential therapeutic use in the treatment of glaucoma. One of these compounds is the endogenous peptide endothelin (ET).

The endothelins (ETs) are a 21 amino acid residue peptide family with potent vasoconstrictive properties. The ETs were first isolated in 1988 from supernatants of porcine vascular endothelial cells (Yanagisawa *et al.*, 1988). Since their discovery, the ETs have been extensively studied for their involvement in a variety of diseases, such as atherosclerosis, myocardial infarctions, pulmonary hypertension, migraines and ocular disorders (Remuzzi and Benigni, 1993). At present, ET research has resulted in the discovery of

three distinct endogenous isoforms of ET; endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), with only ET-1 and ET-3 being widely distributed in mammalian tissues (Yanagisawa *et al.*, 1988). Receptor research has resulted in the molecular characterization and pharmacological recognition of two receptor subtypes for ET; ET_A and ET_B (Bax and Saxena, 1994). A possible new receptor subtype, ET_C, is present in Xenopus dermal melanophores and identified in bovine endothelial cells by pharmacological studies (Bax and Saxena, 1994; Emori *et al.*, 1990). The pharmacologic use and physiologic role of ETs is of particular interest to eye researchers. In several species, rabbit, rat and humans, it has been shown that the ETs are present in various concentrations in the iris, retina, choroid and ciliary body (MacCumber *et al.*, 1991). Endothelins are potentially important mediators in ocular pathologic conditions, and are reported to be involved in the local regulation of IOP, ocular blood vessel tone and iris smooth muscle tone (MacCumber *et al.*, 1991). The reported effects of ET in ocular tissues includes constriction of retinal blood vessels (Nyborg *et al.*, 1991; Sakaue, *et al.*, 1991), either pupillary constriction or dilation depending on whether ET-1 or ET-3 is applied *in vivo* or *in vitro* and reductions of IOP (MacCumber *et al.*, 1991). The potential use of ET-1 and ET-3 in the regulation of IOP has been studied both *in vivo* and *in vitro* in several species. The mechanism of action for IOP reduction, however, is yet unknown.

The main objective of the present study was to determine the cellular mechanism of ET receptor interactions in ocular tissues. In particular, endothelin's effect on second messenger systems, such as phospholipase C (PLC) and calcium (Ca^{2+}), and their interactions with phospholipase A_2 (PLA_2) in human ciliary muscle (HCM) cells were investigated. The effects of the three isoforms of endothelin on PLC and calcium mobilization were evaluated as well as determining the mechanism of action and receptor subtype involved. In addition, the effects of ET-1 on prostaglandin (PG) production were studied with emphasis on the interactions between the PLC and PLA_2 coupled events. The effects of ET-1 on calcium mobilization in the HCM cells were then compared to the effect on transformed human nonpigmented ciliary epithelium (HNPE) cells. Immunocytochemical techniques were utilized to determine if ET-1 is present and released from either the ciliary muscle or ciliary epithelium. The present study provides for a better understanding of the biochemical changes after ET stimulation, which may be responsible for the contraction of the ciliary muscle and enhanced outflow facility, leading to a reduction in IOP.

Overview of the Endothelins

Discovery

The vascular endothelium has been a productive area of research in the last twenty years. The vascular endothelium releases various substances

which mediate both relaxation and vasoconstrictor responses. The vasoconstrictor response is mediated through the secretion of substances which are able to contract the underlying smooth muscle. These substances are identified as endothelium-derived contractile factors (EDCFs). Differences in the releasing stimuli and chemical nature of the factors lead to the discovery of the potent vasoconstrictive, 21 amino acid peptide, endothelin by Yanagisawa *et al.* in 1988. Endothelin was first isolated and purified from supernatants of cultured porcine aortic endothelial cells (Yanagisawa, *et al.*, 1988). Yanagisawa *et al.* reported ET to be the most potent mammalian vasoconstrictor peptide known (Yanagisawa, *et al.*, 1988). The ET isolated from the porcine aortic endothelial cells was a 21 amino acid peptide residue with two disulfide bridges linking Cys^{1}-Cys^{15} and Cys^{3}-Cys^{11} (Figure 1).

Isoforms of Endothelin

Three structurally and pharmacologically distinct peptides have been prepared based on molecular cloning techniques determined from three genes (Inoue, *et al.*, 1989). These three ETs have been designated as ET-1, ET-2 and ET-3. The original porcine/human endothelin was renamed ET-1; the human peptide with two amino acid substitutions relative to ET-1 [Trp^{6}, Leu^{7}] was named ET-2; and the peptide sequence first found in rat with six amino acid substitutions relative to ET-1 [Thr^{2}, Phe^{4}, Thr^{5}, Tyr^{6}, Lys^{7}, Try^{14}] was named ET-3 (Hiley, 1995). Expression of the three isoforms of endothelin

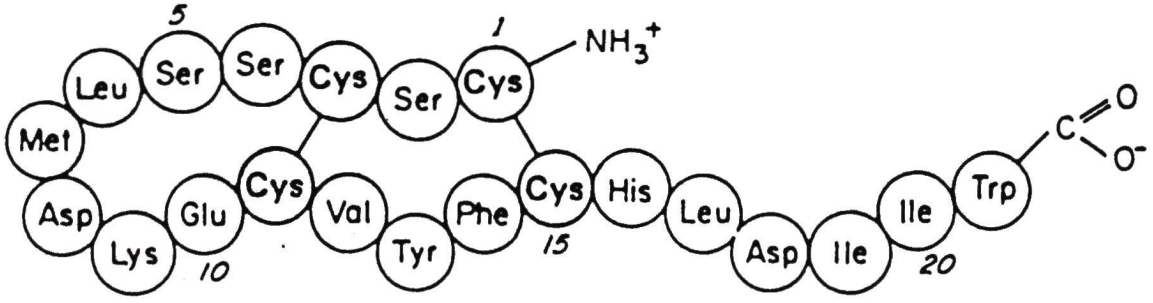


Figure 1. Structure of Endothelin-1.

has been found to occur in various proportions and in many tissues. The family of ETs appear to be related to a family of toxins, the sarafotoxins S6, found in the snake venom of the Israeli burrowing asp *Atractaspis engaddensis* (Lee and Chiappinelli, 1988; Takasaki, *et al.*, 1988). Due to the high degree of homology with the ETs, the sarafotoxins are included as members of the ET superfamily, with sarafotoxin S6c designated as an ET_B receptor agonist. Although the sarafotoxins are homologous with the ETs, they are not endogenous in mammals.

Synthesis and Release of Endothelin

Endothelin is synthesized as a precursor peptide, preproendothelin, a 203 amino acid peptide. The preproendothelin is proteolytically cleaved by dibasic amino acid endopeptidases to produce an intermediate peptide of 37-41 amino acids, designated big-endothelin (Hiley, 1995). This biologically inactive intermediate big-endothelin (Big ET) is subsequently processed to the active 21 amino acid peptide by a specific endothelin converting enzyme (ECE) which cleaves the Trp^{21}-Val^{22} bond (ET-1 and ET-2) or the Trp^{21}-Ile^{22} bond (ET-3) (Battistini and Botting, 1995). Several isozymes of the ECE have been identified and recently classified into ECE-1a, ECE-1b and ECE-2 based on their localization and biochemical characterization (Battistini and Botting, 1995). Processing of Big-ET into ET is necessary for the biological activity of endothelin to be fully expressed (Kimura, *et al.*, 1988), regardless of which

specific ECE is responsible for the cleavage.

Endothelins are synthesized by selected endothelial and epithelial cells and act in a paracrine fashion on nearby smooth muscle or connective tissue. It has been suggested that ET is released as it is synthesized (Hiley, 1995), however, the exact mechanism of this release is unclear. Regulation of the synthesis and release of ET is affected by a variety of factors. The factors which appear to stimulate the synthesis of ET include: thrombin, ionophore A23187, adrenaline (Yanagisawa, *et al.*, 1988) and transforming growth factor-β (TGF-β) (Kurihara *et al.*, 1989). Agents which stimulate the release of endothelin include: thrombin, angiotensin II, vasopressin, phorbol esters, the calcium ionophore ionomycin (Emori, *et al.*, 1989), fluid dynamic shear stress (Yoshizumi, *et al.*, 1989), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Battistini and Botting, 1995).

Receptors for Endothelin

Receptor research has resulted in the molecular characterization and pharmacological recognition of two distinct receptor subtypes for ET; ET_A and ET_B (Bax and Saxena, 1994). The ET_A receptor subtype is relatively selectively activated by ET-1 and ET-2, whereas the ET_B receptor subtype is activated nonselectively by all three isoforms. These receptor subtypes have been cloned in mammals and their physiological and pharmacological profiles have been extensively characterized in a number of species and tissues.

Recently, a new receptor subtype, ET_C, with high affinity for ET-3 was identified in *Xenopus* dermal melanophores and reported in pharmacological studies in bovine endothelial cells (Bax and Saxena, 1994; Emori, *et al.*, 1990). Two novel reverse transcription (RT) PCR transcripts have been identified in human lung and other tissues suggesting the presence of other ET receptors or subclasses (Battistini and Botting, 1995). To date, pharmacological evidence exists that further suggests the existence of subtypes of the ET_B receptor, ET_{B1} and ET_{B2} (Warner, *et al.*, 1993) and the ET_A receptor, ET_{AX} (Kumar, *et al.*, 1994). What role these subclasses of receptors may play physiologically remains unclear.

Pharmacological Effects of Endothelin

The endothelins induce a number of biological/pharmacological effects including hemodynamic actions, strong inotropic and chronotropic actions on the myocardium, mitogenesis and regulation of various endocrine functions. Endothelin is one of the most potent vasoconstrictors yet to be identified and contracts both vascular and nonvascular smooth muscle (Simonson and Dunn, 1990). This family of homologous peptides, ET-1, ET-2 and ET-3, has been shown to have a variety of biological actions and in general differ quantitatively but not qualitatively in their pharmacological profiles (Le Monnier de Gouville, *et al.*, 1989). In a variety of isolated tissues, including arteries, veins, trachea, duodenum, urinary bladder and uterus, the ETs have

potent contractile activity (Le Monnier de Gouville, *et al.*, 1989). *In vivo*, the ETs possess potent vasodilator and vasoconstrictor properties through direct and indirect actions. Through the formation of arachidonic acid metabolites, the ETs appear to produce inflammation and bronchoconstriction (Le Monnier de Gouville, *et al.*, 1989). Endothelin's hemodynamic actions are perhaps its best characterized pharmacological effect. Endothelin causes an initial depressor response followed by a sustained pressor effect (Simonson and Dunn, 1990). The pressor responses to ET appear to be mediated by the ET_A receptor subtype, although in some vascular beds activation of ET_B receptors can also cause vasoconstriction (Kleha, *et al.*, 1994). Endothelin also increases the plasma level of a variety of vasoactive hormones, including atrial naturetic factor (ANF), renin, aldosterone and catecholamines (Simonson and Dunn, 1990). Localization of ET binding sites within the CNS also suggests that ETs may act as neurotransmitters or neuromodulators (Simonson and Dunn, 1990). Due to its hemodynamic actions, ET has been extensively studied for its involvement in a variety of diseases such as atherosclerosis, myocardial infarctions, pulmonary hypertension and migraines. More recently, interest has formed on ETs role in ocular disorders.

Ocular Actions of Endothelin

Distribution of Endothelin in Ocular Tissues

Binding and immunolocalization studies have shown that the ETs are widely distributed in ocular tissues. Initial studies by MacCumber, *et al.*, (1989) detected ET binding sites and mRNA in rat ocular tissues. High concentrations of ET binding sites and mRNA were detected in the rat iris, especially in the stroma which is rich in small blood vessels. High densities of ET binding sites were also found in the endothelial layer of the cornea and Descemet's membrane, as well as the choroid and retina (MacCumber, *et al.*, 1989). In rabbits, ET-1- and ET-3-like immunoreactivity was highest in the iris and ciliary body, with lower amounts in the choroid and retina, and the lowest amounts in the cornea (MacCumber, *et al.*, 1991). Autoradiographic studies by Osborne (1993) showed specific radioactive ET-1 binding sites in the rabbit iris, ciliary processes and corneal endothelium. In human ocular tissue, immunoreactivity to ET-1 and ET-3 was found in all ocular tissues except the cornea, which showed no immunoreactivity to ET-3, with the highest distribution of ET present in the choroid and iris/ciliary body (Chakravarthy, *et al.*, 1994). In the ocular tissues of the various species, the ET-3 concentrations are higher than that of ET-1, with apparently no ET-2 peptide immunoreactivity detected in any ocular tissues (MacCumber, *et al.*, 1991; Eichhorn and Lütjen-Drecoll, 1993; Charkravarthy, *et al.*, 1994). The

physiological implication of these differences in ET concentrations in ocular tissues has yet to be elucidated.

Ocular Effects of Endothelins

Endothelins are potentially important mediators in ocular pathologic conditions, and are reported to be involved in the local regulation of IOP, ocular blood vessel tone and iris smooth muscle tone (MacCumber, *et al.*, 1991). The reported effects of ET in ocular tissues includes constriction of retinal blood vessels (Nyborg, *et al.*, 1991; Sakaue, *et al.*, 1991), either pupillary constriction or dilation depending on whether ET-1 or ET-3 is applied *in vivo* or *in vitro* and reductions of IOP (MacCumber, *et al.*, 1991).

Endothelin's Effect on Intraocular Pressure

It is not clear as to whether ET regulates IOP via actions on the ciliary process to decrease aqueous humor production, acts directly on the ciliary muscle to increase outflow, or acts via prostaglandin mediated increases in uveoscleral outflow. Intracameral injections of ET-1 into the animal eye (mainly rabbit), at low concentrations, such as 4 pmol (10 ng) or less, caused a dose-dependent increase in IOP, which was likely mediated by PGs, since cyclooxygenase inhibitors could prevent such an effect (Granstam *et al.*, 1991). At higher concentrations, such as 1 nmol (2 μg) or higher, ET-1 produced a biphasic IOP response, initially increasing and subsequently

lowering IOP (MacCumber *et al.*, 1991; Azuma, 1993; Okada *et al.*, 1994; Taniguchi *et al.*, 1994). The initial rise in IOP was reported as transient compared to the prolonged ocular hypotensive phase. Recently, Sugiyama and colleagues reported that intravitreal injections of ET-1 at concentrations of 0.05 μg and 0.15 μg, caused a prolonged reduction in IOP without an initial rise in IOP, whereas ET-1 concentrations above 0.5 μg caused the initial IOP rise followed by a prolonged reduction in IOP (K. Sugiyama, *et al.*, 1995). The initial rise in IOP appears to be mediated by prostaglandin E_2 (PGE_2) and possibly other cyclooxygenase products, since indomethacin pretreatment significantly suppressed the ET-1 induced elevation in PGE_2 concentration, as well as the initial rise in IOP (K. Sugiyama, *et al.*, 1995). Therefore, these authors speculated that 0.5 μg of ET-1 may be a threshold dose for an initial IOP rise (K. Sugiyama, *et al.*, 1995).

Endothelin's effect on IOP appears to be mediated by both the ET_A and ET_B receptor subtypes. In rabbits, intravitreal injections of the ET_A-selective antagonist, 97-139 (155 μg) prior to ET-1 (0.5 μg) significantly reduced both the initial IOP rise and prolonged reduction in IOP caused by ET-1 (K. Sugiyama, *et al.*, 1995). Moreover, the ET_B receptor selective agonist, sarafotoxin S6c, has been shown to cause sustained ocular hypotension after intravitreal injections in rabbits without the initial rise in IOP (Haque, *et al.*, 1995; K. Sugiyama, *et al.*, 1995). Thus, both the ET_A and ET_B receptors may be involved in the ET-1 mediated effects on IOP.

The mechanism of ET's effect on IOP is unclear. Recently, Taniguchi and colleagues suggested that in the rabbit, activation of the ET_A receptor can lower aqueous humor formation, whereas activation of the ET_B receptors increases outflow facility (Taniguchi, *et al.*, 1996). In the monkey, Erickson-Lamy, *et al.*, reported that injections of ET-1 into the anterior chamber increased the outflow facility 22% - 71%, probably by a direct effect on the ciliary muscle (Erickson-Lamy, *et al.*, 1991). Whether ET mediates increases in outflow via direct actions on outflow pathways or contraction of the ciliary muscle is unclear (Eichhorn and Lütjen-Drecoll, 1993). In the perfused anterior segment of the bovine eye, where the ciliary muscle was removed, ET-1 (2-20 nM) dose-dependently decreased the outflow rate and increased outflow resistance (Wiederholt, *et al.*, 1995). Since ET has been shown to cause contraction of bovine ciliary muscle strips and isolated trabecular meshwork strips (Lepple-Wienhues, *et al.*, 1991), these authors speculated that although contraction of the ciliary muscle dominates the overall effect on outflow facility in the human, and probably the bovine eye, there may be antagonism between ET-1 mediated contraction of the trabecular meshwork and the ciliary muscle (Wiederholt, *et al.*, 1995). The ciliary muscle is not only responsible for accommodation, but also plays an important role in aqueous humor dynamics. Its effect on both accommodation and aqueous humor dynamics are mediated through muscle contractions (Kaufman, *et al.*, 1984). It has been shown that tension on the ciliary muscle can influence

aqueous outflow and accommodation (Kaufman and Barany, 1976). Therefore, ET-1 induced contractions of the ciliary muscle may lead to changes in IOP.

Endothelin's Effect on Ocular Blood Vessel Tone

Disturbances of ocular blood vessel flow are involved in many ophthalmic diseases and could contribute to the ophthalmic complications seen in hypertension, diabetes, Raynaud's syndrome and migraines (Haefliger, *et al.*, 1994). Local blood flow through the eye is determined by a variety of factors; perfusion pressure, tone of resistance vessels and vascular blood viscosity (Lüscher and Vanhoutte, 1990). Blood vessel tone depends on the contractile state of smooth muscle and is regulated by a variety of endothelium-derived relaxing factors, such as nitric oxide, and contracting factors, such as ET-1 and arachidonic acid products (Palmer, *et al.*, 1987; Lüscher and Vanhoutte, 1990). In isolated human, porcine and bovine ophthalmic and retinal arteries, ET-1 evokes strong contractions (Haefliger, *et al.*, 1992; Haefliger, *et al.*, 1993; Nyborg, *et al*, 1991; Yao, *et al.*, 1991). Interestingly, tachyphylaxis is reported to occur in most blood vessels after repeated exposure to ET-1, typical of agonist-induced ET-receptor down-regulation (Haefliger, *et al.*, 1994). Most likely, this down-regulation of ET receptors is a protective mechanism of the ophthalmic arteries against repeated or prolonged exposure to this potent vasoconstrictor

(Haefliger, *et al.*, 1992). In the perfused porcine eye, a biphasic response to ET was observed. At very low concentrations (10^{-12} M), ET-1 increased ophthalmic flow, but higher doses (10^{-10} M), ET-1 severely reduced the flow for prolonged periods of time (Meyer, *et al*, 1993). Endothelin-3 produced a similar response with low concentrations of ET-3 evoking vasodilatation and higher concentrations causing vasoconstriction, although to a lesser degree than ET-1 (Meyer, *et al*, 1993). Interestingly, the vasodilatory effects appear to be mediated through the release of PGs, since pretreatment with indomethacin blocks the vasodilation, via ET_B receptor activation and the vasoconstriction through ET_A receptors, which can be blocked by pretreatment with the ET_A antagonist FR-139317 (Meyer, *et al*, 1993). Thus, ET appears to induce vasoconstriction or vasodilation depending on the concentration of peptide and receptor population activated. In the cat, intravitreal injection of ET-1 (0.4 nM) induced a 34% reduction in retinal blood flow, but did not affect blood flow in the ciliary body, iris and choroid (Granstam, *et al.*, 1992). Other studies have shown that intravitreal injections of ET dose-dependently constrict retinal blood vessels (Sakaue, *et al.*, 1991) and decrease optic nerve blood flow in the cat (Nishimura, *et al.*, 1996). In the rabbit eye, intravitreal injections of ET produced prolonged, marked effects on the anterior ciliary circulation, as well as constriction of the retinal vasculature (Lüscher, *et al.*, 1992; Sakaue, *et al.*, 1991, Cioffi, *et al.*, 1995). Other routes of administration of ET such as intravenous

injections, intravitreal injections, chronic perineural injections and intraluminal perfusions have shown that ET can cause vasoconstriction in ocular structures (T. Sugiyama, *et al.*, 1995; Cioffi, *et al.*, 1995; Meyer *et al.*, 1993). The effect of ET on ocular blood flow may have important clinical significance as a potential contributor to a variety of optic neuropathies.

Endothelin's Effect on Pupil Size

The effects of ET on pupil size varies among the animal species tested and the route of administration. In the rabbit, the effects of ET on pupil size depend on whether ET-1 and ET-3 are applied *in vivo* or *in vitro*. *In vivo*, intravitreal injection of ET-1 (approximately 1 nM) caused pupillary dilation with blockade of the light reflex, whereas ET-3 caused a slight miosis without inhibiting the light response. However, *in vitro*, ET-1 and ET-3 acted directly on the iris sphincter to cause pupillary constriction (MacCumber, *et al.*, 1991). Endothelin-induced contraction of the iris sphincter and dilator muscles appear to mediate the effects on pupil size, since indomethacin pretreatment did not modify the pupil effect (MacCumber *et al.*, 1991). In the cat, ET-1 appears to be a potent miotic. Intracameral administration of 0.04 pmol of ET-1 in the cat caused a significant reduction in pupil diameter. Within minutes, the pupil size was reduced by greater than 50%, with further reductions in pupil size seen by increasing the dose to 0.4 pmol and 4 pmol (Granstam, *et al.*, 1992). The potent miotic effect was abolished by

indomethacin pretreatment indicating the involvement of arachidonic acid metabolites. Indeed, these authors found that intracameral injection of ET-1 increased the concentration of PGE_2 in aqueous humor (Granstam, *et al.*, 1992). In contrast to the rabbit or cat, ET-1 appears to have no effect on the pupil size in primates. In the cynomolgus monkey, perfusion of the anterior chamber with ET-1 (10^{-10} M to 10^{-7} M) had no effect on the pupil diameter (Erickson-Lamy, *et al.*, 1991).

Signal Transduction Pathways of Endothelin Stimulation

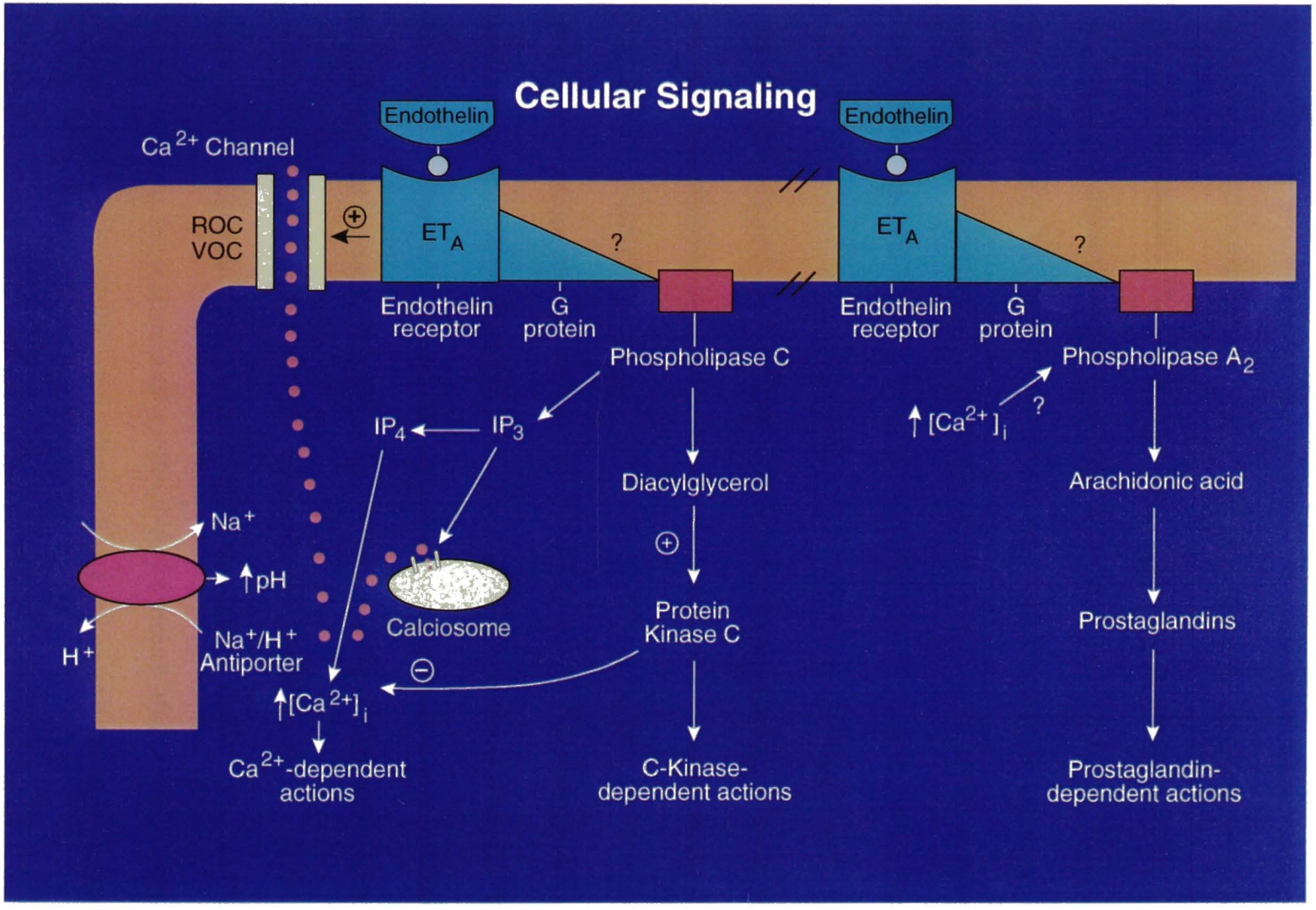
Overview

The cellular signaling mechanism(s) by which ETs exert their biological effects are unknown. Several signal transduction pathways, however, have been proposed based on second messenger studies in various tissues and cell lines. In smooth muscle cells, fibroblasts, retinal pericytes and glomerular mesangial cells, ET appears to bind to receptors on the plasma membrane and through interaction with a putative G protein, activates PLC. This transmembrane event evokes a phosphoinositide cascade producing two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 causes calcium to be released from intracellular stores, thereby increasing intracellular calcium concentrations ($[Ca^{2+}]_i$) and the DAG activates protein kinase C (PKC) which appears to trigger a negative feedback signal on calcium (Simonson and Dunn, 1990). Endothelins also

appear to stimulate PLA_2 via a G protein and/or increasing $[Ca^{2+}]_i$, thus causing membrane phospholipids to release arachidonic acid. The arachidonic acid is converted to bioactive eicosanoids (Simonson and Dunn, 1990). In addition to the stimulation of PLC and PLA_2, ET appears to gate multiple types of calcium channels by a mechanism which is poorly characterized. The two main types of calcium channels which regulate calcium influx are voltage operated channels (VOC) or receptor operated channels (ROC). It is not clear whether these channels are opened directly through a receptor or indirectly through some internal diffusible second messenger to stimulate calcium influx and produce a sustained increase in intracellular calcium (Simonson and Dunn, 1990). Figure 2 summarizes the signal transduction pathways ET may utilize to exert its biological effects.

Effects on Signal Transduction Pathways in Ocular Tissues

Endothelin's effect on second messenger systems have been studied in a variety of ocular tissues. Table 1, pages 28-29, provides a summary of the signal transduction pathways of ET stimulation in ocular tissues.


Cellular Signaling
Endothelin
Endothelin
Ca 2+ Channel
ROC
VOC
ET A
ET A
?
?
Endothelin receptor
G protein
Endothelin receptor
G protein
Phospholipase C
Phospholipase A2
IP4
IP3
[Ca2+]i
?
Diacylglycerol
Arachidonic acid
Na+
pH
H+
Na+/H+ Antiporter
Calciosome
Protein Kinase C
Prostaglandins
[Ca2+]i
Ca2+-dependent actions
C-Kinase-dependent actions
Prostaglandin-dependent actions

Ciliary Muscle

In HCM cell cultures, ET has been shown to produce dose-dependent reversible membrane depolarization and cause a dose-dependent rise in intracellular calcium (Korbmacher *et al.*, 1989; Stahl, *et al.*, 1992). Recent findings by Matsumoto, *et al.* (1996) showed that ET-1 stimulated PLC and increased $[Ca^{2+}]_i$ in cultured HCM cells via the ET_A receptor subtype. ET-1 was also shown to increase the production of PGE_2 and cAMP in these cells (Matsumoto, *et al.*, 1996). Endothelin-1 appears to induce contraction of HCM cells, as a time-dependent decrease of the cell surface area was observed (Pang and Yorio, 1996). In isolated human ciliary muscle strips, however, conflicting results have been reported for the effect of ET-1 on contraction of the ciliary muscle. Lepple-Wienhues, *et al.* (1992) reported that human ciliary muscle strips, tested within 48-72 hours after enucleation, contracted upon application of ET-1 (50 nM). This agrees with the findings that ET-1 contracts isolated ciliary muscle from Rhesus monkey (Millar, *et al.*, 1995) and bovine (Lepple-Wienhues, *et al.*, 1991; Kamikawatoko, *et al.*, 1995; Abdel-Latif, *et al.*, 1996). However, Abdel-Latif, *et al.* (1996) observed no contractile response after the addition of ET-1 (0.1 μM) in human ciliary muscle obtained within 7 to 14 hours after death. The reason for the difference in results from Abdel-Latif, *et al.* (1996) versus Lepple-Wienhues, *et al.* (1992) are not clear. It may be related to how the tissues were acquired during dissection.

In ciliary muscle isolated from the bovine eye, ET-1 stimulates PLC, triggers PGE_2 release, activates adenylyl cyclase and contracts ciliary muscle strips (Lepple-Wienhues, *et al.*, 1991; Kamikawatoko, *et al.*, 1995; Abdel-Latif, *et al.*, 1996). The effects of ET-1 on cAMP accumulation and contraction in bovine ciliary muscle appear to be mediated by the ET_A receptor subtype (Abdel-Latif, *et al.*, 1996; Kamikawatoko, *et al.*, 1995). In contrast, ET-1 stimulation of cat and dog isolated ciliary muscle results in increased release of PGE_2 and activation of adenylyl cyclase, with no effect on PLC or contraction (Abdel-Latif, *et al.*, 1996).

Ciliary Processes

In human ciliary processes, all three isoforms of endothelin, ET-1, ET-2 and ET-3, are reported to be effective inhibitors of forskolin-stimulated cAMP production (Bausher, 1995). Similar results were observed in rabbit ciliary processes, where basal and forskolin stimulated cAMP production was inhibited by each of the ETs, with maximum inhibition of about 75-80% (Bausher, 1995). In addition, ET-1 and ET-2 inhibited isoproterenol- and VIP-stimulated cAMP production (Bausher, 1995). This author suggests involvement of the ET_B receptor in the ET-1, ET-2 and ET-3 inhibition of forskolin-stimulated cAMP synthesis in the rabbit ciliary processes, based on the pharmacological profile of inhibition which was observed (Bausher, 1995). Similar results have been observed in the iris/ciliary body tissues,

where ET-1 reduces the forskolin-induced elevation of cAMP, without altering basal levels of cAMP (Osborne, 1993). In the rabbit, ET-1, ET-2 and ET-3 also stimulate the production of inositol phosphates in the iris/ciliary processes with the rank order for stimulation being ET-1 > ET-2 > ET-3 (Osborne, 1993).

Iris Sphincter

Compared to other ocular tissues, a relatively large number of studies have been conducted to investigate the effects of ET on the mammalian iris sphincter smooth muscle. The species studied include rabbit, bovine, cat, dog, monkey, human, pig and rat. In the rabbit iris sphincter, ET-1 was found to be a potent agonist of IP_3 production, DAG formation, cAMP formation and contraction (Abdel-Latif and Zhang, 1991a). Endothelin-1 was also found to contract the iris dilator muscle in rabbits, however, to a lesser extent than it contracts the iris sphincter (Abdel-Latif and Zhang, 1991a). Additionally, Ishikawa, *et al.* (1993) reported that ET-1, ET-2 and ET-3 caused a slow contractile response in both the iris sphincter and the dilator muscle. The effects of the ET homologs, ET-1, ET-2, ET-3 and the ET_B-selective agonist, sarafotoxin-S6c (SRTX-c) were also tested on rabbit iris sphincter contraction, IP_3 production and cAMP formation. ET-1, ET-2 and ET-3 elicited a dose-dependent increase in muscle contraction, stimulated IP_3 formation with a rank order of potency of ET-1 = ET-2 > > ET-3 and increased

cAMP formation (El-Mowafy and Abdel-Latif, 1994). SRTX-c had no effect on either contraction of the iris sphincter or stimulation of IP_3, however, this ET_B-selective agonist induced the highest increase in cAMP formation implying involvement of the ET_B receptor (El-Mowafy and Abdel-Latif, 1994). The ET_A receptor antagonist, BQ-123 moderately antagonized the ET-1 and ET-3 induced contraction and inhibited the increase in IP_3 production produced by ET-1, ET-2 and ET-3 (El-Mowafy and Abdel-Latif, 1994). These data suggest the presence of both the ET_A and ET_B receptor subtypes in the iris sphincter. The ET_A receptor subtype appears to be involved in the contractile response and the stimulation of IP_3 formation for all homologs of ET, whereas the ET_B receptor subtype appears to be coupled to cAMP formation (El-Mowafy and Abdel-Latif, 1994). Endothelin-1 also appears to be a potent activator of phospholipase D (PLD) in the rabbit iris sphincter muscle (Zhang and Abdel-Latif, 1992). Furthermore, the effect of ET-1 on PLD activity appears to be independent of ET-1's activation of PLC, increase in intracellular calcium or activation of PLA_2 (Zhang and Abdel-Latif, 1992).

In the bovine iris sphincter, ET-1 and ET-2 were equipotent in stimulating IP_3 production and contraction, whereas ET-3 had little effect on both responses and SRTX-c was without effect (El-Mowafy and Abdel-Latif, 1994). Compared to the rabbit iris sphincter, however, the effect of ET on IP_3 formation and contraction produced a considerably weaker response in the bovine iris sphincter (El-Mowafy and Abdel-Latif, 1994). Endothelin's

effect on cAMP formation in the bovine sphincter, however, is much greater than observed in the rabbit for all homologs of ET as well as SRTX-c, with the rank order of potency being SRTX-c > ET-3 = ET-2 = ET-1 (El-Mowafy and Abdel-Latif, 1994). As in the rabbit, the ET_A receptor subtype appears to be involved in the contractile response and the formation of IP_3 for all homologs of ET, whereas the ET_B receptor subtype appears to be coupled to cAMP formation (El-Mowafy and Abdel-Latif, 1994). In addition to the above effects of ET-1 on the bovine iris sphincter, ET-1 also stimulates PLA_2 and increases the production of PGs and related compounds (Yousufzai and Abdel-Latif, 1993).

In the cat iris sphincter, ET-1 stimulates IP_3 accumulation, cAMP formation and contraction (Abdel-Latif and Zhang, 1991a). Unlike other species, however, the effects of ET-1 on PLC, adenylyl cyclase and contraction may be mediated by the activation of PLA_2 and the subsequent elevated release of PGs. Endothelin-1 increased the production of PGE_2 in the cat iris sphincter and pretreatment of the tissue with the cyclooxygenase inhibitor, indomethacin, blocked all of the actions of ET-1 (Yousufzai, *et al.*, 1995). These authors suggest that the ET-1 induced release of PGE_2 acts via the prostaglandin EP_2 receptor to increase cAMP, and activates the EP_1 receptor to stimulate PLC, calcium mobilization and the resulting contraction. Additional studies are needed to substantiate this hypothesis since indomethacin does not only inhibit cyclooxygenase, but can inhibit protein

kinases, phosphodiesterases and interfere with the binding of various prostanoids to their receptor.

In the iris sphincter of the Rhesus monkey and humans, ET-1 only appears to stimulate cAMP formation with no effect on IP_3 accumulation or contraction (Abdel-Latif and Zhang, 1991a). This finding in primates is in contrast to the other mammalian species studied in which ET-1 also activates PLC and induces contraction of the iris sphincter smooth muscle of the rabbit, cat, dog, pig and bovine (Abdel-Latif and Zhang, 1991a; El-Mowafy and Abdel-Latif, 1994). In the rat iris sphincter, the only effect reported is that ET-1 induces contraction of the iris sphincter via the ET_A receptor (Shinkai, *et al.*, 1994).

Trabecular Meshwork

In bovine trabecular meshwork strips ET-1 is a potent contracting agent, inducing contraction in a dose dependent manner (Lepple-Wienhues, *et al.*, 1991). Removal of extracellular calcium reduced the contractile response indicating that ET-1-induced contraction of the bovine trabecular meshwork strips appears to be partially calcium-dependent (Lepple-Wienhues, *et al.*, 1991). In cultured bovine trabecular meshwork cells, ET-1 causes depolarization of the plasma membrane and induced substantial dose-dependent increases in intracellular calcium (Lepple-Wienhues, *et al.*, 1992b; Kohmoto, *et al.*, 1993). ET-1 also increased intracellular pH (pH_i) in bovine

trabecular meshwork cells, apparently mediated by the activation of the Na^+-H^+ antiporter (Kohmoto, *et al.*, 1993). ET-1 induced alkalinization by stimulation of Na^+-H^+ exchange may contribute to the promitogenic actions of ET (Simonson and Dunn, 1990), theoretically promoting cell growth in the trabecular meshwork. Minimal work with endothelin has been done in cultured human trabecular meshwork cells, however, electrophysiological studies have shown that ET-1 induces depolarization of the plasma membrane (Lepple-Wienhues, *et al.*, 1994).

Cornea

In the rabbit cornea, ET-1 increases the production of inositol phosphates in a time- and concentration-dependent manner (Osborne, *et al.*, 1993b; Takagi, *et al.*, 1994). The turnover of phosphoinositides is not significantly increased by ET-1 in the de-epithelialized rabbit cornea, indicating that this effect is mediated by the cornea epithelial cells. Endothelin-1 also causes a slight increase in the accumulation of cAMP in the rabbit cornea which is calcium-dependent (Takagi, *et al.*, 1994). In cultured rabbit cornea epithelial cells, ET increases $[Ca^{2+}]_i$ with a rank order of ET-1 > ET-2 > ET-3, an action which appears to be mediated via the ET_A receptor (Takagi, *et al.*, 1994).

Ocular Blood Vessels

Endothelin-1 causes a potent, reversible contraction of the human ophthalmic artery, bovine retinal small arteries, and porcine ophthalmic and ciliary arteries (Haefliger, *et al.*, 1993; Nyborg, *et al.*, 1991; Meyer, *et al.*, 1993). In cultured bovine retinal microvascular pericytes, ET-1 and ET-2 stimulated increases in $[Ca^{2+}]_i$, however, ET-3 had no effect on calcium (Ramachandran, *et al.*, 1993). Endothelin-1 also contracts bovine retinal microvascular pericytes, most likely through activation of PLC and increases in intracellular calcium (Charkravarthy, *et al.*, 1992; De La Rubia, *et al.*, 1992). Additionally, the *in vitro* growth of cultured bovine retinal pericytes is increased by factors secreted from endothelial cells, which in vascular pericytes, was shown to be ET-1 (Yamagishi, *et al.*, 1993).

Table 1: Signal Transduction Pathways of Endothelin Stimulation in Ocular Tissues

Location	Species		PLC	$[Ca^{2+}]_i$	Contraction	PLA_2/PG	AC	Other Effects	Reference
Ciliary Muscle	Human	cell	S	S	S	S	S		Korbmacher '89; Stahl '92; Matsumoto '96
		tissue	- / S		- / S	S	S		Abdel-Latif '96; Lepple-Wienhues '92
	Bovine	tissue	S		S	S	S		Lepple-Wienhues '91; Kamikawatoko '95; Abdel-Latif '96
	Cat	tissue	-		-	S	S		Abdel-Latif '96
	Dog	tissue	-		-	S	S		Abdel-Latif '96
	Monkey	tissue			S		S		Millar '95
Ciliary Process	Human	tissue					I		Bausher '95
	Rabbit	tissue	S				I		Bausher '95; Osborne '93
Iris Sphincter	Rabbit	tissue	S		S	S	S	increases PLD	Abdel-Latif '91a, b; Mowafy '94; Zhang '92
	Bovine	tissue	- / S		- / S	S	S		Abdel-Latif '91a; El-Mowafy '94; Yousufzai '93
	Cat	tissue	S		S	S	S		Abdel-Latif '91a; Yousufzai '95
	Monkey	tissue	-		-		S		Abdel-Latif '91a
	Human	tissue	-		-		S		Abdel-Latif '91a
	Dog	tissue	S		S		S		Abdel-Latif '91a
	Pig	tissue	S		S		S		Abdel-Latif '91a
	Rat	tissue			S				Shinkai '94

Table 1: Signal Transduction Pathways of Endothelin Stimulation in Ocular Tissues

Location	Species		PLC	$[Ca^{2+}]_i$	Contraction	PLA_2/PG	AC	Other Effects	Reference
Iris Dilator	Rabbit	tissue	S		S				Abdel-Latif '91a; Ishikawa '93
Trabecular Meshwork	Bovine	cell		S				depolarization increases pH_i	Lepple-Wienhues '92b; Komoto '94
		tissue			S				Lepple-Wienhues '91
	Human	cell						depolarization	Lepple-Wienhues '94
Cornea Epithelium	Rabbit	cell		S					Takagi '94
		tissue	S				S		Takagi '94
Cornea Endothelium	Rabbit	tissue	S				-		Osborne '93
Ocular Blood Vessels	Human	tissue			S				Haefliger '92
	Bovine	pericyte	S	S	S			proliferation increases PKC	Chakrava '92; De La Rubia '92
		tissue			S				Nyborg '91
	Pig	tissue			S				Haefliger '93; Meyer '95

Abbreviations: "S" = stimulation; "I" = inhibition; "-" = no effect
PLC = phospholipase C; $[Ca^{2+}]_i$ = intracellular calcium; PLA_2 = phospholipase A_2; PG = prostaglandins; AC = adenylyl cyclase; PLD = phospholipase D; PKC = protein kinase C

Modified with permission from the review by Pang and Yorio, 1996.

Experimental Rationale

Primary open-angle glaucoma affects millions of people and is the leading cause of blindness today. The currently acceptable pharmacological treatments for glaucoma involve either lowering the production of aqueous humor or increasing its outflow facility. Several new types of compounds are currently being studied for their potential therapeutic use in the treatment of glaucoma, including the endogenous peptide ET. The ETs are a family of regulatory peptides, first discovered in 1988 from supernatants of porcine endothelial cells (Yanagisawa, *et al.*, 1988). The discovery of an endogenous peptide with potent vasoconstrictive properties prompted extensive research into their involvement in a variety of diseases, including ocular disorders. The ETs have been shown to be widely distributed in ocular tissues and researchers have shown their effects in the eye to include constriction of retinal blood vessels, pupillary constriction and dilation, and reductions of IOP. This family of regulatory peptides represent excellent candidates for regulation of ciliary muscle and trabecular meshwork function and their actions may offer insight into the regulation of aqueous humor dynamics and IOP.

The objective of this dissertation is to investigate the cellular mechanism of ET receptor interactions in ocular tissues. The ciliary muscle is an intraocular muscle that is not only responsible for accommodation, but also plays an important role in aqueous humor dynamics. Endothelin has

been shown to stimulate ciliary muscle contractions, which could have important implications in the regulation of aqueous humor outflow and IOP. The specific aims of this dissertation are focused on ET's effect on second messengers such as PLC and calcium, and their interactions with PLA_2 in ciliary muscle cells. **It is hypothesized that in HCM cells, ET-1, via the ET_A receptor and a pertussis toxin sensitive G-protein, activates PLC, which in turn stimulates calcium mobilization. Independent of this pathway, ET-1 also activates PLA_2 and increases the release of prostaglandins.** These two pathways provide a cellular second messenger balance that influences ciliary smooth muscle contraction.

In order to pursue the objectives of this dissertation, the study of the cellular mechanism of ET's receptor interactions in the ciliary muscle and ciliary epithelium was divided into five specific aims. **Specific Aim I** was designed to determine what receptor or receptors are involved in ET-1 stimulated calcium mobilization and whether other ET isoforms, ET-2 and ET-3, produce similar effects. Identification of the mechanism(s) of calcium mobilization as either increased intracellular calcium release or activation of a calcium channel were also investigated. **Specific Aim II** focused on ET-1's effect on PGE_2 production and the interactions between the PLC and PLA_2 coupled events. **Specific Aim III** was designed to determine if the ET-1 stimulation of PLC is G-protein linked, while **Specific Aim IV** focused on the effects of ET-1 on calcium mobilization in ciliary epithelial cells as compared

to ciliary muscle cells. **Specific Aim V** investigated the localization of ET in the ciliary muscle and ciliary epithelium.

The findings of this research will aid in the understanding of the mechanism of action whereby endothelins regulate aqueous humor dynamics and IOP. These findings may also offer insight into the development of new ocular selective agents acting at the ET receptors.

CHAPTER II

METHODS

Cell Culture Model of Human Ciliary Muscle (HCM)

The human ciliary muscle cell strain established by Tamm *et al.*, (1991) was obtained from Alcon Laboratories, Inc. (Fort Worth, TX) and cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 4 mM L-glutamine (Gibco), and 50 μg/ml penicillin/streptomycin (Gibco) in humidified air containing 5% CO_2. The cells were grown to confluency and subcultured by trypsinization using 0.0625% trypsin-0.5 mM ethylenediamine-tetraacetic acid (Gibco). Cells of passages 10-12 were used in subsequent experiments.

All procedures relating to cell culturing, harvesting, freezing and thawing were conducted in aseptic conditions under laminar airflow using aseptic solutions and supplies, including sterile pipettes and centrifugation tubes. The cell passing procedure consisted of removing the culture medium, washing the cells with phosphate-buffered saline (D-PBS) solution, (containing 2.7 mM KCl, 1.1 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4) and adding the 0.0625% trypsinizing solution. After 5-10 minutes of

incubation at 37 °C, once the cells had lifted off the plastic culture dish, the trypsinizing solution was removed and placed in a 50 ml polypropylene centrifuge tube containing and equal amount of DMEM containing serum. The flask was washed with DMEM containing serum, removed and pooled with the cell suspension. The cell suspension was centrifuged for 5 minutes at 3500 rpm (Beckman table top centrifuge). The supernatant was removed and cells were resuspended in the appropriate amount of culture medium for splitting. Aliquots of the suspension were than added to the culture flasks prefilled with the appropriate amount of culture medium, mixed thoroughly by pipetting up and down and the flask was transferred to the incubator (Forma Scientific). Extra cells were frozen by resuspending the centrifuged cells in pre-cooled freezing medium in 10% dimethylsulfoxide (DMSO) from Gibco Laboratories (1 ml of freezing medium for every 0.1 ml of packed cells). The aliquoted cells were immediately frozen at -80 ^{0}C using 1 ml cryo-store vials (Perfecta Scientific). After 18-24 hours (overnight) they were transferred to -140 ^{0}C where they were stored until use.

Thawing procedures consisted of fast cell defrosting in a 30 ^{0}C waterbath, resuspension of the cells in 10 ml DMEM, followed by 5 minutes centrifugation at 3500 rpm to remove the freezing medium. After removal of the supernatant, the cells were resuspended in the appropriate amount of culture medium and added to culture flasks containing DMEM. The flasks were transferred to the incubator after the cell suspension and medium were

well mixed. Culture medium was changed 24 hours after starting a new cell culture from previously frozen cells. Otherwise, culture medium was changed as needed, usually every 2-3 days.

Cell Culture Model of Transfected Human Nonpigmented Ciliary Epithelium (HNPE)

A pure cell line of cultured human nonpigmented ciliary epithelial cells (HNPE), immortalized by transfection with the simian virus 4013 (SV-40) were used. The ODM-2 cells were kindly provided by M. Coca-Prados, M.D., Yale University School of Medicine, New Haven, CT. The cells were subcultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 4 mM L-glutamine (Gibco), and 50 μg/ml penicillin/streptomycin (Gibco) in humidified air containing 5% CO_2. Upon confluence, the cells were subcultured by trypsinization using 0.0625% trypsin-0.5 mM ethylenediamine-tetraacetic acid (Gibco). Cells of passages 15-18 were used in subsequent experiments.

All procedures relating to cell culturing, harvesting, freezing and thawing were conducted in aseptic conditions under laminar airflow using aseptic solutions and supplies, including sterile pipettes and centrifugation tubes. The cell passing, freezing and maintenance procedures were the same as described above for the HCM cells.

Chemicals and Radiochemicals

ET-1, goat anti-rabbit IgG-FITC and quinacrine were purchased from Sigma (St. Louis, MO). ET-2, ET-3, BQ610, IRL-1620, IRL-1038, rabbit anti-ET-1 and rabbit anti-Big ET-1 were purchased from Peninsula (Belmont, CA). U73122, $AACOCF_3$, manoalide, HELSS and pertussis toxin were purchased from Biomol (Plymouth Meeting, PA). Isotetrandrine was obtained from Signal Transduction, Inc. (San Diego, CA) and myo-[2-^{3}H]-inositol from American Radiolabeled Chemicals, Inc. (ARC) (St. Louis, MO). The radioimmunoassay kit for PGE_2 was obtained from New England Nuclear Research (Boston, MA). Fura-2-acetoxymethylester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR). A23187-4Bromo and Floursave reagent were purchased from Calbiochem (LaJolla, CA). Thapsigargin and BQ788 were obtained from Research Biochemicals International (RBI) (Natick, MA).

Intracellular [Ca^{2+}] Measurement

Measurement of intracellular calcium was performed using Dynamic Video Imaging. For these experiments, cells were grown on 0.1 mm thick and 25 mm diameter glass coverslips (#0; Biophysica Technologies, Baltimore, MD) in 6-well polystyrene plates to semiconfluency. All procedures pertaining to growing and harvesting were similar for cells grown on coverslips and cells grown in culture flasks. Coverslips were sterilized by

autoclaving for 30 minutes at 120 °C. The culture medium was changed to serum-free DMEM approximately 18-24 hours before the calcium concentration measurements. On the day of the experiment, the cells were incubated for 30-45 minutes in a HEPES buffer (140 mM NaCl; 4.7 mM KCl; 1.2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM glucose; 10 mM HEPES; pH 7.2) containing 3 μM of a calcium-fluorescent dye, fura-2 acetoxymethylester (Fura-2/AM; Molecular Probes, Eugene, OR). After the incubation, the coverslip was rinsed twice with the same HEPES buffer.

For viewing and measurement of $[Ca^{2+}]_i$, the coverslip was mounted in a temperature controlled microincubator-perfusion chamber (Medical Systems Corp., Greenvale, NY) and placed on the stage of a Nikon Diaphot inverted fluorescent microscope (Tokyo, Japan). The chamber was filled with 3 ml of the HEPES buffer and kept at 37°C during the experiment. Light from the high-intensity Xenon source was directed through a digitally controlled electronic shutter/filter changer wheel containing 340 and 380 nm band pass excitation filters (Omega Optical, Brattlegborro, VT.) into the Nikon inverted microscope. Excitation light was deflected with a 405 nm dichroic mirror through a 40x objective lens (Nikon CF Fluor, Nikon, Garden City, NY) onto the cells. Emitted light was collected through a 510 nm band pass emission filter by a high resolution image intensifier coupled to a video camera (both from Video Scope International, Washington, DC). The signal output from the camera was connected to a digital image-processing board controlled by

IMAGE 1/FLUOR software (Universal Imaging, West Chester, PA). Intracellular fluorescence intensity of 510-nm emission wavelength, excited by alternating 340 and 380 nm excitation wavelengths, was measured and the ratio (340/380) image was computer-generated by dividing the 380 image into the 340 image on a pixel-by-pixel basis. Ratio images were continuously updated on a color monitor every 3.7 seconds and the graphical output displayed as changes in ratio over time. Each ratio measurement was stored every 3.7 seconds in a log-file, written to a hard disk for later analysis.

The $[Ca^{2+}]_i$ was calculated from the intensity ratio of fluorescence at these two excitation wavelengths according to the equation of Grynkiewicz (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = Kd \times (R - R_{min})/(R_{max} - R) \times (F_{min}/F_{max})$$

Kd is the dissociation constant for the fura-2-Ca^{2+} complex in HEPES buffer at 37 ^{o}C and equaled 225 nM. R_{min} and R_{max} were determined from *in vitro* dye calibration obtained using 1 μM A23187-4bromo in the presence of 1.2 mM extracellular calcium (R_{max}) or 10 mM EGTA (R_{min}). F_{min} and F_{max} are the minimum and maximum fluorescence. Sigma Plot Scientific Graphing Software, version 2.0 (Jandel Corporation) was used to convert fluorescence ratio data to intracellular calcium concentrations and to plot changes in $[Ca^{2+}]_i$ in nM over time.

In a typical calcium mobilization experiment, a baseline measurement

NaCl; 26 mM $NaHCO_3$; 5.4 mM KCl; 1.8 mM $CaCl_2$; 1 mM NaH_2PO_4; 0.8 mM $MgSO_4$; 5.5 mM glucose; pH = 7.3), supplemented with 10 mM LiCl. The cells were washed three time with the buffer and then equilibrated in 1 ml buffer for 10 minutes. In agonist experiments, 10 μl of agonist were added to the indicated wells to the desired final concentration and incubated for 90 minutes. In antagonist experiments, the cells were incubated with the antagonist for 15 minutes prior to the addition of 10 μl of agonist. At the end of the incubation period, the assay was terminated by replacing the medium with 1 ml of 0.1 M formic acid. The cells were scraped from their wells and transferred into 16x100 glass test tubes. The wells were washed three times with 1 ml H_2O and each wash was added to the test tube containing the cells. The test tubes were centrifuged at 2500 rpm for 10 minutes (Beckman table top centrifuge). The cell lysate was removed to assay for inositol phosphates and 100 μl of 1N NaOH was added to dissolve the pellet for protein determinations. The cell lysate was then loaded onto an anion exchange column (AG 1-X8 anion exchange resin in formate form, 1 ml; BioRad, Hercules, CA). The eluate and a 7 ml H_2O wash were discarded. Glycerophosphoinositols were eluted first with a 5 mM sodium tetraborate and 60 mM sodium formate solution. The columns were washed with 7 ml of H_2O and the inositol phosphates, IP_2 and IP_3 were then eluted with a 0.1M formic acid and 1M ammonium formate solution. The eluate was collected directly into scintillation vials and 12 ml of Monoflow 4 was added. The

radioactivity in the eluate was counted with a β-scintillation counter. Phospholipase C activity was estimated from the production of radioactive inositol phosphates.

Prostaglandin E_2 Assay

Prostaglandin E_2 activity was assayed using a commercially available RIA kit (New England Nuclear Research, Boston, MA) following the procedure described by Matsumoto *et al.* (1996). Human ciliary muscle cells were grown in 24-well polystyrene plates to confluency. On the day of the experiment, the confluent monolayers of cells were rinsed with 4 x 1 ml of serum free DMEM-F12 (1:1) nutrient mixture (JRH Biosciences, Lenexa, KS). Five minutes after the rinse, 10 μl of ET-1 was added to each well, and the cells were incubated at room temperature for 10 minutes. In antagonist experiments, 10 μl of agonists were added 15-30 minutes after the addition of antagonists. At the end of incubation, 0.2 ml aliquots of medium were transferred into polypropylene test tubes on an ice bath and the PGE_2 concentration in each aliquot was determined by radioimmunoassay according to the manufacturer's recommended procedure. Details of the assay are as follows: All steps to prepare samples and standards were done on an ice bath at 0 °C with cold reagents. Two dilutions of each sample were prepared for analysis since the samples could not be refrozen for later analysis if the [PGE_2] did not fall within the standard curve. The first sample

dilution consisted of 50 μl of sample and 50 μl of assay buffer. The second sample dilution consisted of 10 μl of sample and 90 μl of assay buffer. Serial dilutions of the stock PGE_2 standard provided in the RIA kit were prepared for the standard curve according to the following table:

Stock #	Assay Buffer	PGE_2	Conc.
0	194 ul	+ 6 ul PGE_2 Stock	300 pg/0.1 ml
I	200 ul	+ 100 ul Stock 0	100 pg/0.1 ml
II	270 ul	+ 30 ul Stock 0	30 pg/0.1 ml
III	270 ul	+ 30 ul Stock I	10 pg/0.1 ml
IV	270 ul	+ 30 ul Stock II	3 pg/0.1 ml
V	270 ul	+ 30 ul Stock III	1 pg/0.1 ml
VI	270 ul	+ 30 ul Stock IV	0.3 pg/0.1 ml

Duplicate 100 μl aliquots of the stock PGE_2 solutions were transferred into polypropylene tubes as follows:

0 Standard	Assay buffer
3 pg/ml	Stock VI
10 pg/ml	Stock V
30 pg/ml	Stock IV
100 pg/ml	Stock III
300 pg/ml	Stock II
1000 pg/ml	Stock I
Blank	Assay buffer
Total Counts	None

The radioactive assay tracer provided in the RIA kit [^{125}I]-PGE_2 was diluted 1:20 with cold assay buffer and 0.1 ml of the diluted tracer was added to all samples and standards, vortexing immediately after the addition of tracer. The lyophilized antibody provided in the RIA kit was diluted with 13 ml of assay buffer and 0.1 ml was added to all samples and standards, except the tubes containing the blank standard and total counts. All test tubes were vortexed and incubated overnight in the refrigerator. After incubation, 1 ml of cold precipitating reagent provided in the RIA kit was added to all samples and standards, except the tubes for total counts, and vortexed immediately. The test tubes were incubated for 30 minutes and centrifuged at 4 ^{0}C and 3000 rpm for 30 minutes. The supernatant was decanted off into a radioactive waste container and the pellet counted in the Gamma counter using 1 minute counts. The net counts per minute (cpm) were calculated by subtracting the counts per minute of the blank from each sample and standard. The $\%B/B_0$ was calculated for each sample and standard according to the following formula:

$$\%B/B_0 = (\text{Net CPM} / \text{avg. 0 standard}) \times 100\%$$

The standard curve was obtained by plotting the average $\%B/B_0$ for each standard against the concentration of PGE_2. Prostaglandin E_2 concentrations of the samples were calculated from interpolation of the standard curve.

Protein Determination

Protein concentrations were determined using the method described by Bradford in 1976. In brief, protein standard solutions containing 3 to 30 μg protein in a volume up to 150 μl were pipetted into 12x75 mm glass test tubes. The volume of the test tube was adjusted to 300 μl with water. Three milliliters of Bradford protein reagent [0.01% (w/v) Coomassie brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid] were added to the test tube and vortexed. The optical density (Absorption) at 595 nm was measured in 1 ml cuvettes against a reagent blank containing 300 μl of water and 3.0 ml Bradford-reagent. The protein concentration was plotted against the corresponding absorbance resulting in a standard curve from which the unknown protein concentration could be calculated.

Immunocytochemistry Technique

Localization of ET-1 in ciliary muscle and ciliary epithelium was investigated using immunocytochemistry. Human ciliary muscle cells (passages 11-14) and HNPE cells (passages 12-18) were grown on 0.1 mm thick and 25 mm diameter glass coverslips (#0; Biophysica Technologies, Baltimore, MD) in 6-well polystyrene plates to approximately 90% confluency. All procedures pertaining to growing and harvesting are similar for cells grown on coverslips and cells grown in culture flasks. Coverslips were sterilized by autoclaving for 30 minutes at 120 ^{o}C. The culture medium

was changed to serum-free DMEM approximately 8 hours before the immuno-fluorescence experiment. On the day of the experiment, the cells were washed 3 times, at 5 minute intervals, with 2 ml of tris-buffered saline (TBS) with 2% bovine serum albumin (BSA), pH 7.5 (0.05M Tris, 0.15M NaCl, 2% BSA). After the final rinse, cells were incubated for 30 minutes in either TBS + 2% BSA solution (fluorescent controls and unstimulated cells) or 10 nM TNF-α (cytokine stimulated cells). At 30 minutes, the incubation media was removed and the cells were treated with 2 ml of a pre-cooled (-20 ^{0}C) 1:1 mixture of methanol:acetone for 5 minutes to fix the cells. After the 5 minute incubation, the fixative mixture was evaporated and the cells were rapidly rinsed 2 times with TBS + 2% BSA solution. The unstimulated cells and cytokine stimulated cells were then incubated with 500 μl of primary antibody (rabbit anti-ET-1 or rabbit anti-Big ET-1, Peninsula Laboratories, Belmont, CA) diluted 1:500 in TBS + 2% BSA solution, at 4 ^{0}C for 15 hours. Cross-reactivity for anti-ET-1 with Big ET-1 was 17% while anti-Big ET-1 showed cross-reactivity of $<$0.01% with ET-1. After the incubation, the cells were rinsed 3 times with TBS + 2% BSA solution to block the non-specific antigen receptors. The secondary antibody, goat anti-rabbit IgG-FITC (Sigma, St. Louis, MO) was diluted 1:200 with TBS + 2% BSA solution and the cells were incubated in secondary antibody for 30 minutes at 37 ^{0}C. The coverslips were rinsed 3 times with TBS + 2% BSA solution and then quickly with deionized H_2O. A drop of Fluorsave reagent

(Calbiochem, LaJolla, CA) was added to the center of a slide and the coverslip was carefully inverted onto the drop of Fluorsave. The slide was air dried for 20-30 minutes allowing the Fluorsave to harden. The cells were viewed under a Nikon Diaphot fluorescence microscope (Tokyo, Japan) at wavelength 492 nm [fluorescein isothiocyanate (FITC) labeled sections are illuminated by blue light]. The labeled antigen was visible as a bright green glow (wavelength 520 nm). Fluorescence was visualized at an objective lens magnification of 40X and camera magnification of 100-150X. Photographs were captured on Kodak P3200 ASA black and white film. Four exposures were taken per treatment and the results were first observed on a contact sheet. Prints for each treatment were selected based on the overall trend observed in the four exposures taken for that treatment. Each photograph depicted the same percentage (85-90%) of cell confluency compared to those taken for all treatments. The photographs (5"X7") were scanned using a Hewlett-Packard DeskScan II and stored as TIFF files for gray-scale measurements using Image-1 software (Universal Imaging, West Chester, PA). Gray-scale measurements were made in order to detect changes in the fluorescence between the background fluorescence and unstimulated or stimulated cells. A rectangular measuring tool (450 pixels X 100 pixels) from the imaging software was used to calculate the black-white-gray ratio on a scale from zero (white) to 255 (black) level. Each 5"X7" photograph was sampled three times by the rectangular tool and the average gray scale

value was calculated. The gray-scale value was then compared between the background fluorescence and the unstimulated or stimulated cells. The mean background fluorescence was defined as 100% and the results of the unstimulated and stimulated cells were represented as percent change from background (%Fluorescence = treatment/background X 100%). Any % fluorescence value greater than 100% (background fluorescence) represents less fluorescence (darker) while any value less than 100% indicates an increase in fluorescence (brighter).

Data Analysis

All data are presented as mean ± S.E.M.; N refers to the number of individual studies performed for that parameter. Statistical analyses were performed using Jandel SigmaStat for windows (version 2.0) supplied by Jandel Corporation (Sausalito, CA) or the Pharmacological Calculation System (PCS version 4.0). Appropriate statistical analyses were applied to each set of data with $p<0.05$ considered statistically significant. The specific test used for analysis is indicated within the text of the results. In antagonist studies, all treatment comparisons were made against the response to ET even if presented in separate tables. All graphical presentations were plotted using SigmaPlot for windows (version 2.0) Scientific Graphing Software supplied by Jandel Corporation (Sausalito, CA). TableCurve 2D for windows (version 2.03, Jandel Corporation, Sausalito, CA) was used for interpolation from standard curves.

CHAPTER III

RESULTS

Specific Aim I

Endothelin Stimulated Calcium Mobilization: Receptor Identification and Cellular Signaling Pathway

Dynamic video imaging was used to measure the effect of the three isoforms of endothelin, ET-1, ET-2 and ET-3, on changes in intracellular calcium $[Ca^{2+}]_i$ in HCM cells. Once the HCM cells reached a steady baseline, doses of ET ranging from 1 nM to 100 nM were tested for their effect on the mobilization of calcium. In experiments where multiple doses of agonists were tested, a new steady baseline was obtained prior to the addition of the next dose.

Endothelin-1 and ET-2 increased $[Ca^{2+}]_i$ in HCM cells, whereas ET-3 at doses from 1 nM to 100 nM had no effect on $[Ca^{2+}]_i$ (Table 2, Figure 3). Dose-response curves for ET-1 and ET-2 stimulation in HCM cells were attempted, however, repeated agonist stimulation evoking calcium mobilization in the HCM cells resulted in decreased responses or no response to higher doses of ET. For example, in one experiment the HCM cells were

Table 2. Effects of Endothelin on Intracellular Calcium Levels in Human Ciliary Muscle Cells.

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-2 (1 nM)	15	149 ± 31	32 ± 8
ET-3 (1 nM)	11	6 ± 1	-1 ± 1

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

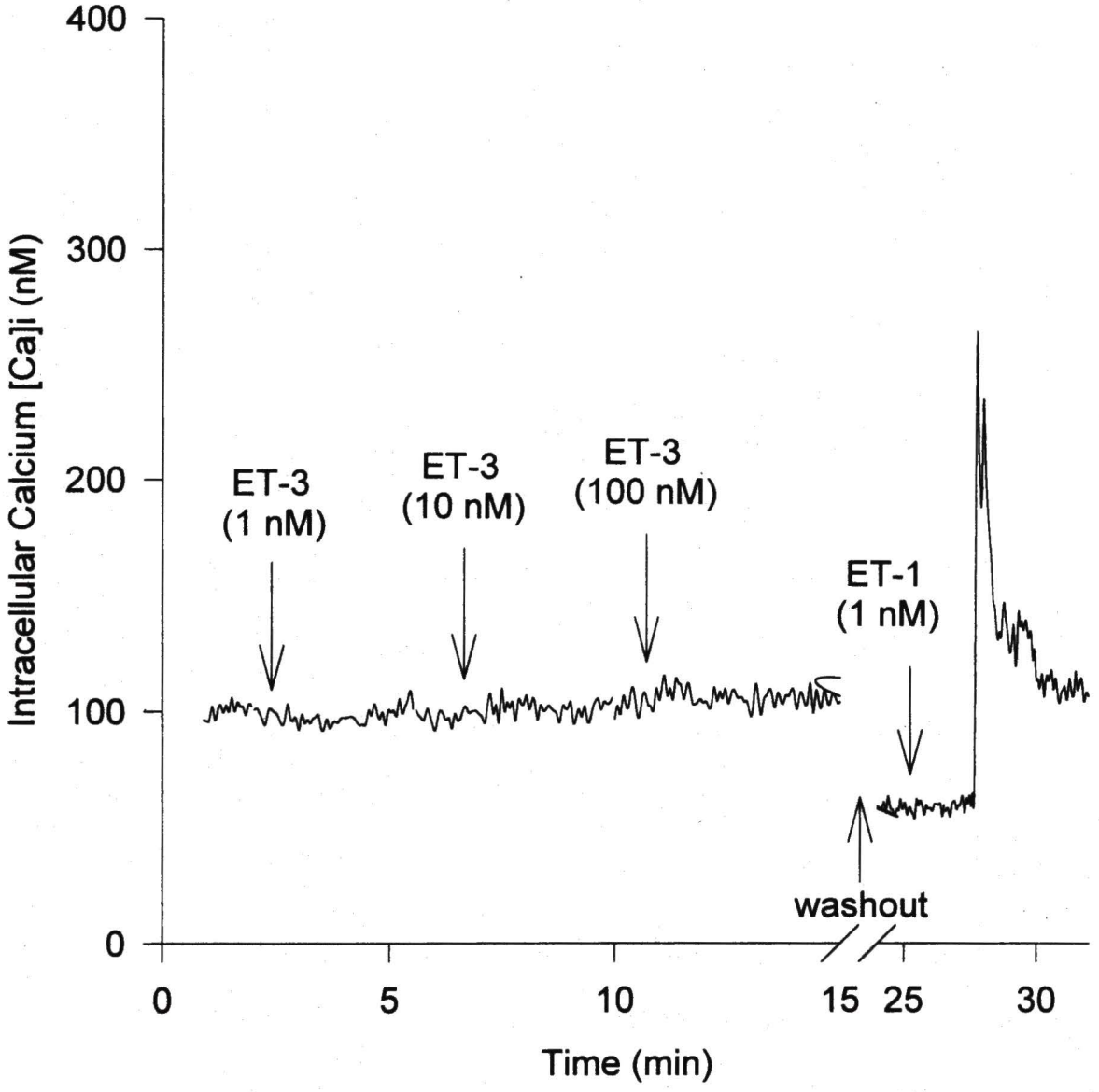


Figure 3. Effect of ET-3 on $[Ca^{2+}]_i$ in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$ after addition of 1, 10 and 100 nM ET-3.

dosed with 1 nM of ET-1 increasing the $[Ca^{2+}]_i$ with a mean peak of 139 ± 23 nM ($n = 7$) above the resting level. Upon subsequent stimulation with 10 nM of ET-1, the increase in $[Ca^{2+}]_i$ was significantly lower with a mean peak of 68 ± 16 nM ($n = 7$) above the resting level. Even when washouts between doses were done, lasting approximately 10-15 minutes, the same effect was observed. There appears to be a receptor desensitization or down regulation with repeated exposure to ET in HCM cells, rendering dose-response studies within the same experiment unreliable.

The time-dependent changes in $[Ca^{2+}]_i$ following the addition of ET-1 (1 nM) and ET-2 (1 nM) resulted in an increase in $[Ca^{2+}]_i$ reaching its peak 2 to 4 minutes after the addition of the peptide, which was followed by a sustained elevation in $[Ca^{2+}]_i$ lasting up to 15 minutes (Figure 4). The mean resting $[Ca^{2+}]_i$ in these cells was 56 ± 2 nM (mean ± SEM, $n = 70$). The addition of 1 nM ET-1 caused an increase in $[Ca^{2+}]_i$ with a mean peak $[Ca^{2+}]_i$ of 240 ± 22 nM ($n = 12$) above the resting level and the addition of 1 nM ET-2 caused a mean peak increase in $[Ca^{2+}]_i$ of 149 ± 31 nM ($n = 15$) above the resting level (Table 2). A sustained elevation in $[Ca^{2+}]_i$, or a plateau phase, was observed upon stimulation with both ET-1 and ET-2. To quantify the plateau phase a one minute post-peak $[Ca^{2+}]_i$ was selected to capture the most stable post-stimulatory time of the plateau.

Dynamic video imaging was used to determine the receptor subtype involved in the ET stimulated calcium mobilization. Various agonists and

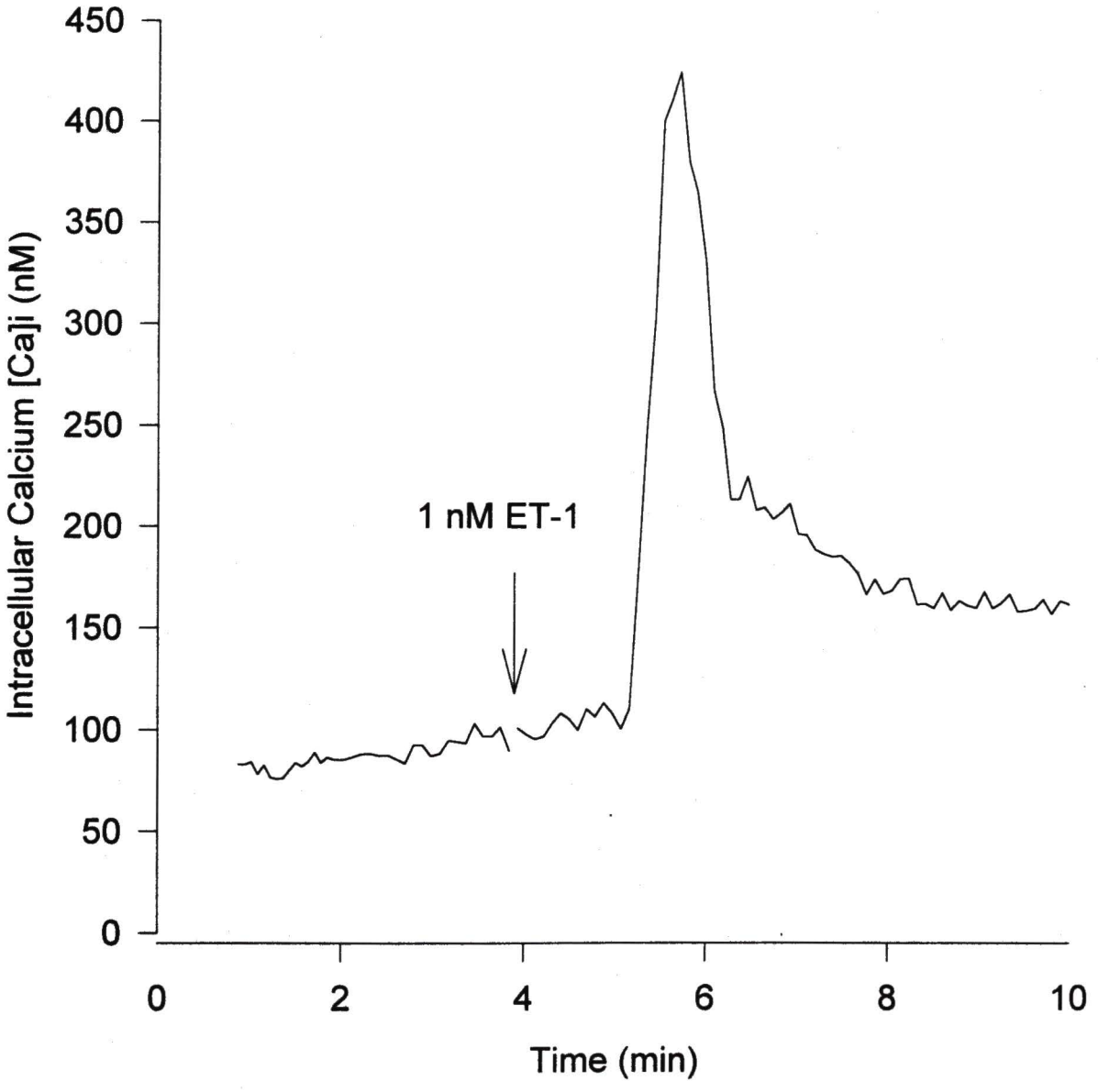


Figure 4. Effect of ET-1 on $[Ca^{2+}]_i$ in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$ after addition of 1 nM ET-1.

antagonists of the ET_A and ET_B receptor were utilized to determine their effect on the ET-1 and ET-2 mediated increases in intracellular calcium. The following compounds were used: BQ610 (10 nM), an ET_A receptor subtype-selective antagonist, $K_i = 10$ nM (Ishikawa, K. *et al.*, 1993; Matsumoto, *et al.*, 1996); IRL-1620 (1 nM - 100 nM), an ET_B subtype-selective agonist (Takai, *et al.*, 1992; Watanabe, *et al.*, 1992; Karaki, *et al.*, 1993); IRL-1038 (50 nM -100 nM), an ET_B subtype-selective antagonist, K_i = 6-11 nM (Urade, *et al.*, 1992); and BQ788 (50 nM) an ET_B subtype-selective antagonist, K_i = 4 nM (Ishikawa, K. *et al.*, 1994) . In antagonist studies, ET was dosed 5 minutes after the addition of the antagonist to determine if calcium mobilization was blocked. In experiments where multiple doses of agonists were tested, a new steady baseline was obtained prior to the addition of the next dose.

The effect of both ET-1 and ET-2 on calcium mobilization appears to be mediated by the ET_A receptor subtype. BQ610, an ET_A receptor subtype-selective antagonist, at 10 nM, completely blocked the ET-1 (1 nM) and ET-2 (1 nM) increase in $[Ca^{2+}]_i$, with mean peak $[Ca^{2+}]_i$ to ET-1 of 9 ± 2 nM ($n = 15$) and 4 ± 0.5 nM for ET-2 addition ($n = 6$), and a mean plateau $[Ca^{2+}]_i$ of -2 ± 1 nM and 2 ± 0.6 nM, respectively (Table 3). Even with equal doses of antagonist and agonist, BQ610 (10 nM) blocked the ET-1(10 nM) stimulation of calcium mobilization. No significant increase in $[Ca^{2+}]_i$ was seen at the 1:1 antagonist:agonist dose as compared to the 10:1

Table 3. Effects of ET-1 and ET-2 Following the Addition of BQ610 (ET_A Receptor Antagonist) on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + BQ610 (10 nM)	15	9 ± 2*	-2 ± 1*
ET-1 (10 nM) + BQ610 (10 nM)	15	27 ± 4*	10 ± 2*
ET-2 (1 nM)	15	149 ± 31	32 ± 8
ET-2 (1 nM) + BQ610 (10 nM)	6	4 ± 0.5*	2 ± 0.6

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist with antagonist studies)

antagonist:agonist dose (Table 3).

To verify that endothelin stimulates calcium mobilization primarily via the ET_A receptor subtype, IRL-1620, an ET_B receptor subtype-selective agonist, was tested for its effect on calcium mobilization in HCM cells. IRL-1620 had no effect on $[Ca^{2+}]_i$ at doses ranging from 1 nM to 100 nM (Figure 5). The results are summarized in Table 4. Mean peak and plateau $[Ca^{2+}]_i$ when stimulated with 1 nM IRL-1620 were 11 ± 2 nM and 3 ± 2 nM ($n = 13$), respectively. Mean peak and plateau $[Ca^{2+}]_i$ when stimulated with 10 nM IRL-1620 were 12 ± 2 nM and 6 ± 2 nM ($n = 13$), respectively. Mean peak and plateau $[Ca^{2+}]_i$ when stimulated with 100 nM IRL-1620 were 6 ± 1 nM and 0.1 ± 1 nM ($n = 13$), respectively.

The effect of IRL-1038, an ET_B receptor subtype-selective antagonist, on the ET-1 and ET-2 stimulated calcium mobilization, was also investigated. Initially, the HCM cells were pretreated with 100 nM IRL-1038 prior to the addition of ET-1 (1 nM). IRL-1038 (100 nM) partially blocked the ET-1 (1 nM) mediated calcium mobilization (Table 5). Therefore, the dose of IRL-1038 was reduced to 50 nM and the experiment was repeated. IRL-1038 (50 nM) still partially blocked the ET-1 (1 nM) and ET-2 (1 nM) mediated Ca^{2+} mobilization with mean peak $[Ca^{2+}]_i$ of 87 ± 23 nM (n = 6) and 59 ± 15 nM ($n = 4$), respectively and mean plateau $[Ca^{2+}]_i$ of 23 ± 7 nM and 46 ± 15 nM, respectively (Table 5). It was expected that IRL-1038 would have no effect on the ET-1 and ET-2 stimulated increase in $[Ca^{2+}]_i$. Prior

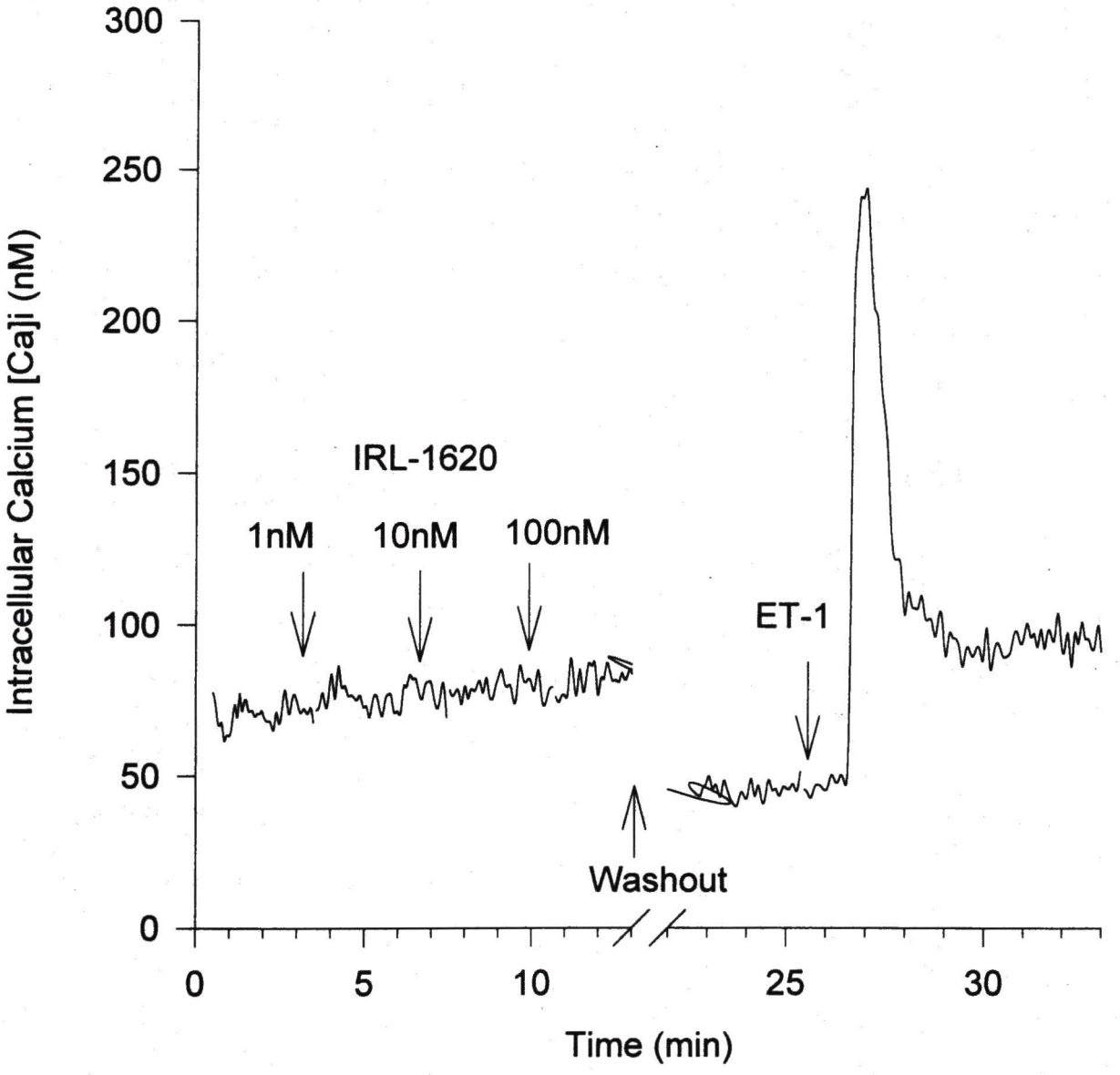


Figure 5. Effect of IRL-1620, an ET_B receptor subtype-selective agonist on $[Ca^{2+}]_i$ in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$ after addition of IRL-1620.

Table 4. Effects of IRL-1620 (ET_B Receptor Agonist) on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
IRL-1620 (1 nM)	13	11 ± 2*	3 ± 2*
IRL-1620 (10 nM)	13	12 ± 2*	6 ± 2*
IRL-1620 (100 nM)	13	6 ± 1*	0.1 ± 1*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 response and IRL-1620 responses)

Table 5. Effects of ET-1 and ET-2 Following the Addition of IRL-1038 or BQ-788 (ET_B Receptor Antagonists) on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + IRL-1038 (100 nM)	8	43 ± 5*	23 ± 2*
ET-1 (1 nM) + IRL-1038 (50 nM)	6	87 ± 23*	23 ± 7*
ET-1 (1 nM) + BQ-788 (50 nM)	5	129 ± 19*	81 ± 13
ET-2 (1 nM)	15	149 ± 31	32 ± 8
ET-2 (1 nM) + IRL-1038 (50 nM)	4	59 ± 15	46 ± 15
ET-2 (1 nM) + BQ-788 (50 nM)	9	146 ± 30	74 ± 19

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist with antagonist studies)

experiments showed that the ET_A receptor antagonist, BQ610 completely blocked the ET-1 and ET-2 stimulated calcium mobilization, and the ET_B receptor agonist, IRL-1620 did not increase $[Ca^{2+}]_i$ at doses as high as 100 nM. Therefore, the specificity of IRL-1038 was investigated. From binding studies, the K_i for IRL-1038 at ET_B receptors is 10 fold higher than at ET_A receptors (Urade, *et al.*, 1992), therefore it is possible that some ET_A receptor antagonism was occurring. Another ET_B receptor antagonist, BQ-788 was tested for its effect on the ET-1 and ET-2 stimulated calcium mobilization. BQ-788 (50 nM) showed some inhibition of the ET-1 (1 nM) mediated calcium mobilization with mean peak and plateau $[Ca^{2+}]_i$ of 129 ± 19 nM and 81 ± 13 nM, respectively ($n = 5$), but had no effect on the ET-2 mediated calcium mobilization with mean peak and plateau $[Ca^{2+}]_i$ of 146 ± 30 nM and 74 ± 19 nM, respectively ($n = 9$) (Table 5).

To determine the source(s) of the ET-1 stimulated $[Ca^{2+}]_i$, a series of experiments were conducted using dynamic video imaging to identify whether the mechanism of ET-1 stimulated calcium mobilization was the consequence of increased intracellular release of calcium or activation of a calcium channel in the membrane. Thapsigargin was used to deplete intracellular stores of calcium prior to treating the cells with 1 nM ET-1. The calcium channel blockers, nickel and nifedipine (selective for L-type channels) were administered prior to the addition of ET-1 to test the effect of calcium channels. The effect of external calcium on the ET-1 stimulated calcium

mobilization was also investigated.

Thapsigargin (1 μM) was used to deplete IP_3-sensitive intracellular stores of calcium prior to treating the cells with ET-1 (1 nM). Once the intracellular stores of calcium in the HCM cells were depleted, the cells were allowed to reach a new steady state prior to the addition of the ET-1 (Figure 6). Endothelin-1 (1 nM) caused a slight increase in $[Ca^{2+}]_i$, with oscillations in calcium levels observed for all cells. The mean peak increase in $[Ca^{2+}]_i$ after depletion of the intracellular stores with thapsigargin, was 43 $\pm$ 6 nM ($n = 9$) above the new steady state level and the mean plateau $[Ca^{2+}]_i$ was -15 $\pm$ 6 nM relative to the new steady state level (Table 6). These results indicated that a large portion of the ET-1 induced calcium mobilization depends on the release of calcium from IP_3-sensitive intracellular stores.

An alternative mechanism of the ET-1 induced increase in $[Ca^{2+}]_i$ is through calcium channels. It appears that endothelin gates multiple types of calcium channels and it has been proposed that the mechanism by which endothelin increases calcium may be through an indirect, modulatory mechanism in which endothelin increases the activity of L-type calcium channels (Simonson and Dunn, 1990). Therefore, the calcium channel blockers, nickel and nifedipine, which are selective for L-type channels, were administered prior to the addition of ET-1 to test their effect on the ET-1 stimulated calcium mobilization. When the HCM cells were pretreated for 10 minutes with 5 mM NiCl prior to the addition of ET-1, the ET-1 mediated

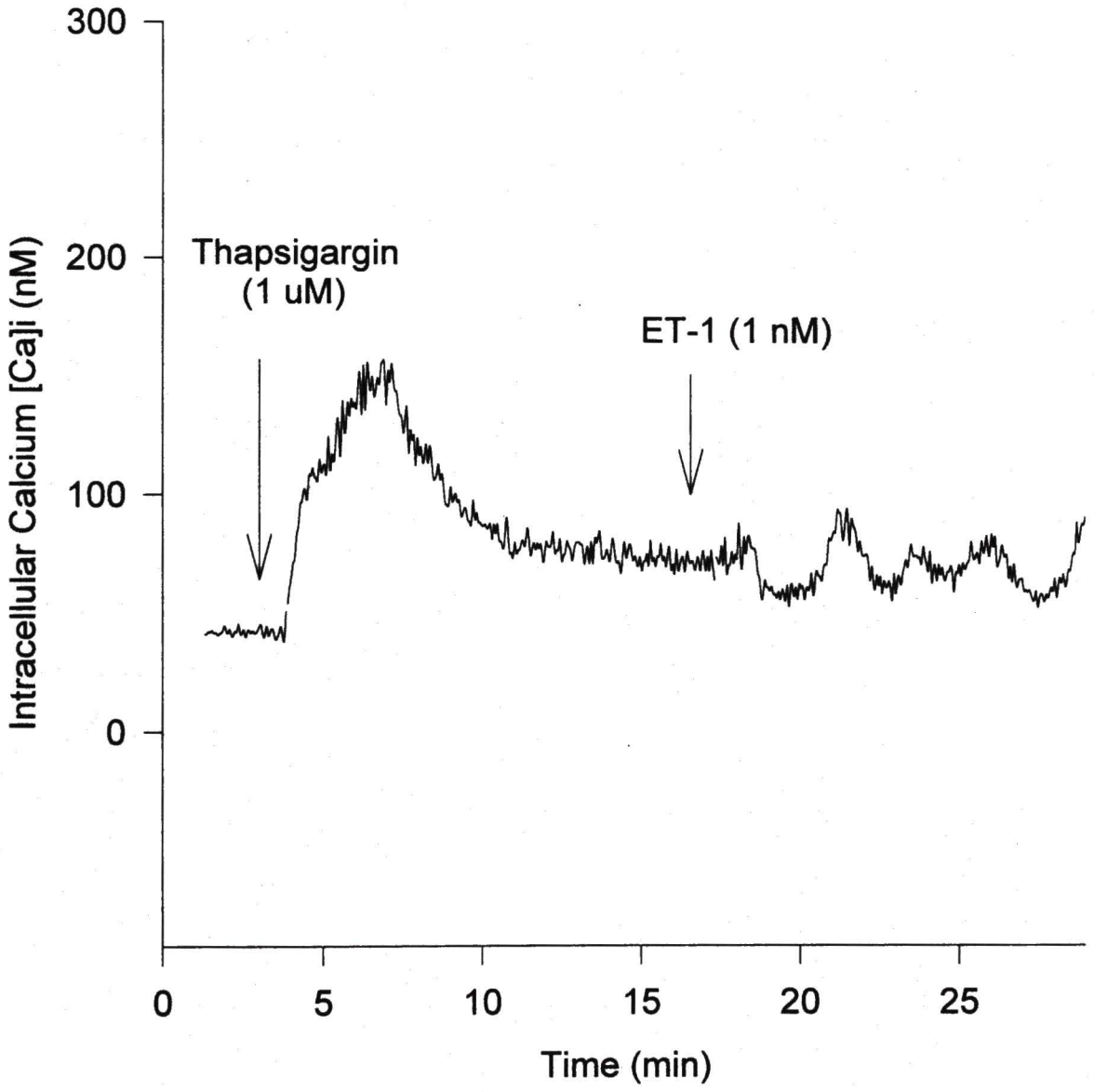


Figure 6. Effect of ET-1 on $[Ca^{2+}]_i$ after depletion of the intracellular stores of Ca^{2+} with thapsigargin in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

Table 6. Effects of ET-1 Following the Addition of Thapsigargin on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + Thapsigargin (1 μM)	9	43 ± 6*	-15 ± 6*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist with pretreatment)

calcium mobilization appeared to be inhibited at both 1 nM ET-1 and 10 nM ET-1 (Table 7). However, the NiCl itself stimulated calcium mobilization in the HCM cells prior to the addition of ET-1. Therefore, the concentration of NiCl was reduced to 1 mM and the experiment was repeated. Again, the NiCl (1 mM) produced some increase in $[Ca^{2+}]_i$ prior to the addition of ET-1 (1 nM) and inhibited the ET-1 stimulated calcium mobilization with a mean peak increase in $[Ca^{2+}]_i$ of 28 $\pm$ 5 nM ($n=9$) and a mean plateau of 3 $\pm$ 2 nM (Table 7). Pretreatment of the HCM cells with nifedipine (1 μM) for 10 minutes prior to the addition of ET-1, decreased the ET-1 (1 nM) mediated increase in $[Ca^{2+}]_i$ relative to ET-1 alone with a mean peak increase in $[Ca^{2+}]_i$ of 96 $\pm$ 18 nM ($n=10$) and a mean plateau of 41 $\pm$ 6 nM (Table 8). Although the increase in $[Ca^{2+}]_i$ was blunted in the presence of nifedipine, the typical biphasic calcium response to ET-1, a rapid increase in $[Ca^{2+}]_i$ followed by a sustained elevation in $[Ca^{2+}]_i$, was preserved in most cells (Figure 7).

It appears that the ET-1 stimulated increase $[Ca^{2+}]_i$ in HCM cells results from a receptor activated release of intracellular stores of calcium as well as an influx of calcium across the plasma membrane. Therefore, the effect of external calcium on the ET-1 stimulated calcium mobilization was also investigated. In the HCM cells, ET-1 (1 nM) in the presence of extracellular calcium elicited the typical biphasic increase in $[Ca^{2+}]_i$ consisting of a rapid, transient increase followed by a lesser but sustained increment

Table 7. Effects of ET-1 Following the Addition of Nickel Chloride (NiCl) on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + NiCl (5 mM)	9	27 ± 8*	2 ± 2*
ET-1 (10 nM) + NiCl (5 mM)	4	9 ± 1*	2 ± 2*
ET-1 (1 nM) + NiCl (1 mM)	9	28 ± 5*	3 ± 2*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist with pretreatment)

Table 8. Effects of ET-1 Following the Addition of Nifedipine on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + Nifedipine (1 μM)	10	96 ± 18*	41 ± 6*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist with pretreatment)

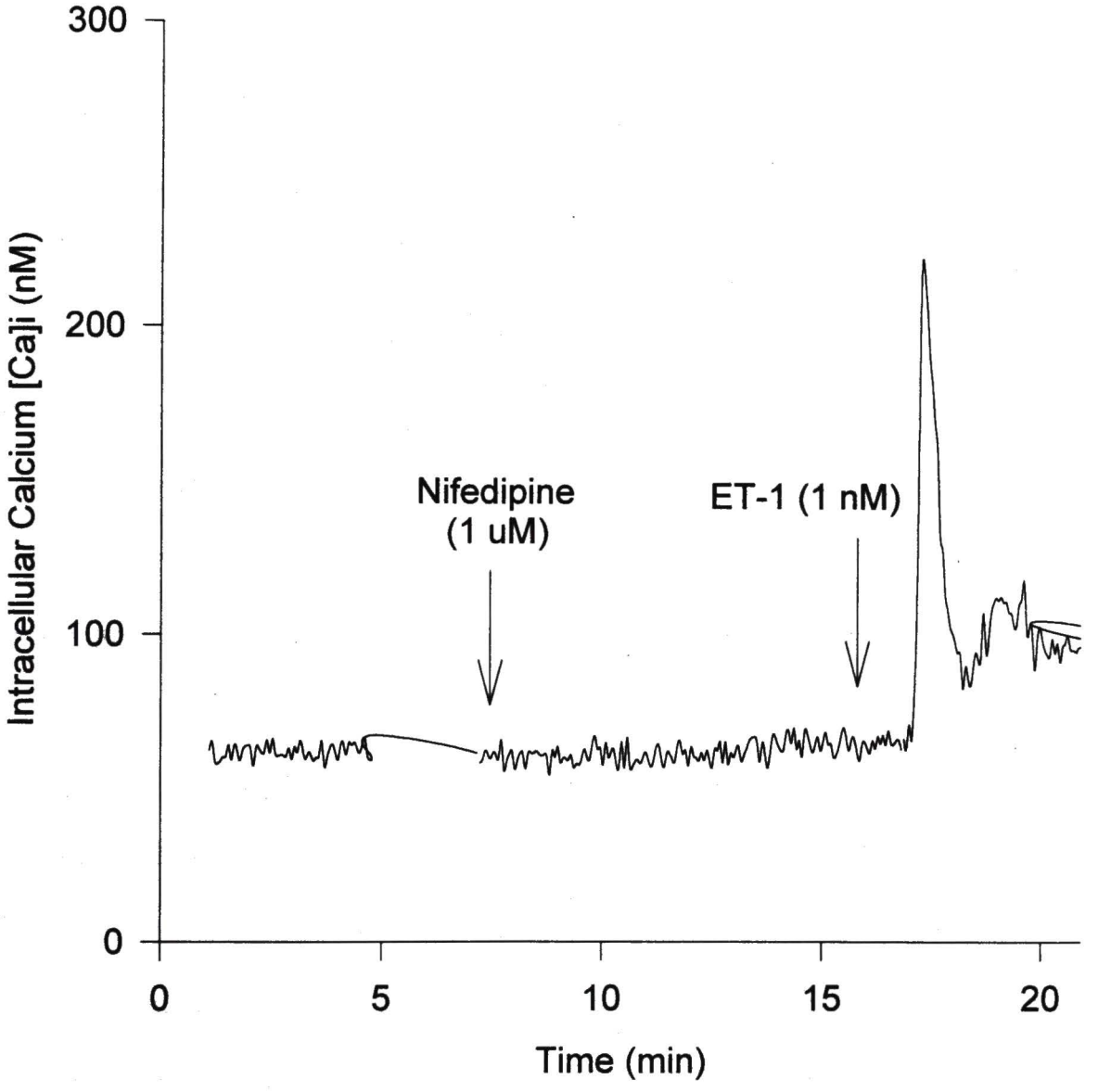


Figure 7. Effect of ET-1 on $[Ca^{2+}]_i$ after blockade of Ca^{2+} channels with nifedipine in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

(Figure 8). In this set of experiments, the addition of 1 nM ET-1 caused an increase in $[Ca^{2+}]_i$ with a mean peak $[Ca^{2+}]_i$ of 851 ± 161 nM ($n = 20$) above the resting level and a mean plateau $[Ca^{2+}]_i$ of 219 ± 27 nM above the resting level (Table 9). To test the effect of the ET-1 stimulated calcium mobilization in the absence of extracellular calcium, HEPES buffer without $CaCl_2$ was used in the perfusion chamber. In the absence of extracellular calcium, the ET-1 (1 nM) stimulated increase in peak $[Ca^{2+}]_i$ was significantly blunted with a mean peak $[Ca^{2+}]_i$ of 160 ± 15 nM ($n = 17$) and the sustained plateau phase was eliminated with mean plateau $[Ca^{2+}]_i$ values of -10 ± 4 nM relative to the resting level (Figure 9; Table 9). This data suggests that in calcium-free medium, the ET-1 induced increases in $[Ca^{2+}]_i$ are attenuated and the sustained increase in $[Ca^{2+}]_i$, or plateau phase, is abolished.

Increases in intracellular calcium typically result from receptor stimulation of PLC and release of IP_3 in many smooth muscle preparations. Therefore, the role of PLC activity in the ET-1 mediated increase in $[Ca^{2+}]_i$ was investigated, as well as the direct effect of ET-1 on PLC activity. Dynamic video imaging was used to determine the role of PLC activity in the ET-1 mediated increase in intracellular calcium concentrations. The cells were pretreated with the PLC inhibitor, U73122 (1 μM) to test its effect on ET-1 stimulated calcium mobilization. As expected, the ET-1 stimulated increase in $[Ca^{2+}]_i$ appears to be mediated through the PLC signaling pathway in HCM cells.

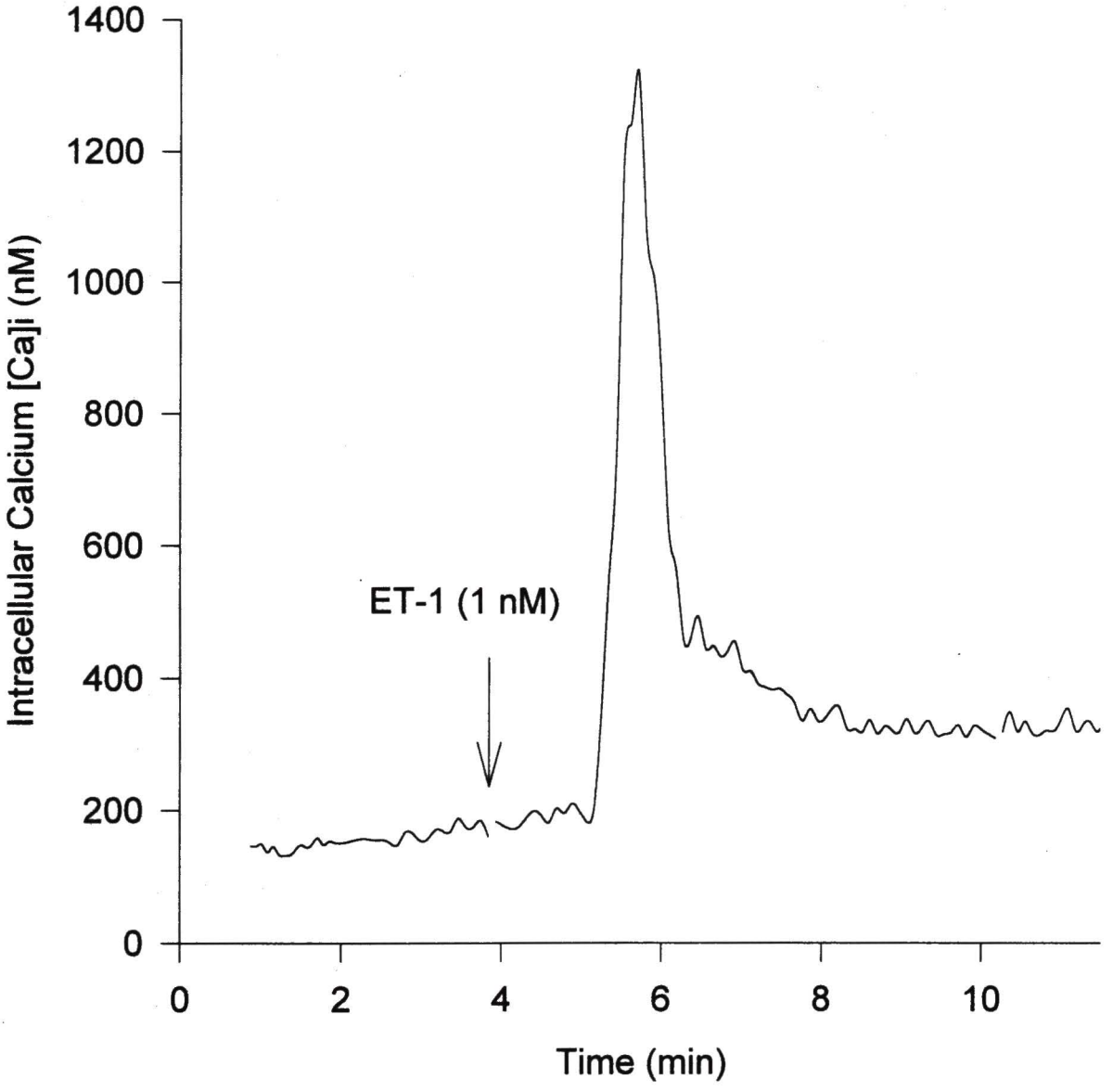


Figure 8. Effect of ET-1 on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

Table 9. Effects of ET-1 in Ca^{2+}-Free Media on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM) in Ca^{2+} Buffer	20	851 ± 161	219 ± 27
ET-1 (1 nM) in Ca^{2+}-Free Buffer	17	160 ± 15*	-10 ± 4*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist in Ca^{2+} buffer and agonist in Ca^{2+}free buffer)

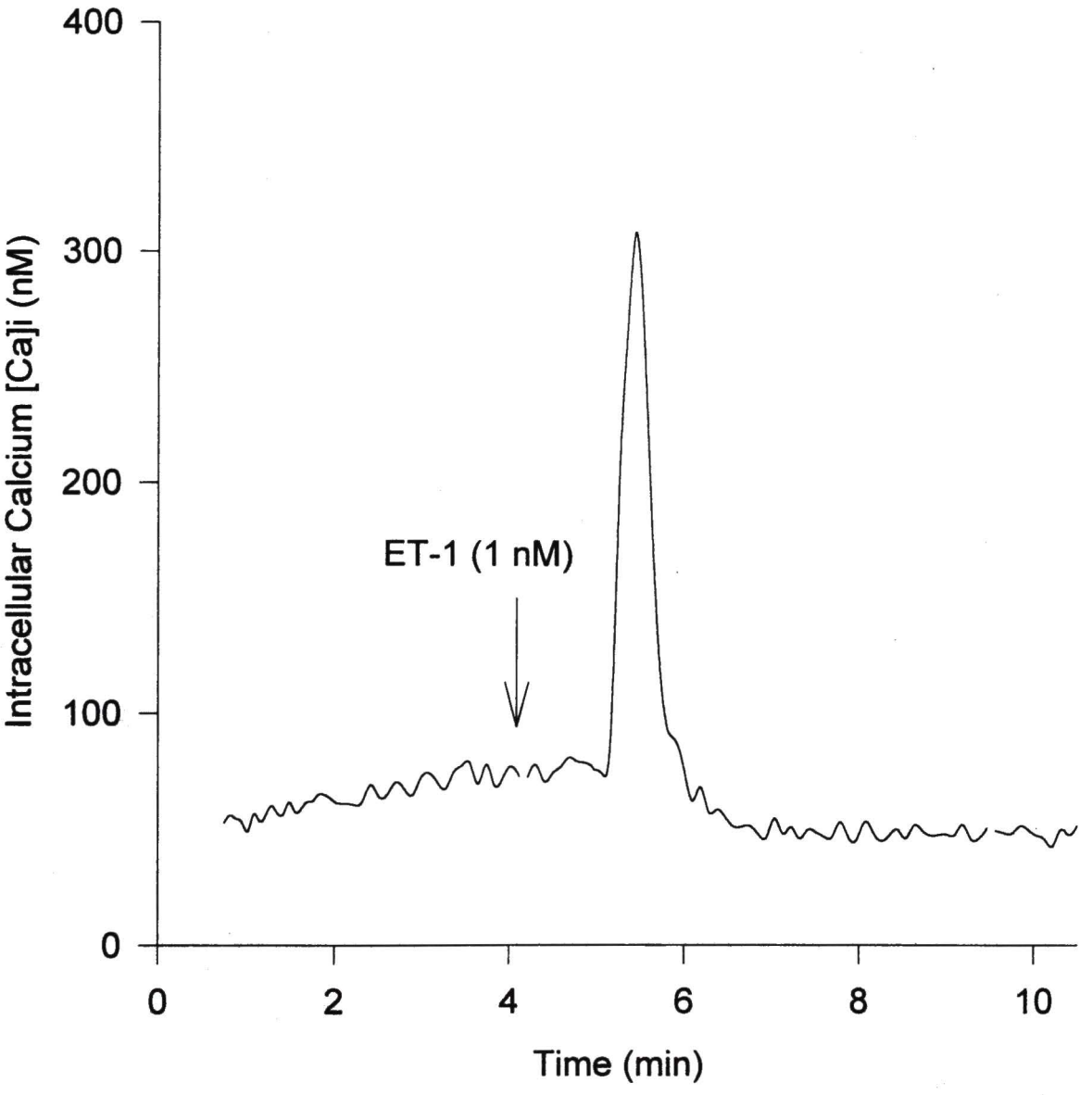


Figure 9. Effect of ET-1 on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

Pretreatment of the HCM cells for 10 minutes with 1 μM U73122, a PLC inhibitor, completely abolished the ET-1 stimulated calcium mobilization with a mean peak increase in $[Ca^{2+}]_i$ of 4 $\pm$ 1 nM ($n = 15$) and mean plateau value of 1 $\pm$ 1 nM (Table 10). This result indicated that stimulation of PLC activity in the HCM cells is integral to the ET-1 stimulated calcium mobilization.

The direct effect of ET-1 on PLC activity was then investigated. A time course study was first conducted to determine the optimal incubation time for the production of inositol phosphates following stimulation with 100 nM ET-1. Incubation times for the time course study were 0 min, 30 min, 60 min and 90 min. The effect of ET-1 on the accumulation of inositol phosphates was then tested. Two doses of ET-1 (1 nM and 100 nM) were utilized in the PLC activity measurements based on the dose-response study and EC_{50} reported by Matsumoto, *et al.* (1996). Additionally, pretreatment of the cells with the PLC inhibitor, U73122 was tested for its effect on the production of inositol phosphates. To elucidate the involvement of ET receptor subtypes in the activation of PLC by ET-1, the ET_A subtype-selective antagonist, BQ610, was used to block this effect. The effect of two PLA_2 inhibitors, $AACOCF_3$ (cytosolic PLA_2 inhibitor) and isotetrandrine (G-protein linked PLA_2 inhibitor), on ET-1 stimulated PLC activity was also investigated.

The time course of ET-1 induced PLC stimulation is shown in Figure 10. After 100 nM ET-1 treatment, accumulation of inositol phosphates

Table 10. Effects of the PLC Inhibitor (U73122) on Intracellular Calcium Levels in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + U73122 (1 μM)	15	4 ± 1*	1 ± 1*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET + pretreatment)

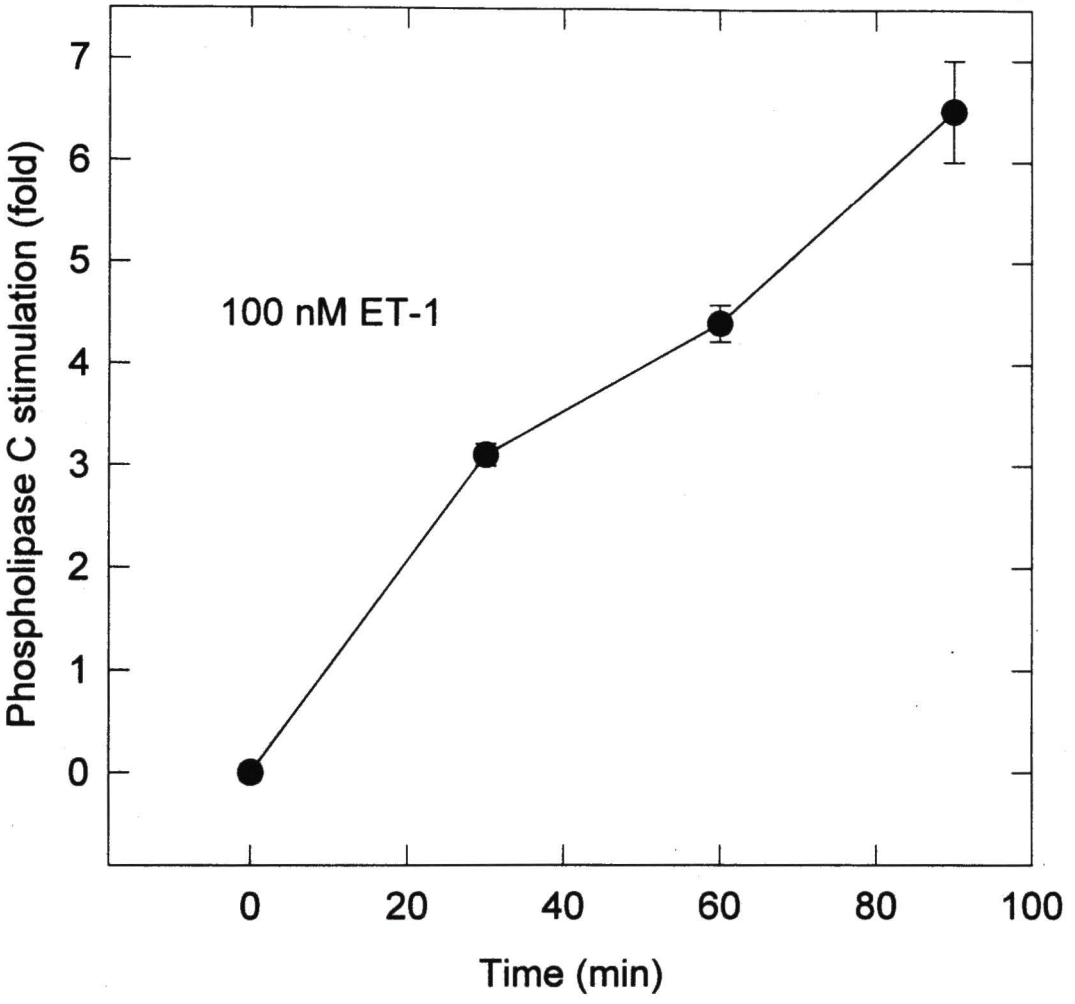


Figure 10. Representative time course of 100 nM ET-1 induced PLC stimulation in human ciliary muscle cells. Similar results were obtained in 3 independent studies.

increased continuously for at least 90 minutes, indicating no significant depletion of the enzyme substrate or desensitization of the receptor during this period. Ninety minutes was then chosen as the incubation time in the following studies of PLC stimulation. Endothelin-1 at 1 nM and 100 nM concentrations increased PLC activity in HCM cells 5.2 ± 0.2 fold (mean ± SEM, $n=3$) and 7.4 ± 1.4 fold (mean ± SEM, $n=9$), respectively.

Pretreatment with 1 μM U73122, a PLC inhibitor, significantly inhibited the ET-1 (1 nM and 100 nM) mediated increase in PLC activity (Table 11). To elucidate the involvement of endothelin receptor subtypes in the activation of PLC by ET-1, the ET_A subtype-selective antagonist, BQ610, was used to block this effect. The PLC stimulation induced by ET-1 at 1 nM was completely blocked by pretreatment with BQ610 (Table 11). The PLC stimulation induced by 100 nM ET-1 was also significantly inhibited by pretreatment with BQ610 (Table 11). As expected, pretreatment of the HCM cells with the PLA_2 inhibitors, $AACOCF_3$ and isotetrandrine, prior to the addition of ET-1, did not significantly inhibit PLC activity (Table 12). Therefore, it appears that in HCM cells a major mechanism of the ET-1 stimulated calcium mobilization is via receptor activation of PLC activity.

Table 11. Effects of an ET_A Antagonist (BQ610) and a PLC Inhibitor (U73122) on PLC Activity in Human Ciliary Muscle Cells

Treatment	n	PLC
ET-1 (1 nM)	3	100 ± 3.7%
ET-1 (1 nM) + BQ610 (1 μM)	3	0.3 ± 0.7%*
ET-1 (1 nM) + U73122 (1 μM)	3	69 ± 1.9%*
ET-1 (100 nM)	3	100 ± 6.5%
ET-1 (100 nM) + BQ610 (1 μM)	3	55 ± 6.0%*
ET-1 (100 nM) + U73122 (1 μM)	3	60 ± 2.3%*

Data are presented as mean values ± SEM. PLC activity with ET-1 (1 nM or 100 nM) defines 100%.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Table 12. Effects of PLA_2 Inhibitors ($AACOCF_3$ and Isotetrandrine) on PLC Activity in Human Ciliary Muscle Cells

Treatment	n	PLC
ET-1 (100 nM)	3	100 ± 6.5%
ET-1 (100 nM) + $AACOCF_3$ (1 μM)	3	84 ± 6.8%
ET-1 (100 nM) + Isotetrandrine (1 μM)	3	129 ± 8.5%*

Data are presented as mean values ± SEM. PLC activity with ET-1 (100 nM) defines 100%.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Specific Aim II

ET-1 Stimulated PGE_2 Production: Mechanism of Action

Endothelin-1 has been shown to increase the production of PGE_2 via the ET_A receptor subtype in a dose-dependent manner in HCM cells (Matsumoto *et al.*, 1996). In cultured vascular smooth muscle and endothelial cells, ET has been reported to release arachidonic acid and increase PG formation by stimulating PLA_2 (Rubanyi and Polokoff, 1994). To determine if the ET-1 mediated changes in intracellular calcium are dependent on enhanced prostaglandin production, experiments were designed to see if inhibitors of the PLA_2 pathway had any effect on the ET-1 stimulated calcium mobilization.

Dynamic video imaging was utilized to measure the calcium response to ET-1 in the presence and absence of the cyclooxygenase inhibitor, ibuprofen. The HCM cells were incubated with 10 μM ibuprofen for 15 minutes prior to the addition of ET-1 (1 nM). Matsumoto, *et al.* (1996) reported that 10 μM indomethacin blocked the ET-1 mediated increase in PGE_2 production, therefore, the same dose of ibuprofen (10 μM) was chosen to measure its effect on the ET-1 mediated calcium response. Additionally, the calcium response to ET-1 was measured after the cells were pretreated with various PLA_2 inhibitors. The PLA_2 inhibitors tested include; quinacrine (PLA_2 inhibitor), manoalide (non-specific inhibitor of PLA_2, PLC and calcium

channels), $AACOCF_3$ (cytosolic PLA_2 inhibitor), isotetrandrine (G-protein linked PLA_2 inhibitor), and HELSS (haloenol lactone suicide substrate - calcium-independent PLA_2 inhibitor). The IC_{50} these PLA_2 inhibitors are reported to be in the micromolar range, therefore, 1000 fold above the 1 nM agonist concentration, or 1 μM of inhibitor was used. The cells were incubated with the inhibitors for 15-30 minutes prior to the addition of ET-1.

The cyclooxygenase inhibitor, ibuprofen (10 μM) had no effect on the ET-1 (1 nM) mediated calcium mobilization, with mean peak and plateau increases in $[Ca^{2+}]_i$ of 235 $\pm$ 56 nM and 76 $\pm$ 13 nM ($n=9$), respectively (Table 13). The calcium response to ET-1 was then measured after the HCM cells were pretreated with various PLA_2 inhibitors. Manoalide (1 μM), a non-specific inhibitor of PLA_2, PLC and calcium channels, attenuated the ET-1 stimulated calcium mobilization. The mean peak increases in $[Ca^{2+}]_i$ following ET-1 administration was 108 $\pm$ 32 nM ($n=21$) and the plateau phase was abolished with mean plateau $[Ca^{2+}]_i$ of -1.4 $\pm$ 3 nM (Table 14). This differs from that seen with the selective PLC inhibitor, U73122, in which both ET-1 induced peak and plateau increases in $[Ca^{2+}]_i$ were blocked (Table 14). Therefore, it is unlikely that PLA_2 is involved in the ET-1 stimulated calcium mobilization and that the partial blockade of calcium mobilization by manoalide is most likely due to inhibitory effects on calcium channels (Wheeler, *et al.*, 1987). The other PLA_2 inhibitors, $AACOCF_3$ (cytosolic PLA_2 inhibitor), isotetrandrine (G-protein linked PLA_2 inhibitor) and

Table 13. Effects of Cyclooxygenase Inhibitor (Ibuprofen) on $[Ca^{2+}]_i$ Levels in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + Ibuprofen (10 μM)	9	235 ± 56	76 ± 13

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET + pretreatment)

Table 14. Effects of the PLC Inhibitor (U73122), Non-selective PLA_2, PLC and Ca^{2+} Channel Inhibitor (Manoalide), PLA_2 Inhibitors ($AACOCF_3$, Isotetrandrine and HELSS) on $[Ca^{2+}]_i$ in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + U73122 (1 μM)	15	4 ± 1*	1 ± 1*
ET-1 (1 nM) + Manoalide (1μM)	21	108 ± 32*	-1.4 ± 3*
ET-1 (1 nM) + $AACOCF_3$ (1 μM)	7	147 ± 15	40 ± 4*
ET-1 (1 nM) + Isotetrandrine (1 μM)	7	128 ± 16	32 ± 6*
ET-1 (1 nM) + HELSS (1 μM)	6	148 ± 23	45 ± 11*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET + pretreatment)

HELSS (calcium-independent PLA_2 inhibitor) attenuated the ET-1 (1 nM) mediated increase in $[Ca^{2+}]_i$, but did not alter the typical biphasic calcium response seen with ET (Table 14 and Figures 11-13). The mean ET-1 stimulated increases in peak and plateau $[Ca^{2+}]_i$ after pretreatment with $AACOCF_3$ (1 μM) were 147 $\pm$ 15 nM and 40 $\pm$ 4 nM ($n=7$), respectively. The mean ET-1 stimulated increases in peak and plateau $[Ca^{2+}]_i$ after pretreatment with isotetrandrine (1 μM) were 128 $\pm$ 16 nM and 32 $\pm$ 6 nM ($n=7$), respectively. The mean ET-1 stimulated increases in peak and plateau $[Ca^{2+}]_i$ after pretreatment with HELSS (1 μM) were 148 $\pm$ 23 nM and 45 $\pm$ 11 nM ($n=6$), respectively. Quinacrine was found to possess intrinsic fluorescence which interfered with the calcium mobilization assay, therefore its effects on ET-1 induced calcium mobilization are unknown. These results indicate that the ET-1 stimulated changes in intracellular calcium are not dependent on PLA_2 activation and enhanced PG production, but that some interactions between the PLC and PLA_2 signaling pathways may be occurring.

The direct effect of ET-1 on PGE_2 activity was then investigated. A time course study was first conducted to determine the optimal incubation time for the production of PGE_2 following stimulation with 100 nM ET-1. Incubation times for the time course study were 1.25 min, 2.5 min, 5 min and 10 min. The time course of ET-1 induced PGE_2 production is shown in Figure 14. After 100 nM ET-1 treatment, the rate of PGE_2 release into the

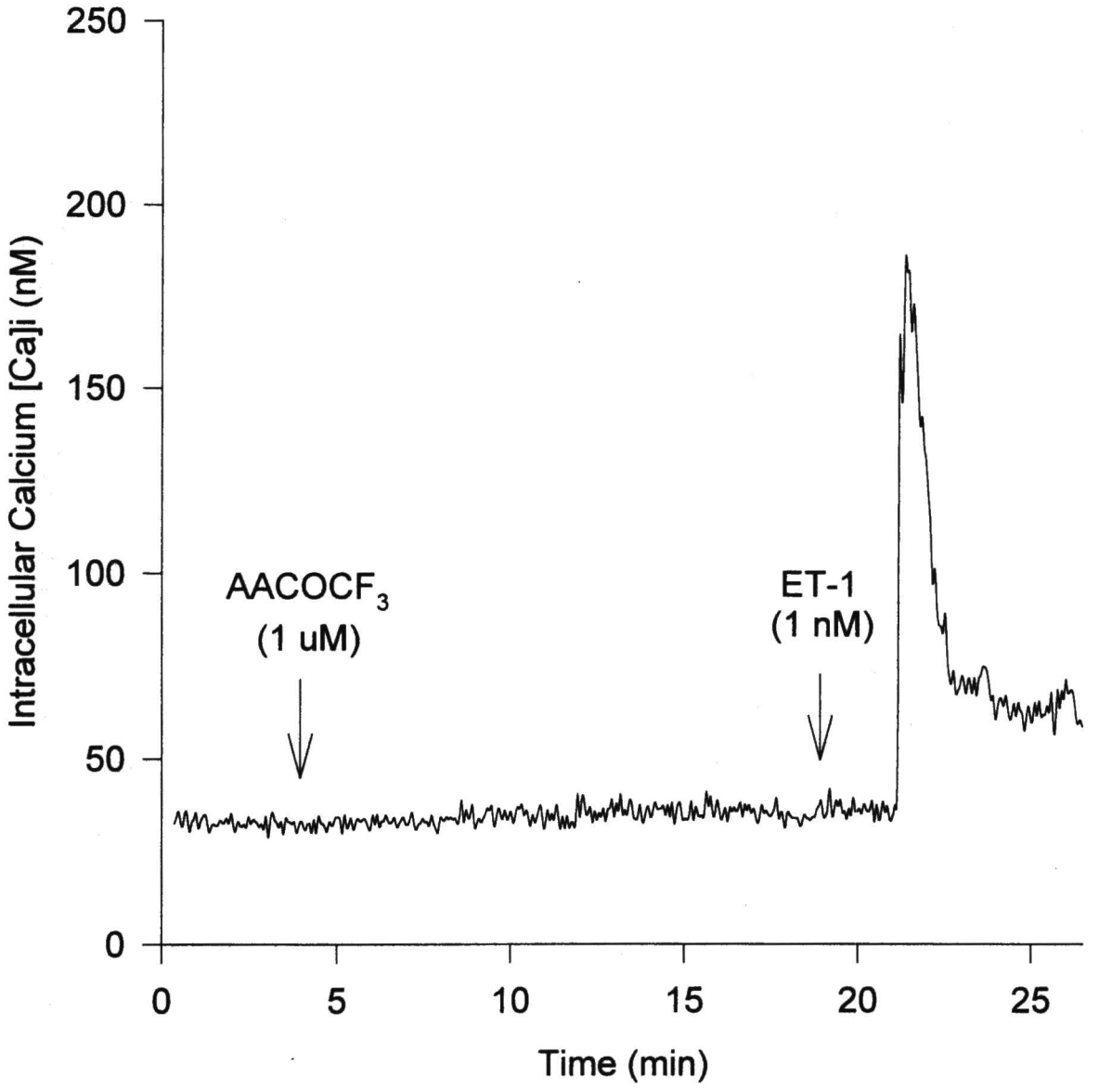


Figure 11. Effect of ET-1 on $[Ca^{2+}]_i$ after inhibition of PLA_2 with $AACOCF_3$ (cytosolic PLA_2 inhibitor) in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

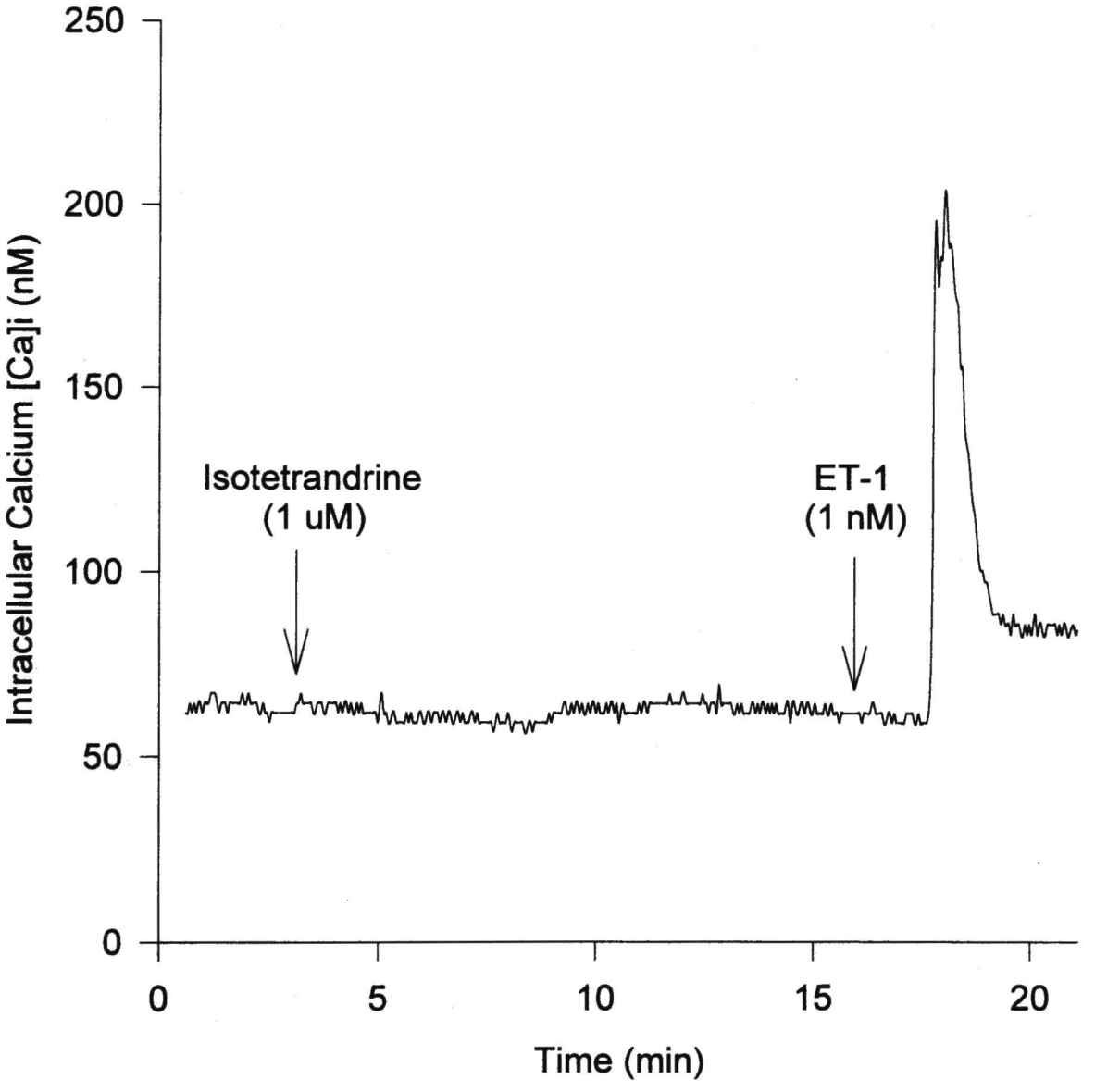


Figure 12. Effect of ET-1 on $[Ca^{2+}]_i$ after inhibition of PLA_2 with isotetrandrine (G-protein linked PLA_2 inhibitor) in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

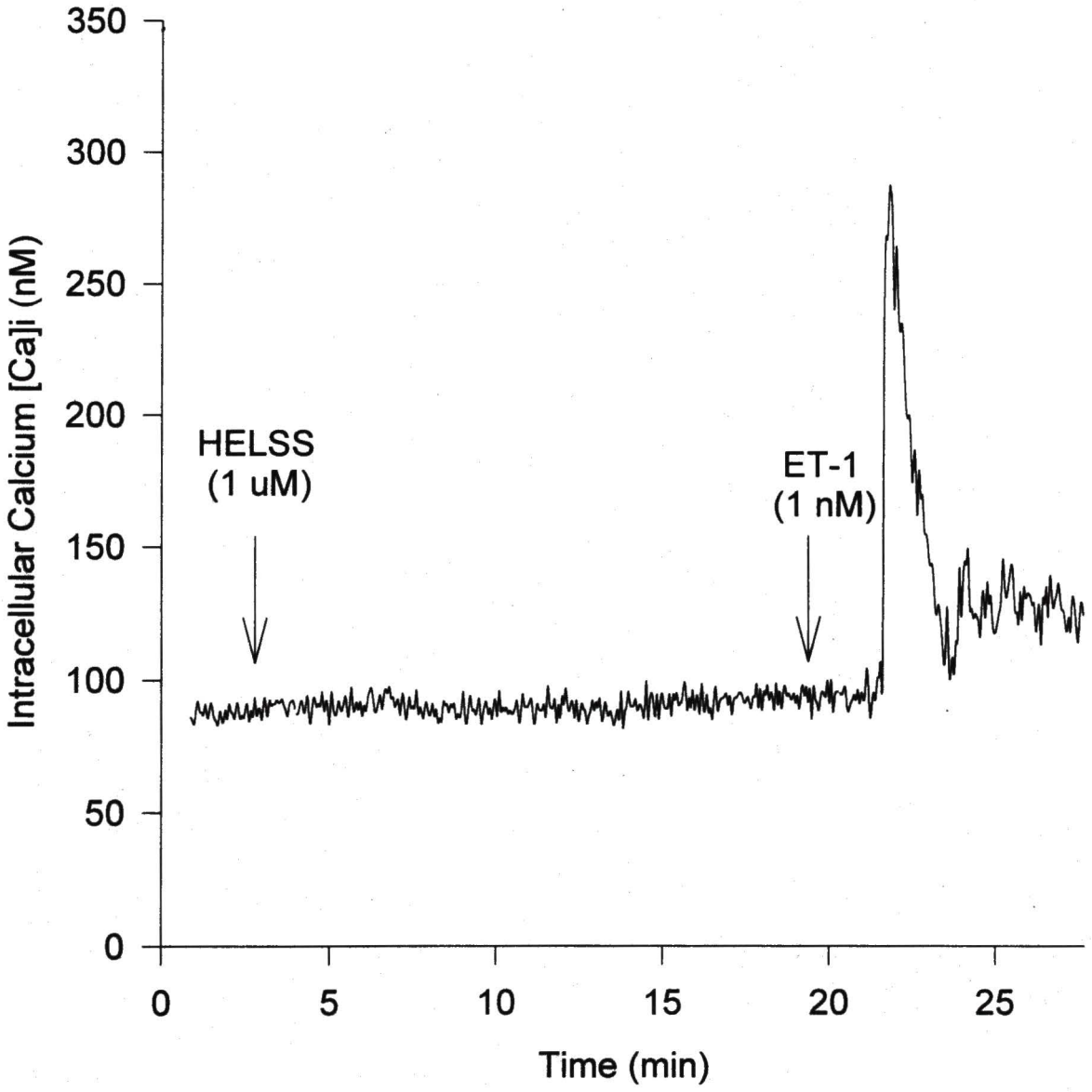


Figure 13. Effect of ET-1 on $[Ca^{2+}]_i$ after inhibition of PLA_2 with HELSS (Ca^{2+}-independent PLA_2 inhibitor) in human ciliary muscle cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$.

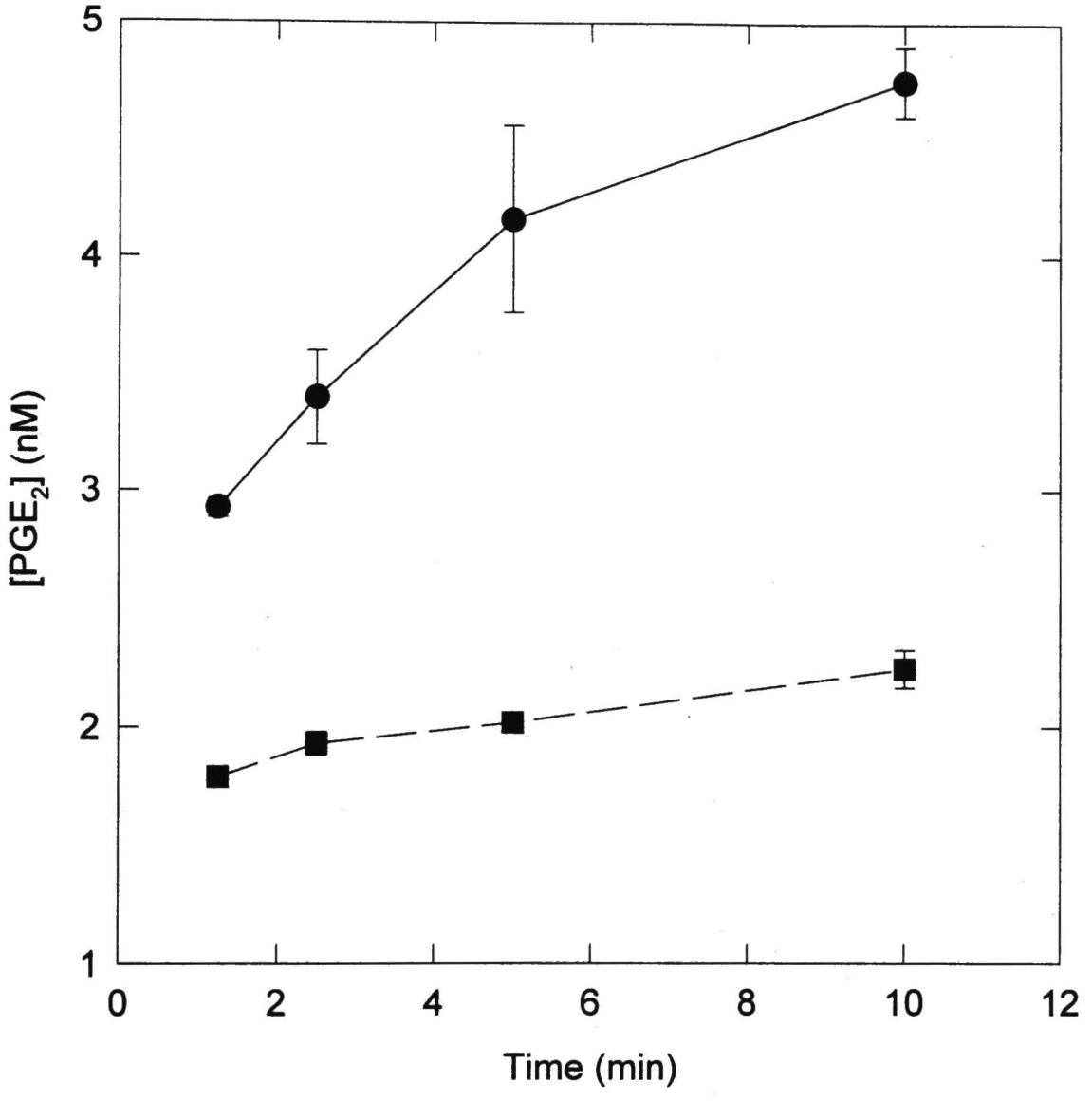


Figure Legend: ● = ET-1 stimulated [PGE_2]; ■ = basal [PGE_2]

Figure 14. Time course of 100 nM ET-1 induced PGE_2 production in human ciliary muscle cells. Each data point represents the mean increase in [PGE_2] from 3 independent studies.

culture medium increased rapidly with time up to 5 minutes then increased more slowly up to 10 minutes. Basal release of PGE_2 into the culture medium was also measured at each time point and found to increase slightly over time. This time course corresponds well to the ET-1 stimulated release of arachidonic acid and PGs from rabbit iris sphincter reported by Abdel-Latif, *et al.* (1991). Ten minutes was then chosen as the incubation time in the following studies of PGE_2 stimulation. The resting concentration of PGE_2 in the medium, within a 10 minute period, was 2.5 ± 0.5 nM (mean ± SEM, $n = 7$). At 100 nM, ET-1 increased the concentration of PGE_2 in culture medium to 4.8 ± 0.6 nM ($n = 12$). The dose of ET-1 utilized in the studies of PGE_2 stimulation was based on the concentration-response curve of ET-1 induced PGE_2 formation and the EC_{50} in HCM cells reported by Matsumoto, *et al.* (1996). The ET-1 (100 nM) stimulated PGE_2 levels obtained were comparable to those reported by Matsumoto, *et al.* (1996).

The following experiments were then conducted to delineate whether the ET-1 increases in PGE_2 formation are dependent on PLC activated increases in intracellular calcium or if ET-1 activates PLA_2 independent of PLC. The production of PGE_2 was measured in the presence and absence of the cyclooxygenase inhibitors, indomethacin (10 μM) and ibuprofen (10 μM). The dose of the cyclooxygenase inhibitors used in the PGE_2 assay corresponds with the dose used by Matsumoto, *et al.* (1996). Additionally, the ET-1 stimulated production of PGE_2 was measured after the cells were

pretreated with the PLC inhibitor, U73122, as well as various PLA_2 inhibitors. The dose response effect of U73122 (0.1 μM to 10 μM) on ET-1 (100 nM) stimulated PGE_2 production was tested, as well as the dose response effect of one concentration of U73122 (1 μM) on doses of ET-1 ranging from 1 nM to 100 nM). The PLA_2 inhibitors tested include; manoalide - 1μM (non-specific inhibitor of PLA_2, PLC and calcium channels), quinacrine - 10 μM and 50 μM (PLA_2 inhibitor), $AACOCF_3$ - 1 μM (cytosolic PLA_2 inhibitor) and isotetrandrine - 1 μM to 20 μM (G-protein linked PLA_2 inhibitor). The HCM cells were incubated for 30 minutes with the various PLA_2 inhibitors prior to the addition of 100 nM ET-1.

Pretreatment of the HCM cells with the PLC inhibitor, U73122, at doses of 1 to 100 fold higher than ET-1 (100 nM) had no effect on the ET-1 stimulated increase in PGE_2 formation (Table 15). Furthermore, a dose-response study with a constant dose of U73122 (1 μM) and doses of ET-1 from 1 nM to 100 nM showed that the dose response effect of ET-1 mediated PGE_2 formation is preserved (Table 16). The effects of various PLA_2 inhibitors on the ET-1 stimulated PGE_2 production were then investigated. Quinacrine, a selective inhibitor of PLA_2, significantly decreased the levels of ET-1 (100 nM) stimulated PGE_2 production when the HCM cells were pretreated with either 10 μM or 50 μM quinacrine, inhibiting the PGE_2 formation approximately 60% (Table 17). Manoalide, an inhibitor of PLA_2, as well as PLC and calcium channels, at 1 μM, completely

Table 15. Effects of Various Doses of the PLC inhibitor, U73122, on ET-1 Stimulated PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	6	0.0 ± 7.1%
ET-1 (100 nM)	6	100.0 ± 14.5%
ET-1 (100 nM) + U73122 (0.1 μM)	3	126.7 ± 8.8%
ET-1 (100 nM) + U73122 (1 μM)	3	108.4 ± 12.4%
ET-1 (100 nM) + U73122 (10 μM)	3	80.0 ± 1.1%

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

Not Significant (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Table 16. Effects of U73122 (PLC Inhibitor) on Various Doses of ET-1 Stimulated PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	[PGE_2] (nM)
U73122 (1 μM) + ET-1 (1 nM)	3	6.7 ± 0.5
U73122 (1 μM) + ET-1 (10 nM)	3	8.1 ± 1.1
U73122 (1 μM) + ET-1 (100 nM)	3	9.7 ± 0.4

Data are presented as mean values ± SEM.

Values represent triplicate samples from *n* experiments.

Table 17. Effects of Quinacrine (PLA_2 Inhibitor) on PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	3	0.0 ± 0.2%
ET-1 (100 nM)	3	100 ± 10.1%
ET-1 (100 nM) + Quinacrine (10 μM)	3	46.8 ± 1.7%*
ET-1 (100 nM) + Quinacrine (50 μM)	3	41.8 ± 8.2%*

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

blocked the ET-1 (100 nM) mediated increase in PGE_2 production (Table 18). Since the selective PLC inhibitor, U73122 had no effect on PGE_2 production, it appears that ET-1 stimulates PGE_2 formation via activation of PLA_2 and does not require prior activation of PLC. The PLA_2 inhibitor, isotetrandrine (G-protein linked PLA_2 inhibitor), produced some inhibition of PGE_2 production at doses from 1 μM to 20 μM (Table 19). However, the cytosolic PLA_2 inhibitor, $AACOCF_3$ (1 μM), did not affect PGE_2 levels (Table 20). As expected, the ET-1 (1 nM) stimulated increase in PGE_2 concentration was blocked by pretreatment of the cells with the cyclooxygenase inhibitors, ibuprofen (10 μM) or indomethacin (10 μM) (Table 21). These results indicate that the ET-1 stimulated increase in PGE_2 formation appears to be mediated through PLA_2 activation, independent of PLC mediated increases in intracellular calcium.

Table 18. Effects of Manoalide (Non-specific PLA_2, PLC and Ca^{2+} Channel Inhibitor) on PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	6	0.0 ± 7.1%
ET-1 (100 nM)	6	100 ± 14.5%
ET-1 (100 nM) + Manoalide (1 μM)	6	3.2 ± 4.4%*

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Table 19. Effects of Isotetrandrine (G-protein linked PLA_2 Inhibitor) on ET-1 Stimulated PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	6	0.0 ± 7.1%
ET-1 (100 nM)	6	100.0 ± 14.5%
ET-1 (100 nM) + Isotetrandrine (1 μM)	3	73.1 ± 26.0%
ET-1 (100 nM) + Isotetrandrine (10 μM)	3	86.9 ± 39.5%
ET-1 (100 nM) + Isotetrandrine (20 μM)	3	74.8 ± 30.6%

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

Not Significant (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Table 20. Effects of $AACOCF_3$ (Cytosolic PLA_2 Inhibitor) on PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	6	0.0 ± 7.1%
ET-1 (100 nM)	6	100 ± 14.5%
ET-1 (100 nM) + $AACOCF_3$ (1 μM)	6	146.7 ± 24.2%

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

Not Significant (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Table 21. Effects of Cyclooxygenase Inhibitors (Indomethacin and Ibuprofen) on PGE_2 Levels in Human Ciliary Muscle Cells

Treatment	n	PGE_2 Level
Vehicle	6	0.0 ± 7.1%
ET-1 (100 nM)	6	100 ± 14.5%
ET-1 (100 nM) + Indomethacin (10 μM)	3	49.5 ± 1.9%*
ET-1 (100 nM) + Ibuprofen (10 μM)	3	36.8 ± 0.9%*

Data are presented as mean values ± SEM. The mean response of ET-1 defines 100% and that of vehicle defines 0%.

Values represent triplicate samples from *n* experiments.

*$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between ET-1 alone and ET-1 + pretreatment)

Specific Aim III

Role of G-proteins in the ET Stimulation of PLC

In various tissues and cell lines, evidence suggests that ET activates a membrane transduction process through coupling of a cell-surface receptor and G protein. Bacterial toxins from Vibrio cholerae (cholera toxin) and Bordetella pertussis (pertussis toxin) are useful research tools in studies on signal transduction systems. Both cholera toxin (CTX) and pertussis toxin (PTX) catalyse ADP-ribosylation of some members of the GTP-binding protein superfamily (Cassel and Pfeuffer, 1978; Ui, 1984). Cholera toxin specifically and irreversibly activates both major forms of the heterotrimeric G-protein, G_s, which stimulates adenylyl cyclase (Mittag, *et al.*, 1994). Pertussis toxin ADP-ribosylates some G_i type proteins, blocking their function and their activation by G_i-coupled receptor, preventing the inhibition of adenylyl cyclase by G_i (Kurose, *et al.*, 1983). Experiments in vascular smooth muscle and rat mesangial cells show that a pertussis toxin-sensitive G protein appears to couple ET receptors to PLC (Simonson and Dunn, 1990). In HCM cells, ET-1 and ET-2 appear to stimulate calcium mobilization through activation of the ET_A receptor subtype coupled to PLC. Dynamic video imaging was utilized to determine if the changes in intracellular calcium induced by ET-1 and ET-2 are mediated through a pertussis toxin sensitive G-protein, the HCM cells were incubated with 100 ng/ml pertussis toxin for 24

hours prior to stimulation with ET. The dose of pertussis toxin was chosen based on studies in isolated bovine iris sphincter smooth muscle (Yousufzai and Abdel-Latif, 1993).

Blockade of the G_i protein with pertussis toxin inhibited the ET-1 induced increases in $[Ca^{2+}]_i$. The ET-1 (1 nM) stimulated mean peak and plateau increases in $[Ca^{2+}]_i$ after incubation with pertussis toxin were 31 ± 4 nM and -16 ± 4 nM ($n = 11$), respectively (Table 22). The ET-2 (1 nM) stimulated increase in $[Ca^{2+}]_i$ was also abolished after incubation with pertussis toxin (100 ng/ml) with mean peak and plateau increases in $[Ca^{2+}]_i$ of 16 ± 2 nM and -9 ± 3 nM ($n = 7$), respectively (Table 22). These results suggest that the ET-1 and ET-2 induced increases in intracellular calcium are mediated through a pertussis toxin sensitive G protein.

Table 22. Effects of ET-1 and ET-2 on $[Ca^{2+}]_i$ Following 24 Hour Incubation with Pertussis Toxin (PTX) in Human Ciliary Muscle Cells

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	12	240 ± 22	90 ± 7
ET-1 (1 nM) + PTX (100 ng/ml)	11	31 ± 4*	-16 ± 4*
ET-2 (1 nM)	15	149 ± 31	32 ± 8
ET-2 (1 nM) + PTX (100 ng/ml)	7	16 ± 2*	-9 ± 3*

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

*$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between agonist and agonist + PTX studies)

Specific Aim IV

Effects of Endothelin on Calcium Mobilization in Ciliary Epithelium

The ciliary epithelium is a component of the ciliary body considered to be responsible for the secretion of the aqueous humor, and is composed of a double layer of cells, a non-pigmented layer and a pigmented layer. Endothelin-like immunoreactivity has been identified in all ocular tissues, with the highest concentrations of ET in the iris, ciliary body and choroid (MacCumber, *et al.*, 1991; Chakravarthy, *et al.*, 1994). One possibility for the localization of ET in the vicinity of the ciliary body is the local synthesis and secretion of ET from the pigmented and non-pigmented ciliary epithelial cells and ciliary smooth muscle. The effect of ET on the intracellular signaling messenger, calcium, has been investigated in the HCM cells. Dynamic video imaging was used to determine if ET has similar effects on calcium mobilization in the ciliary epithelium. Mobilization of intracellular calcium and changes in $[Ca^{2+}]_i$ induced by the three isoforms of endothelin, in cultured HNPE cells, were characterized. Doses of ET-1, ET-2 and ET-3 from 1 nM to 100 nM were tested for their effect on inducing calcium mobilization.

None of the isoforms of ET induced significant increases in $[Ca^{2+}]_i$ (Tables 23-25). Slight, but insignificant increases in $[Ca^{2+}]_i$ were seen when the HNPE cells were dosed with ET-1 (10 nM) and ET-2 (100 nM), with

Table 23. Dose-Response Effects of ET-1 on Intracellular Calcium Levels in Non-Pigmented Human Ciliary Epithelial Cells.

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-1 (1 nM)	8	5 ± 1	-0.3 ± 2
ET-1 (10 nM)	4	30 ± 6	25 ± 7
ET-1 (100 nM)	4	14 ± 5	9 ± 6

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

Table 24. Dose-Response Effects of ET-2 on Intracellular Calcium Levels in Non-Pigmented Human Ciliary Epithelial Cells.

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-2 (1 nM)	5	5 ± 1	2 ± 0.5
ET-2 (10 nM)	5	6 ± 1	3 ± 1
ET-2 (100 nM)	5	23 ± 6	19 ± 5

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

Table 25. Dose-Response Effects of ET-3 on Intracellular Calcium Levels in Non-Pigmented Human Ciliary Epithelial Cells.

Treatment	n	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ET-3 (1 nM)	5	14 ± 3	8 ± 4
ET-3 (10 nM)	5	5 ± 2	-1 ± 2
ET-3 (100 nM)	5	2 ± 2	-6 ± 2

Data are presented as mean $[Ca^{2+}]_i$ values ± SEM. Peak $[Ca^{2+}]_i$ values = Maximum increase in $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$. Plateau $[Ca^{2+}]_i$ values = 1 minute post-Peak $[Ca^{2+}]_i$ - baseline $[Ca^{2+}]_i$.

mean peak and plateau increases in $[Ca^{2+}]_i$ of 30 ± 6 nM and 25 ± 7 nM, respectively for ET-1 and 23 ± 6 nM and 19 ± 5 nM, respectively for ET-2 (Table 23 and 24). The mean resting $[Ca^{2+}]_i$ in these cells was 46 ± 2 nM (mean ± SEM, $n = 20$). The time-dependent changes in $[Ca^{2+}]_i$ following the addition of ET were gradual increases in $[Ca^{2+}]_i$ and not the typical biphasic calcium response (rapid increase followed by a sustained elevation) seen in HCM cells (Figure 15). These results indicate that in HNPE cells ET has little effect on calcium mobilization.

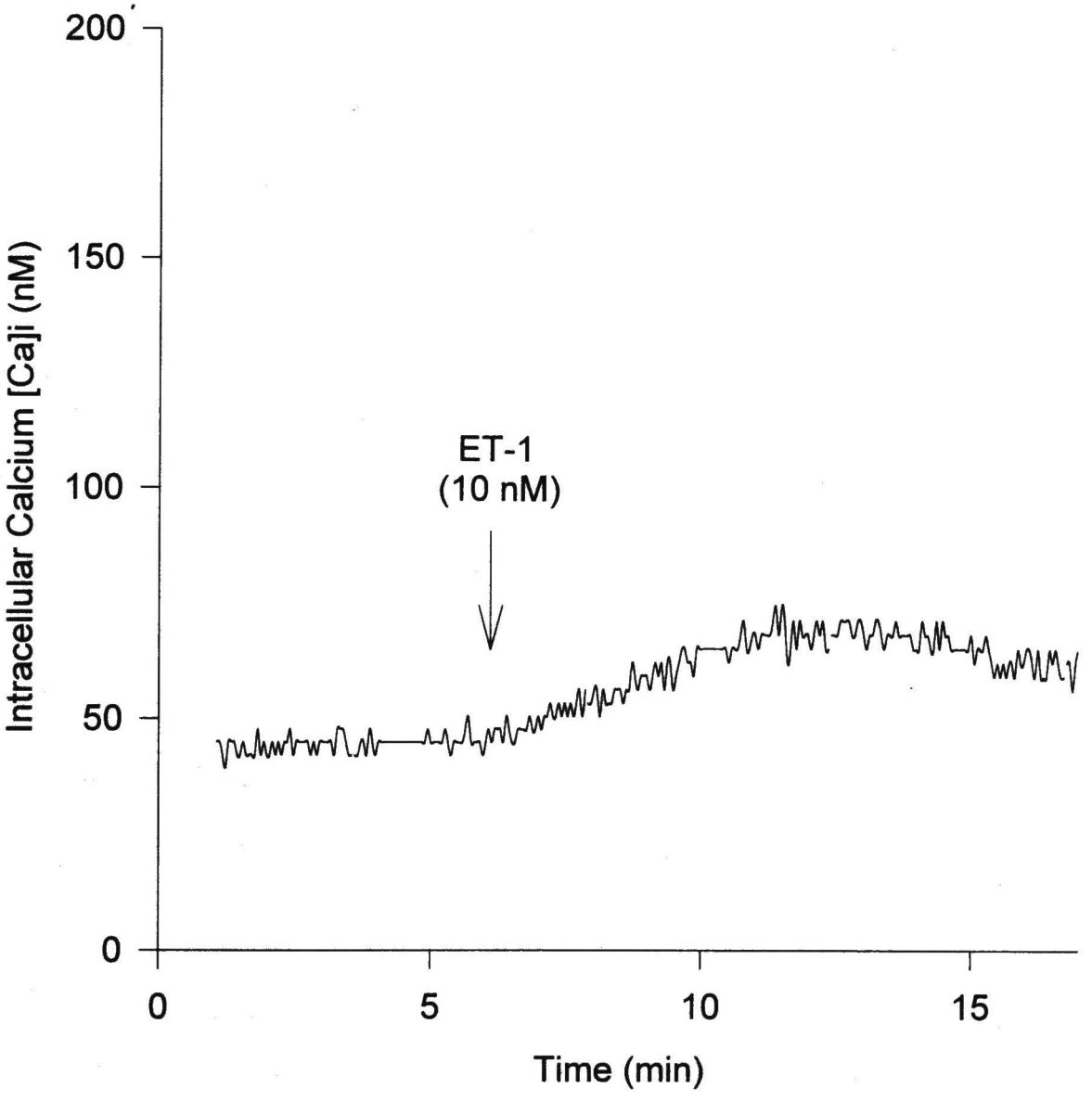


Figure 15. Effect of ET-1 on $[Ca^{2+}]_i$ in non-pigmented human ciliary epithelial cells. A representative trace of time-dependent changes in $[Ca^{2+}]_i$ after addition of 10 nM ET-1.

Specific Aim V

Localization of ET-1 and Big ET-1 in Ciliary Muscle and Ciliary Epithelium

Immunolocalization studies have identified ET-like immunoreactivity in all ocular tissues, with the highest concentrations of ET in the iris, ciliary body and choroid (MacCumber, *et al.*, 1991; Chakravarthy, *et al.*, 1994). In the ocular tissues of various species, ET-1 and ET-3 have been identified, with apparently no ET-2 immunoreactivity detected in any ocular tissues (MacCumber, *et al.*, 1991; Eichhorn and Lütjen-Drecoll, 1993; Charkravarthy, *et al.*, 1994). One possibility for the localization of ET in the vicinity of the ciliary body is the local synthesis and secretion of ET from the pigmented and non-pigmented ciliary epithelial cells and ciliary smooth muscle cells. To determine if ET-1 and Big ET-1 (the inactive precursor peptide for ET) are localized in HCM cells and HNPE cells, indirect immunofluorescence experiments were conducted.

Immunofluorescence experiments for the localization of ET-1 and Big ET-1 in HCM and HNPE cells were conducted in triplicate. All incubations were carried out for 30 minutes prior to fixing the cells with pre-cooled 1:1 methanol:acetone. The fluorescent controls (background fluorescence) and unstimulated cells were left untreated throughout the incubation period. The cytokine stimulated cells were treated with 10 nM TNF-α for the 30 minute incubation. The unstimulated cells and the cytokine

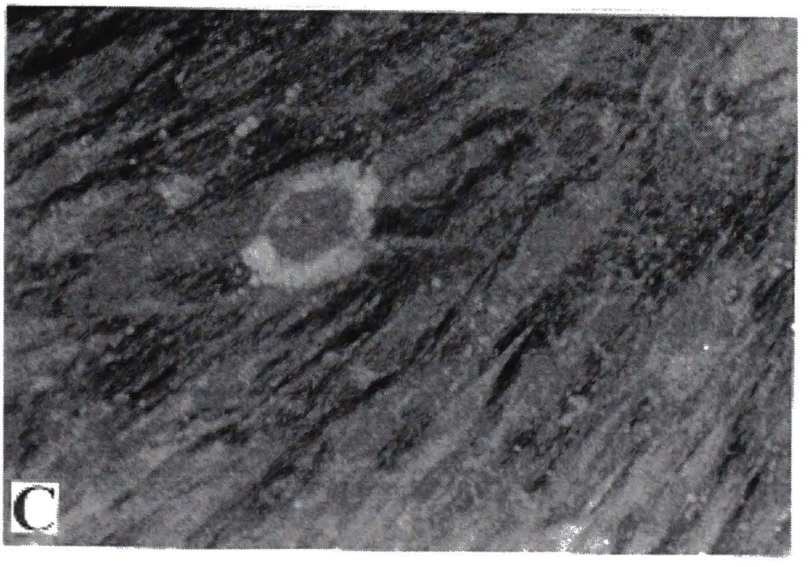
stimulated cells were then treated with primary antibody and secondary antibody as described in the immunocytochemisty procedure in Chapter II - Methods. The fluorescent controls (background fluorescence) were prepared by eliminating the incubation step with the primary antibody and only incubating with secondary antibody (goat anti-rabbit IgG-FITC).

In the HCM cells, a positive staining reaction was exhibited with antibodies against ET-1. In cells incubated with secondary antibody only, the staining was completely blocked, showing only the background fluorescence (Figure 16). A significant amount of ET-1 immunoreactivity was observed in the cytoplasm of HCM cells incubated with both primary antibody to ET-1 and secondary antibody (Figures 16 and 17). Stimulation of the HCM cells with the cytokine, TNF-α, (10 nM) for 30 minutes significantly increased the level of ET-1 immunoreactivity above both the background fluorescence and the fluorescence seen in unstimulated cells (Figures 16 and 17). Therefore, it appears that TNF-α increases the amount of ET-1 in these cells. In contrast to ET-1, only minimal amounts of Big ET-1 were detected in the HCM cells. A slight increase in fluorescence was seen as compared to the background fluorescence, but only in isolated cells (Figure 18). Quantitatively, however, there was no difference from the background fluorescence (Figure 19). The level of Big ET-1 immunoreactivity was increased after stimulation of the HCM cells with the cytokine, TNF-α, (10 nM) for 30 minutes (Figures 18 and 19). The TNF-α stimulated increase in

Figure 16. Indirect Immunostaining of ET-1 in HCM Cells. (A) Background fluorescence = 2^0 antibody only (no antibody to ET-1). (B) Basal ET-1 = 1^0 antibody + 2^0 antibody. (C) TNF-α stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody.


A


B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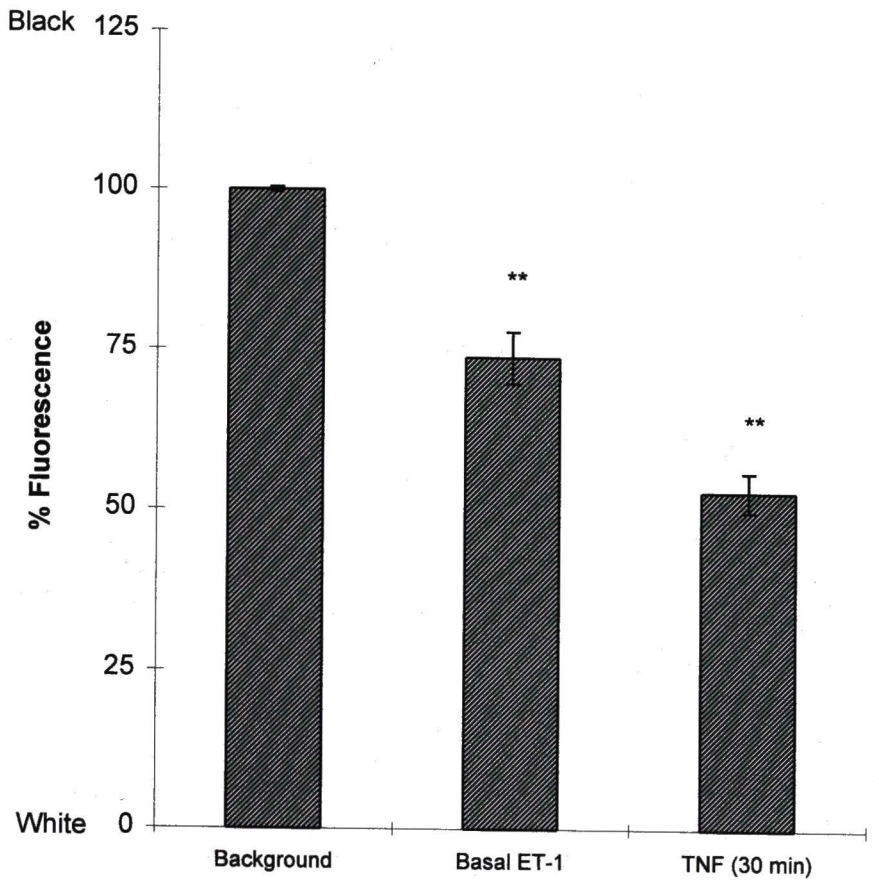


Background = 2^0 antibody only (No antibody to ET-1)

Basal ET-1 = 1^0 antibody + 2^0 antibody

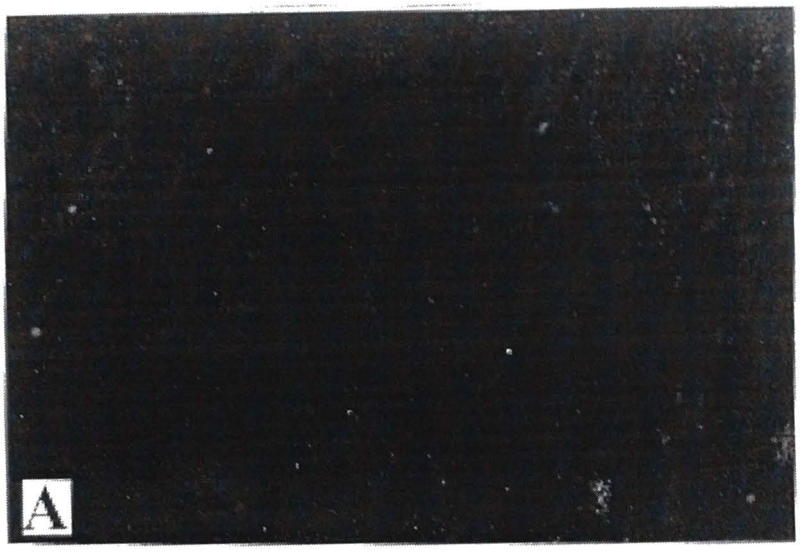
TNF-α Stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody

Figure 17. Localization of ET-1 in HCM Cells. Degree of immuno-fluorescence computed on an average gray scale: the lower the gray scale number the greater the fluorescence.
% Fluorescence = treatment/background X 100 %. Data are presented as mean values ± SEM. *$p<0.05$ (One Way ANOVA with Bonferroni's test for significance between background and basal or stimulated)

Figure 18. Indirect Immunostaining of Big ET-1 in HCM Cells.

(A) Background fluorescence = 2^0 antibody only (no antibody to ET-1). (B) Basal Big ET-1 = 1^0 antibody + 2^0 antibody.

(C) TNF-α stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody.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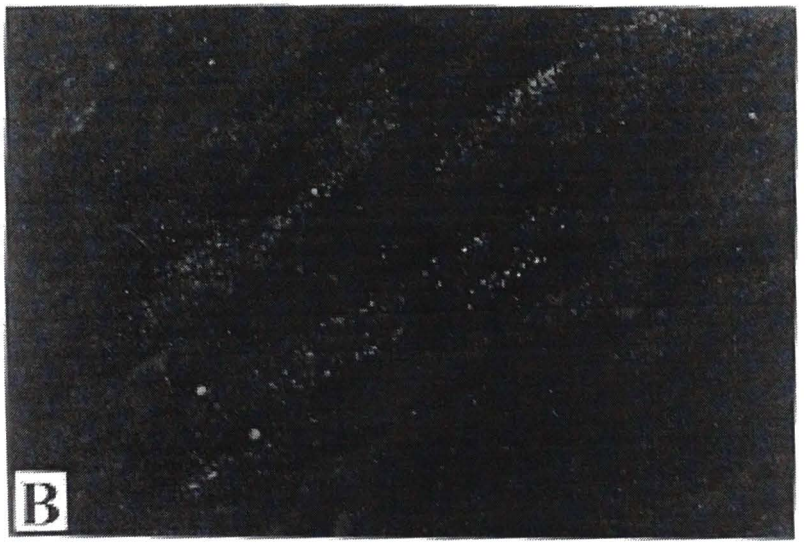
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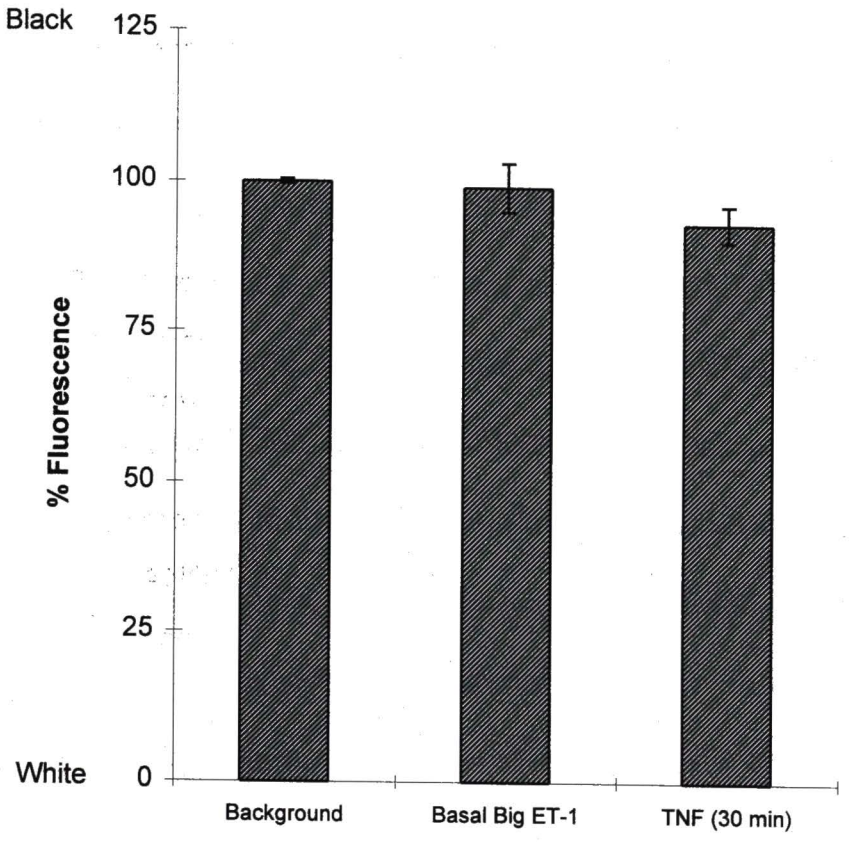


Background = 2^0 antibody only (No antibody to ET-1)

Basal Big ET-1 = 1^0 antibody + 2^0 antibody

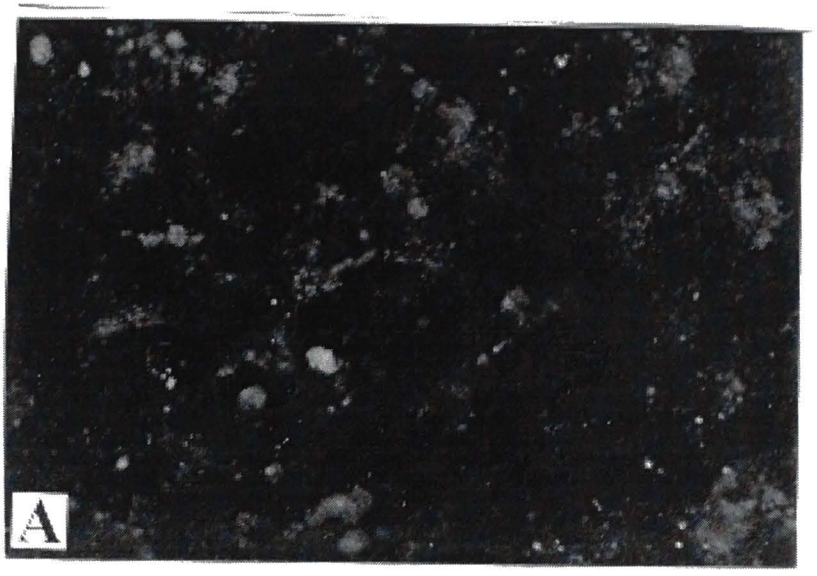
TNF-α Stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody

Figure 19. Localization of Big ET-1 in HCM Cells. Degree of immuno-fluorescence computed on an average gray scale: the lower the gray scale number the greater the fluorescence.
% Fluorescence = treatment/background X 100 %. Data are presented as mean values ± SEM. Not Significant (One Way ANOVA with Bonferroni's test for significance between background and basal or stimulated)

the Big ET-1 immunoreactivity, however, was much less than that observed with TNF-α on ET-1 in these cells.

Like the HCM cells, the HNPE cells exhibited a positive staining reaction with antibodies against ET-1. Relative to the background fluorescence, a significant amount of ET-1 immunoreactivity was observed in the HNPE cells incubated with primary antibody to ET-1 (Figures 20 and 21). Stimulation of the HNPE cells with the cytokine, TNF-α, (10 nM) for 30 minutes, significantly increased the level of ET-1 immunoreactivity above both the background fluorescence and the fluorescence seen in unstimulated cells (Figures 20 and 21). Big ET-1 was also detected in isolated HNPE cells (Figure 22). The level of Big ET-1 immunoreactivity was significantly increased after stimulation with the cytokine, TNF-α, (10 nM) for 30 minutes (Figures 22 and 23). Therefore, it appears that TNF-α effectively increases the amount of both ET-1 and Big ET-1 in HNPE cells.

Figure 20. Indirect Immunostaining of ET-1 in HNPE Cells. (A) Background fluorescence = 2^0 antibody only (no antibody to ET-1). (B) Basal ET-1 = 1^0 antibody + 2^0 antibody. (C) TNF-α stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody.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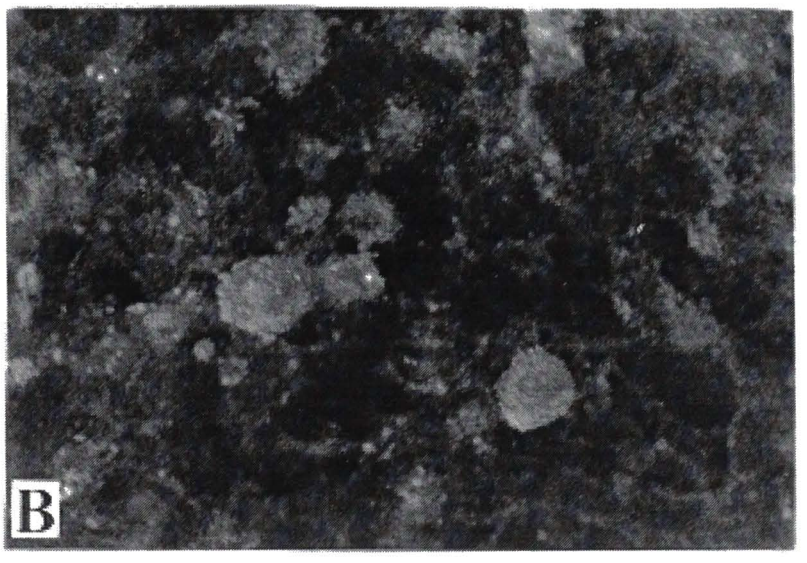
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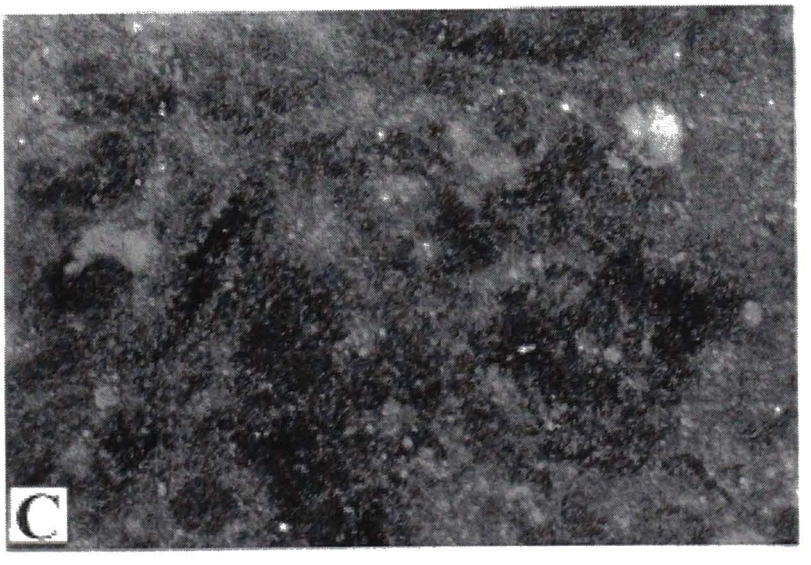
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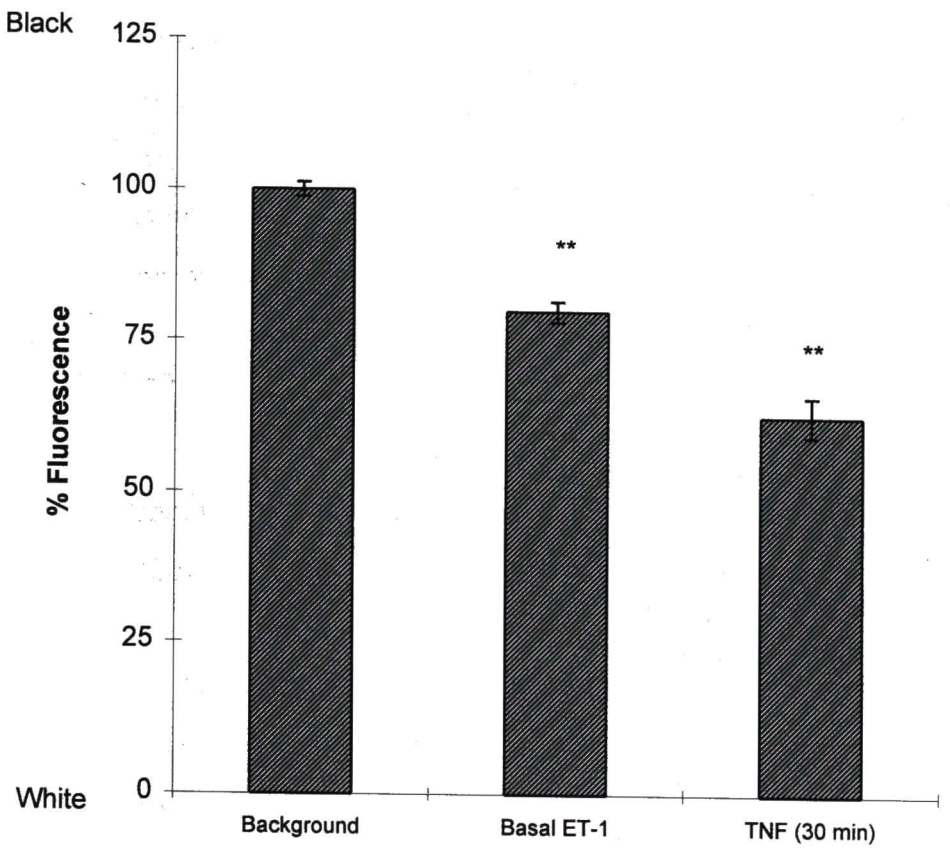


Background = 2^0 antibody only (No antibody to ET-1)

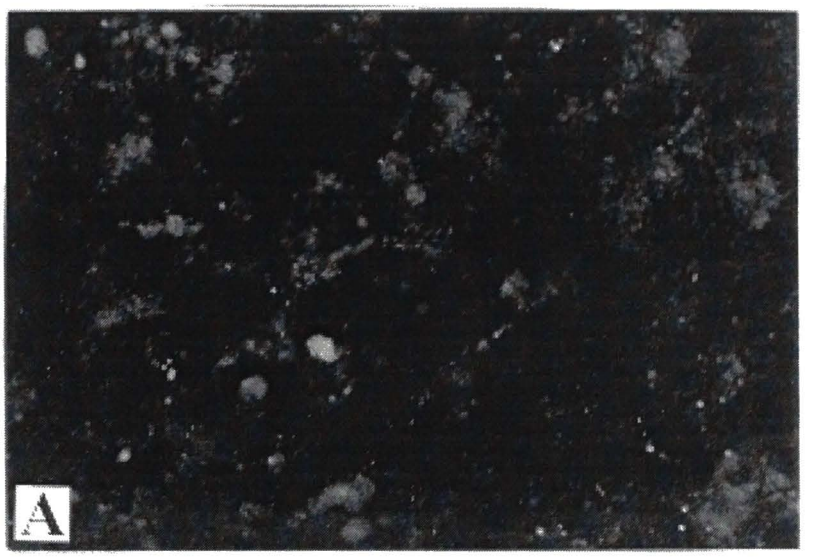
Basal ET-1 = 1^0 antibody + 2^0 antibody

TNF-α Stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody

Figure 21. Localization of ET-1 in HNPE Cells. Degree of immuno-fluorescence computed on an average gray scale: the lower the gray scale number the greater the fluorescence. % Fluorescence = treatment/background X 100%. Data are presented as mean values ± SEM. *$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between background and basal or stimulated)

Figure 22. Indirect Immunostaining of Big ET-1 in HNPE Cells.

(A) Background fluorescence = 2^0 antibody only (no antibody to Big ET-1). (B) Basal ET-1 = 1^0 antibody + 2^0 antibody. (C) TNF-α stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody.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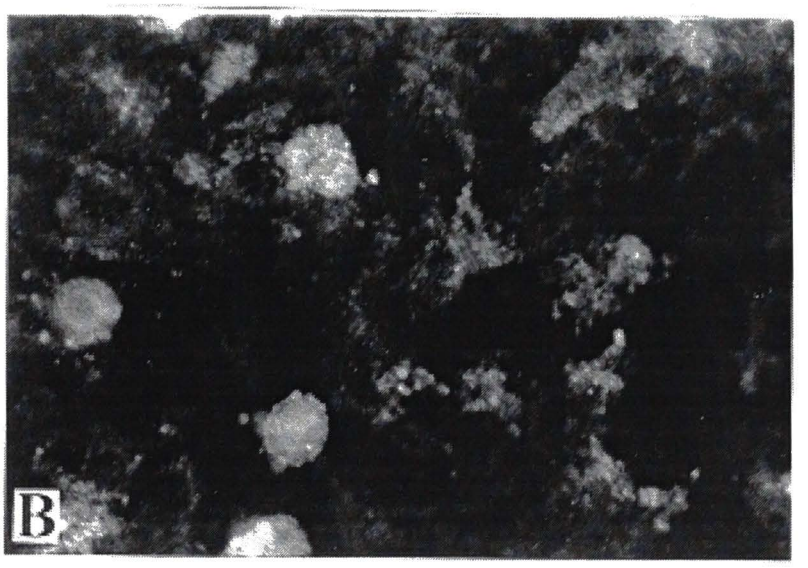
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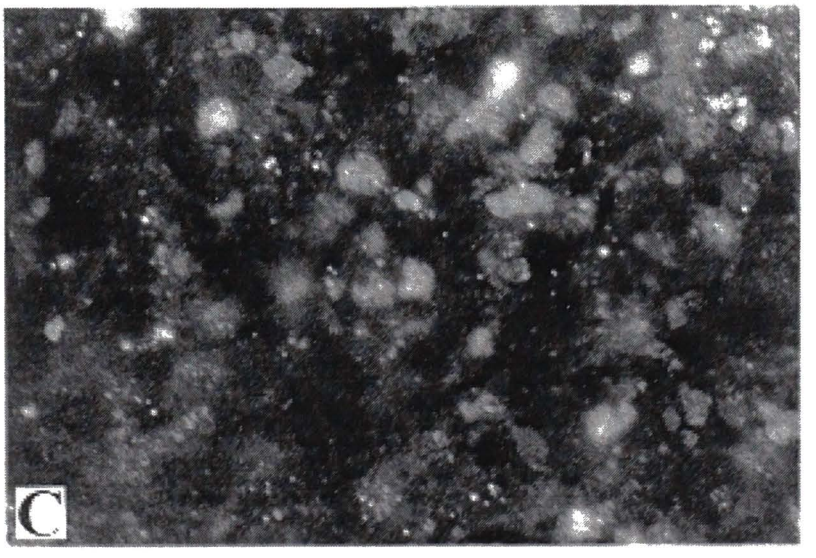
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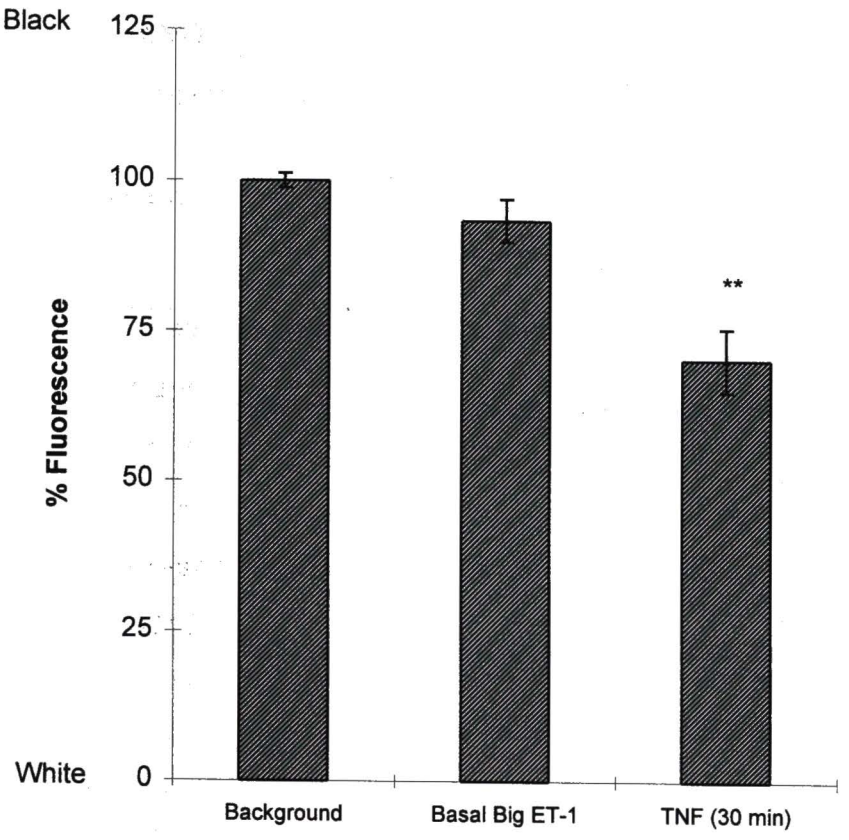

Background = 2^0 antibody only (No antibody to ET-1)

Basal Big ET-1 = 1^0 antibody + 2^0 antibody

TNF-α Stimulated = TNF-α (10 nM for 30 min.) + 1^0 antibody + 2^0 antibody

Figure 23. Localization of Big ET-1 in HNPE Cells. Degree of immuno-fluorescence computed on an average gray scale: the lower the gray scale number the greater the fluorescence.
% Fluorescence = treatment/background X 100%. Data are presented as mean values ± SEM. *$p < 0.05$ (One Way ANOVA with Bonferroni's test for significance between background and basal or stimulated)

CHAPTER IV

DISCUSSION

Specific Aim I

Endothelin Stimulated Calcium Mobilization: Receptor Identification and Cellular Signaling Pathway

In cultured HCM cells, both ET-1 and ET-2 stimulate $[Ca^{2+}]_i$. The calcium response consisted of a rapid, transient increase in $[Ca^{2+}]_i$, 2-4 minutes after the addition of the peptide, followed by a sustained elevation in $[Ca^{2+}]_i$ lasting up to 15 minutes. To quantify this response, peak $[Ca^{2+}]_i$ values, as well as plateau $[Ca^{2+}]_i$ values, one minute post peak (selected to capture the most stable post-stimulatory time), were calculated. This biphasic increase in $[Ca^{2+}]_i$ induced by ET stimulation has been observed in other cell lines. In cultured human and rat glomerular mesangial cells, ET caused a similar biphasic calcium waveform, evoking a rapid, transient increase in $[Ca^{2+}]_i$ (2-5 seconds), followed by a lesser but sustained increment (Simonson and Dunn, 1990).

The HCM cells exhibited cell to cell variability in their calcium response, as not all cells would elicit the same amount of calcium release

and occasionally, one or two cells would not respond at all. The reason for differences in responses between cells is not known, however, this could reflect differences in the cell cycle at the time of measurement. Additionally, oscillations of $[Ca^{2+}]_i$ would occasionally be observed in the HCM cells after stimulation with ET. When these oscillations occurred, the increases in $[Ca^{2+}]_i$ were typically at consistent intervals. This was not a reproducible response and it is not clear what triggers these oscillations. Oscillations have been previously reported in human mesangial cells upon stimulation with ET-1 and ET-2 (Osanai, *et al.*, 1990). The oscillations were proposed to be caused by the initial ET-induced release of calcium stores, triggering a "calcium wave", propagated by a process of calcium mediated calcium diffusion (Osanai, *et al.*, 1990; Simonson and Dunn, 1990). It would be interesting to study these oscillations in HCM cells, if they could be reproducibly produced.

When the HCM cells were dosed with 1 nM ET-1 and ET-2, these agonists induced increases in $[Ca^{2+}]_i$ of 3-20 fold above resting levels. Dose-response curves for ET stimulation in HCM cells were attempted, however, repeated agonist stimulation evoking calcium mobilization in the HCM cells resulted in decreased responses or no response to higher doses of ET. For example, in one experiment the HCM cells were dosed with 1 nM of ET-1 increasing the $[Ca^{2+}]_i$ with a mean peak of 139 $\pm$ 23 nM ($n=7$) above the resting level. Upon subsequent stimulation with 10 nM of ET-1, the increase

in $[Ca^{2+}]_i$ was significantly lower with a mean peak of 68 ± 16 nM ($n=7$) above the resting level. Even when a washout was done between doses, the same effect was observed. There appears to be a receptor desensitization or down regulation with repeated exposure to ET in HCM cells, rendering dose-response studies within the same experiment unreliable. Several other studies investigating various effects of ET have also noted what appears to be a receptor desensitization or down regulation (Hirata, *et al.*, 1988; Lepple-Wienhues, *et al.*, 1991).

The effect of both ET-1 and ET-2 on calcium mobilization appears to be mediated by the endothelin ET_A receptor subtype. BQ610, an ET_A subtype-selective antagonist, completely blocked the increase in $[Ca^{2+}]_i$ and IRL-1620, an ET_B receptor subtype-selective agonist, had no effect on $[Ca^{2+}]_i$. ET_B receptor-subtype selective antagonists did not block the ET mediated calcium mobilization, but some blunting of the peak $[Ca^{2+}]_i$ was observed. With IRL-1038, however, some batch-to-batch variations and possible molecular aggregation were recently reported (Urade, *et al*, 1994). From binding studies the K_i for IRL-1038 at ET_B receptors is 10 fold higher than at ET_A receptors. It may be that IRL-1038 at a dose of 50 nM induced some ET_A receptor antagonism which could account for this effect. The ET_B receptor-subtype selective antagonist, BQ788, however, had less blunting of the peak $[Ca^{2+}]_i$ to ET-1 and no effect on the ET-2 mediated calcium mobilization. Although the results with the ET_B antagonists were not

definitive, the total blockade of the ET calcium response by the ET_A receptor antagonist, BQ-610, as well as no stimulatory effect by the ET_B agonist, IRL-1620, suggests that the calcium response is mediated through an ET_A receptor. Endothelin-1 also appears to be more efficacious than ET-2 in stimulating calcium mobilization. This agrees with the affinity rank order of endothelin for the ET_A receptor of ET-1 > ET-2 > > ET-3 (Masaki, *et al.*, 1994). These findings suggest that the ET induced stimulation of calcium mobilization is mediated by receptor activation at the ET_A receptor subtype. These results agree with the findings of Matsumoto, *et al.* (1996) who reported that ET-1 stimulates PLC and increases $[Ca^{2+}]_i$ in HCM cells via the ET_A receptor subtype.

The signal transduction mechanism(s) responsible for the ET stimulated increase in $[Ca^{2+}]_i$ have not been fully elucidated. In HCM cells, the data suggest that the initial increase in $[Ca^{2+}]_i$ is the result of ET binding to the ET_A receptor to activate PLC and release intracellular stores of calcium, whereas the sustained phase is dependent on extracellular calcium. The use of thapsigargin, a compound which discharges intracellular calcium stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup, *et al.*, 1990), showed that depletion of the IP_3-sensitive intracellular stores of calcium caused ET-1 to only elicited a small calcium response. This small response could be due to calcium mobilization from other sources, or due to some IP_3-stored pools which were not completely depleted by thapsigargin.

The results indicate, however, that a large portion of the ET-1 induced calcium mobilization appears to depend on the release of calcium from IP_3-sensitive intracellular stores. Blockade of the L-type calcium channels with nifedipine, blunted the ET-1 mediated increase in $[Ca^{2+}]_i$, but did not affect the biphasic response. Removal of extracellular calcium, however, attenuated the peak increase in $[Ca^{2+}]_i$ and eliminated the plateau phase of the biphasic calcium response. Therefore, it appears that after the binding of ET to the ET_A receptor, the transient increase in $[Ca^{2+}]_i$ is primary due to release of intracellular stores, whereas extracellular influx is primarily responsible for the sustained phase. This agrees with Putney's capacitative model for calcium mobilization, as well as the observations of other groups. The capacitative model describes calcium entry in which activation of receptors, which are linked to IP_3, trigger the entry of calcium across the plasma membrane (Putney, 1990). It theorizes that in biphasic calcium responses, the depletion of the intracellular calcium store, somehow signals the opening of a pathway for the influx of extracellular calcium to replenish the intracellular pool. The calcium response in HCM cells correlates well with this model. Other groups have also reported a similar pattern of ET induced calcium mobilization where the later sustained level depends on the external calcium ion concentration, suggesting the involvement of calcium channels in the plasma membrane (Takuwa, *et al.*, 1990; Aramori and Nakanishi, 1992; Sakamoto, *et al.*, 1993).

Increases in intracellular calcium typically result from receptor stimulation of PLC and release of IP_3 in many cells including smooth muscle. In HCM cells, the ET-1 induced increases in $[Ca^{2+}]_i$ also appear to be mediated through receptor activation of the PLC signaling pathway. The ET-1 stimulated increase in $[Ca^{2+}]_i$ was completely abolished when the cells were pretreated with U73122, a PLC inhibitor, indicating that stimulation of PLC activity in the HCM cells is integral to the ET-1 stimulated calcium mobilization. Likewise, the ET-1 increase in PLC activity was also significantly inhibited by the PLC inhibitor, U73122. Although IP_3 production was not completely abolished, at similar doses of U73122 calcium mobilization was completely blocked. It is unclear why this difference was observed. It is not known how much IP_3 is needed to activate calcium mobilization, therefore it is possible that complete blockade of PLC activity is not necessary to completely inhibit calcium mobilization. In HCM cells, the threshold amount of PLC inhibition required for blockade of calcium mobilization may be different from that required to completely inhibit PLC activity. The type of assays used in the measurements of calcium mobilization and PLC activity could also factor into the difference in the inhibitory response of U73122. In measurements of intracellular calcium, 1 nM doses of ET-1 were sufficient to produce an instantaneous robust response, therefore the smallest dose which elicited reproducible results was

indicated. In measurements of PLC activity the assay required a longer incubation time (90 minutes) to optimize the accumulations of inositol phosphates, thus allowing for detection of differences between treatments. Additionally, it has been suggested that U73122 may not act through direct enzymatic inhibition, but instead through decreased substrate availability, preventing the formation of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Vickers, 1993). If the mechanism of action of U73122 is in fact through substrate depletion instead of enzyme inhibition, it is possible that the actions of U73122 were most active in blocking PIP_2 pools linked to calcium mobilization. Therefore, it would not be unexpected that complete inhibition of PLC activity was not seen. Additionally, ET-1 appears to stimulate calcium mobilization through receptor activation of PLC. The ET_A receptor antagonist, BQ-610, completely abolished the ET-1 (1 nM) mediated increase in PLC activity suggesting the involvement of the ET_A receptor in activation of PLC. This correlates well with the finding that ET increases $[Ca^{2+}]_i$ by activation of the ET_A receptor.

Therefore, in cultured HCM cells, the ET-1 and ET-2 stimulation of $[Ca^{2+}]_i$ appears to be mediated through receptor activation of the PLC signaling pathway. Binding of ET to the ET_A receptor, activates PLC and the subsequent mobilization of calcium. A biphasic calcium response is elicited with ET stimulation consisting of a transient increase in $[Ca^{2+}]_i$ which

appears to be primary due to release of intracellular stores, followed by a lower sustained phase which appears to be dependent on the influx of extracellular calcium.

Specific Aim II

ET-1 Stimulated PGE_2 Production: Mechanism of Action

The ET receptor has been shown to be coupled to the activation of PLA_2 and PLC in many tissues. It is not known whether ET activates PLA_2 directly via a G-protein and/or indirectly by increasing $[Ca^{2+}]_i$, thus causing membrane phospholipids to release arachidonic acid which are converted to bioactive eicosanoids (Simonson and Dunn, 1990). In cultured vascular smooth muscle and endothelial cells, ET has been reported to release arachidonic acid and increase PG formation by stimulating PLA_2 (Rubanyi and Polokoff, 1994). In isolated mammalian ciliary muscle, ET-1 increased PG release and cAMP accumulation through activation of the ET_A receptor coupled to PLA_2 mediating release of arachidonic acid for PG synthesis (Abdel-Latif, *et al.*, 1996). In HCM cells, ET-1 has been shown to dose-dependently increase the production of PGE_2 and adenylyl cyclase via the ET_A receptor subtype (Matsumoto *et al.*, 1996).

In the present study using HCM cells, the increase in $[Ca^{2+}]_i$ mediated by ET-1 appears to be through the PLC signaling pathway,

independent of PG production, whereas the effect of ET-1 on PGE_2 production appears to be the result of an ET_A receptor coupled to PLA_2. Inhibition of arachidonic acid with the cyclooxygenase inhibitor, ibuprofen, had no effect on the ET-1 mediated calcium mobilization, but ibuprofen and indomethacin both blocked the ET-1 stimulated PGE_2 production. This corresponds to the results of Matsumoto, *et al.* (1996) who reported complete inhibition of PGE_2 production with indomethacin, without affecting the stimulatory effects of ET-1 on PLC activity. Likewise, inhibition of the ET_A receptor-coupled PLC pathway with U73122, abolished the ET-1 stimulated calcium mobilization, as well as the inhibiting PLC activity, but had no effect on the ET-1 mediated increase in PGE_2 production. Various PLA_2 inhibitors were also tested for their effect on the ET-1 stimulated calcium mobilization, as well as PLC activity and PGE_2 production. The mechanisms of PLA_2 inhibition varied among the PLA_2 inhibitors, however, PGE_2 production was inhibited by all except the cytosolic PLA_2 inhibitor, $AACOCF_3$. None of the specific PLA_2 inhibitors tested had an affect on the ET-1 mediated increase in PLC activity, and only a slight attenuation in the ET-1 mediated increase in $[Ca^{2+}]_i$ was observed, without any alteration in the typical biphasic calcium response. Manoalide, a non-specific inhibitor of PLA_2, PLC and calcium channels, blocked the ET-1 stimulated increase in PGE_2 production and attenuated the peak increase in $[Ca^{2+}]_i$ following ET-1 administration, as well as completely abolishing the calcium plateau phase.

This differs from results obtained with the selective PLC inhibitor U73122, in which PGE_2 production was not affected, but both the ET-1 induced peak and plateau increases in $[Ca^{2+}]_i$ were blocked. Therefore, it is unlikely that PLA_2 is involved in the ET-1 stimulated calcium mobilization and the partial blockade of calcium mobilization by manoalide is most likely due to its inhibitory effects on calcium channels (Wheeler, *et al.*, 1987). These findings suggest that the ET-1 stimulated changes in intracellular calcium are not dependent on PLA_2 activation and enhanced PG production. Furthermore, the increase in PGE_2 formation appears to be mediated through PLA_2 activation, independent of PLC mediated increases in intracellular calcium. These results correlate with the findings of Matsumoto, *et al.* (1996) who reported that ET-1 stimulates PLC and adenylyl cyclase through the ET_A receptor subtype and that pretreatment of the HCM cells with indomethacin blocks the effect of ET-1 on adenylyl cyclase, but not the activation of PLC and calcium mobilization. It is interesting to note that in a recent study, Abdel-Latif, *et al.* (1996) reported that ET-1 induced PGE_2 release and cAMP formation in isolated bovine, cat, dog and human ciliary muscle through the ET_A receptor coupled to PLA_2, but only observed an increase in IP_3 and contraction in the isolated bovine ciliary muscle and not the human ciliary muscle. These authors suggested that accessory cells in the intact muscle could regulate the activity of the ciliary muscle cells, accounting for differences in second messengers produced in isolated ciliary

muscle versus ciliary muscle cells. Furthermore, Abdel-Latif *et al.* (1996) found that in the isolated bovine ciliary muscle, the increase in IP_3 production and contraction appears to be mediated via the ET_A receptor directly, through a G-protein linked to PLC activation, independent of the production of arachidonic acid metabolites. It is possible that subclasses of the ET_A receptor are responsible for the separate activation of PLC and PLA_2. Recently, two novel reverse transcription (RT) PCR transcripts have been identified in human lung and other tissues suggesting the presence of other ET receptors or subclasses (Battistini and Botting, 1995) and pharmacological evidence exists that suggest the existence of subtypes of the ET_B receptor, ET_{B1} and ET_{B2} (Warner, *et al.*, 1993). Thus, these results are consistent with the possibility of subtypes of the ET_A receptor. Alternatively, the same ET_A receptor may be coupled to the different enzyme pathways through different G-proteins. With the advent of specific antagonists it may be possible to pharmacologically determine if receptor subtypes for the ET_A receptor are present in human ciliary muscle. This could be substantiated by (RT) PCR studies and identification of the cDNA for these receptors.

Specific Aim III

Role of G-proteins in the ET Stimulation of PLC

In various tissues and cell lines, evidence suggests that ET activates a membrane transduction process through coupling of a cell-surface receptor and G-protein. The bacterial toxin Bordetella pertussis (pertussis toxin) is a useful research tool in studies on signal transduction systems. Pertussis toxin ADP-ribosylates some G_i proteins, blocking their function and their activation by G_i-coupled receptors, preventing the inhibition of adenylyl cyclase by G_i (Kurose, *et al.*, 1983). In the present study preincubation with pertussis toxin in HCM cells inhibited the ET-1 and ET-2 induced increases in $[Ca^{2+}]_i$, suggesting the involvement of a pertussis toxin sensitive G-protein in the signal transduction process. Although PLC is typically reported to be coupled to G_q (Neer, 1995) and PLA_2 to G_i (Johnson and Dhanasekaran, 1989), others have reported the involvement of a pertussis toxin sensitive G-protein coupling of ET receptors to PLC (Reynolds, *et al.*, 1989; Thomas, *et al.*, 1991). In vascular smooth muscle cells, pertussis toxin attenuated ET mediated phosphoinositol turnover (Reynolds, *et al.*, 1989), and in rat mesangial cells, pertussis toxin partially inhibited ET stimulated phosphoinositol hydrolysis and reduced the magnitude of ET stimulated $[Ca^{2+}]_i$ (Thomas, *et al.*, 1991). In HCM cells, the effects of pertussis toxin on the ET mediated calcium response could either be due to direct inhibitory

effects of pertussis toxin on PLC activity, or through indirect actions in elevating cAMP thru increases in PGE_2 formation. The latter action can best be explained by pertussis toxin's blockade of G_i, thus increasing cAMP. The elevated cAMP could activate protein kinase A (PKA), which could phosphorylate PLC or an isoform of PLC, such as PLC-β, decreasing its activity. Further investigation into the identification of the G-proteins associated with the activation of ET_A receptor in HCM cells would be an interesting area to explore.

Specific Aim IV

Effects of Endothelin on Calcium Mobilization in Ciliary Epithelium

The ciliary epithelium is a component of the ciliary body considered to be responsible for the secretion of the aqueous humor. Endothelin-like immunoreactivity has been identified in ocular tissues with some of the highest concentrations found in the ciliary body (MacCumber, *et al.*, 1991; Chakravarthy, *et al.*, 1994). One possibility for the localization of ET in the vicinity of the ciliary body is the local synthesis and secretion of ET from the pigmented and non-pigmented ciliary epithelial cells and ciliary smooth muscle. In HCM cells, both ET-1 and ET-2 have been shown to mediate increases in the intracellular signaling messenger, calcium. In HNPE cells, however, little effect was seen on calcium mobilization by ET-1, ET-2 or ET-

3. It is possible that ET produces effects on second messenger pathways, which were not being measured in this study. For instance, ET has been shown to cause other ionic and biochemical changes, contributing to cellular signaling, such as activation of Na^+-H^+ exchange (Simonson and Dunn, 1990). Ion transport systems in the ciliary epithelium are believed to be involved in the secretion of aqueous humor, therefore it possible that ET acts through second messengers other that calcium in the ciliary epithelium. Alternatively, it is possible that the role of the ciliary epithelium is to synthesize and release ET which acts as a paracrine hormone on the nearby ciliary smooth muscle. In many tissues, due to the proximal location of cells with ET binding sites and those expressing ET mRNA transcripts, it has been suggested that ET acts as a local hormone, synthesized by endothelial or epithelial cells and communicating in a paracrine fashion with nearby smooth muscle cells, fibroblasts or pericytes (Simonson and Dunn, 1990).

Specific Aim V

Localization of ET-1 and Big ET-1 in Ciliary Muscle and Ciliary Epithelium

Immunolocalization studies have identified ET-like immunoreactivity in all ocular tissues, with the highest concentrations of ET in the iris, ciliary body and choroid (MacCumber, *et al.*, 1991; Chakravarthy, *et al.*, 1994). In the ocular tissues of various species, ET-1 and ET-3 have been identified,

with apparently no ET-2 immunoreactivity detected in any ocular tissues (MacCumber, *et al.*, 1991; Eichhorn and Lütjen-Drecoll, 1993; Charkravarthy, *et al.*, 1994). The potential role of ET in the regulation of IOP has been postulated by several authors (Lepple-Wienhues, *et al.*, 1992; Eichhorn and Lütjen-Drecoll, 1993). Endothelin has been shown to stimulate ciliary muscle contractions (Lepple-Wienhues, *et al.*, 1991) and if released locally in the eye, ET-1 could enhance aqueous outflow by contracting the ciliary muscle, thus increasing outflow facility. However, of the two isoforms of ET identified in ocular tissues, the concentration of ET-3 was found to be two to five fold greater than ET-1 (MacCumber, *et al.*, 1991; Chakravarthy, *et al.*, 1994). Therefore, it appears that the deposition of ET in ocular tissue is not completely dependent upon vascular involvement, but instead involves other avascular tissue. The immediate precursor for ET-1 is a 39 amino acid peptide, Big ET-1, which is acted upon by a membrane bound, metallo-endopeptidase called endothelin-converting enzyme (ECE). Processing of Big ET-1 into ET-1 is necessary for the biological activity of endothelin to be fully expressed (Kimura, *et al.*, 1988). One possibility for the localization of ET in the vicinity of the ciliary body is the local synthesis and secretion of ET from the pigmented and non-pigmented ciliary epithelial cells and ciliary smooth muscle cells.

Endothelin-1 and Big ET-1 immunoreactivity was observed in the cytoplasm of HCM and HNPE cells. The basal levels of ET-1 were

approximately equal in the two cell lines. Endothelin-1 immunoreactivity has previously been reported in human ciliary epithelium (Eichhorn and Lütjen-Drecoll, 1993) and in transformed HNPE cells (Lepple-Wienhues, *et al.*, 1992). This is the first time, however, that ET-1 and Big ET-1 immunoreactivity has been detected in the HCM cells, suggesting that these cells have the capability to synthesize both peptides. Small amounts of Big ET-1 immunoreactivity were also detected in HNPE cells. This observation is in contrast to previous studies using radioimmunoassays which did not detect Big ET-like immunoreactivity in human ocular tissue extracts, except in the vitreous (Chakravarthy, *et al.*, 1994). It is possible that the level of extracted Big ET-1 was below that detected by the radioimmunoassay, whereas these immunofluorescent studies were on intact cultured cells. The levels of basal Big ET-1, however, were much lower than the basal levels of ET-1 in both HCM and HNPE cells. It is possible that the majority of ET-1 in HCM and HNPE cells exists in its biologically active, 21 amino acid form. This correlates with the observations in ocular tissues reported by Chakravarthy, *et al.* (1994).

Tumor necrosis factor-α, a proinflammatory cytokine, has been shown in several studies to stimulate ET-1 synthesis *in vitro* and *in vivo* (Marsden and Brenner, 1992; Klemm, *et al.*, 1995). Treatment of the HCM and HNPE cells with TNF-α significantly increased the ET-1 immunoreactivity relative to basal levels of ET-1. Therefore, it appears that TNF-α increases

the amount of ET-1 immuno-like activity in these cells. An increase in Big ET-1 fluorescence was also observed in HCM and HNPE cells treated with TNF-α for 30 minutes. In the HCM cells, although the increase in Big ET-1 was not statistically significant, an increase in fluorescence distribution was observed upon visualization of the cells treated with TNF-α, such that fluorescence appeared throughout the cell cytoplasm. In the HNPE cells, stimulation with TNF-α for 30 minutes significantly increased the level of Big ET-1 immunoreactivity. The general pathway for synthesis of ET-1 has been established, however, a variety of factors could influence rate in which Big ET-1 is converted to ET-1. It is not known the amount of Big ET-1 being tonically synthesized for rapid conversion to ET-1 by the endothelin-converting enzyme (ECE), or if the amount of ECE becomes a rate limiting factor in the conversion. In cultured guinea pig tracheal epithelial cells, TNF-α was found to facilitate release of ET-1 for up to 6 hours, but after 24 hours only transiently increased the level of Big ET-1 (Endo, *et al.*, 1992). It is possible that in the HCM cells, early stimulation by TNF-α first stimulates conversion of the available Big ET-1 to ET-1 and has only a minimal effect on increasing the synthesis of Big ET-1. Whereas chronic stimulation by TNF-α might have delayed effects on stimulating protein synthesis of Big ET-1 for conversion to ET-1. Studies with cultured HNPE cells have shown that enhanced ET-like immunoreactivity, in response to fetal calf serum, thrombin, carbachol and phorbol esters, was prevented by pretreatment with

cyclohexamide, a protein synthesis inhibitor, providing evidence that ET production requires a translational event (Lepple-Wienhues, *et al.*, 1992). Therefore, it appears that TNF-α stimulates the production of Big ET-1 and ET-1 and that cytokines may be involved in the mechanism of ET-1 synthesis and/or release in HCM and HNPE cells. It remains to be determined what intracellular mechanism(s) are responsible for the TNF-α induced ET-1 release and whether TNF-α initiates the transcription and translation of genes which are precursors to ET-1 (preproendothelin and Big ET-1).

CHAPTER V

SUMMARY

Endothelins have been shown to be widely distributed in ocular tissues with the ocular effects to include constriction of retinal blood vessels, pupillary constriction and dilation, and reductions of intraocular pressure. This family of regulatory peptides represent excellent candidates for regulation of ciliary muscle and trabecular meshwork function and their actions may offer insight into the regulation of aqueous humor dynamics and intraocular pressure. The objectives of this dissertation were to investigate the cellular mechanism of endothelin receptor interactions in ocular tissues. The ciliary muscle is an intraocular muscle that is not only responsible for accommodation, but also plays an important role in aqueous humor dynamics. Endothelin has been shown to stimulate ciliary muscle contractions, which could have important implications in the regulation of aqueous humor outflow and IOP. The specific aims of this dissertation were focused on ET's effect on second messengers such as PLC and calcium, and their interactions with PLA_2 in ciliary muscle cells. The hypothesis was that

in HCM cells, ET-1, via the ET_A receptor and a pertussis toxin sensitive G protein, activates PLC, which in turn stimulates calcium mobilization. Independent of this pathway, ET-1 also activates PLA_2 and increases the release of prostaglandins. These two pathways provide a cellular second messenger balance that influences ciliary smooth muscle contraction.

The current study demonstrated that ET-1 and ET-2 stimulate calcium mobilization in HCM cells via an ET_A receptor subtype. It appears that the increase in $[Ca^{2+}]_i$ is the result of ET coupled to PLC via a pertussis toxin sensitive G-protein. A biphasic calcium response is elicited with ET stimulation consisting of a transient increase in $[Ca^{2+}]_i$ which appears to be primarily due to release of intracellular stores, followed by a lower sustained phase which appears to be dependent on the influx of extracellular calcium. Endothelin-1 also appears to stimulate an increase in PGE_2 formation through activation of PLA_2. Furthermore, it appears that the effects of ET-1 on PLC and calcium are independent of the ET-1 effects on PGE_2 production, such that the ET-1 induced increase in $[Ca^{2+}]_i$ are coupled to the PLC signaling pathway, whereas increase in PGE_2 production appears to be the result of an ET_A receptor coupled to PLA_2. Whether there are different subtypes of ET_A receptors or the receptor is coupled through different G-proteins is uncertain. Further studies are needed to clarify these pathways as well as to establish the role that these ET-induced second messengers may play on ciliary muscle function. In addition to the effects of ET on cellular signaling, ET-1 and Big

ET-1 immunoreactivity was observed in both HCM and HNPE cells. Although ET-1 immunoreactivity has previously been reported in human ciliary epithelium (Eichhorn and Lütjen-Drecoll, 1993) and in transformed HNPE cells (Lepple-Wienhues, *et al.*, 1992), this is the first time that ET-1 and Big ET-1 immunoreactivity has been detected in the HCM cells, suggesting that these cells have the capability to synthesize both peptides. Furthermore, the increase in ET-1 and Big ET-1 immunoreactivity upon stimulation with TNF-α suggests that cytokines may be important regulators of ET synthesis and release.

The findings of this research aid in the understanding of the mechanism of action whereby ETs regulate aqueous humor dynamics and intraocular pressure. Through a better understanding of the cellular actions of ET, insight is gained into the development of new ocular selective agents acting at the ET receptor.

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