MECHANISMS OF ACTION OF DRUGS LOWERING INTRAOCULAR PRESSURE OF THE EYE

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DECLARATION

I would like to declare that the experimental work and other research contained within this thesis was undertaken wholly by myself, with the technical assistance of my supervisor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

IOP	Intraocular pressure
AH	Aqueous Humour
CP	Ciliary processes
TM	Trabecular meshwork
TCA	Trichloroacetic Acid
cAMP	Cyclic adenosine-3,5-monophosphate
ATP	Adenosine-5'-triphosphate
IBMX	Isobutyl methylxanthine
POAG	Primary open-angle glaucoma
PACG	Primary angle-closure glaucoma
CPA	N-cyclopentyl adenosine
NECA	5-N-ethyl carboxamide adenosine
CV-1808	8-Phenyl-amino adenosine
CHA	N^6-Cyclohexyladenosine
DPMA	N-^{6}-(2-(3,5-Dimethoxyphenyl)-2(2-methylphenyl)-ethyl adenosine
5-HT	5-Hydroxytryptamine
8-0H-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin)
5-CT	5-Carboxamidotryptamine
I.C inj	Intracameral injection
Art inj	Arterial injection
Art per	Arterial perfusion

SUMMARY

An in vitro preparation of the bovine perfused eye has been used to study the mechanisms of action of drugs on intraocular pressure (IOP) in isolation from the complicating influence of the cardiovascular system, hormones and central nervous system as in most previous reports using the rabbit. This method also avoids the necessity to kill animals for experimental purposes and provides rapid access to the living tissues for biomedical analysis.

In this model the isolated bovine eye is perfused through the long posterior ciliary artery to provide simultaneous monitoring of drug effects on IOP and on the ciliary blood vessels.

In this research most of the drugs have been tested by using various routes of administration in our effort to provide logical and more understandable mechanisms of action for their effect upon IOP. IOP values were measured for 60-90 min following drug administration and were compared against similar experiments in which water was used as a control.

Results obtained with adenosine and its selective agonists cyclohexyl adenosine (CHA), the selective adenosine A_1 receptor agonist and N-^{6}-(2-(3,5-Dimethoxyphenyl)-2(2-methylphenyl)-ethyl adenosine (DPMA), the selective adenosine A_2 receptor agonist showed that they produced small transient effects on arterial perfusion pressure. Their effects on IOP were insignificant, whether inserted into the reservoir as an arterial perfusate solution or when injected into the posterior ciliary artery as a bolus injection.

ATP produced a significant effect on both perfusion pressure and IOP when injected intra-arterially. The increase in perfusion pressure produced immediately after ATP injection probably was due to its vasoconstrictor effect on the uveal vasculature. ATP produced a statistically significant dose-dependent decline in IOP in a dose range of 300 to 3000nmol. The mechanism of action of ATP on IOP is still obscure.

Preliminary experiments with 5-hydroxytryptamine (5-HT) using arterial routes indicate that this drug has no effect on perfusion pressure or IOP. On the other hand intracameral (I.C) injection of 5-HT produced a transient increase in perfusion pressure and a statistically significant decrease in IOP in a dose range of 3 to 30 nmol. 100nmol 5-HT produced a statistically insignificant decrease in IOP, suggesting that 30nmol was the maximum dose of 5-HT to produce decreases in IOP.

Doses of 8-hydroxy-2-(di-n-propylamino)-tetralin) (8-OH-DPAT), the selective 5-HT_{1A} receptor agonist, produced an insignificant effect on bovine IOP by the arterial routes. I.C 8-OH-DPAT produced no effect on perfusion pressure, but significant decline of IOP was produced in a dose-dependent manner.

Single bolus injections of 5-carboxamidotryptamine (5-CT) (20 to 200nmol) produced no effect on perfusion pressure, but produced a statistically significant dose-dependent decrease in IOP. The conclusion is reached that the mechanism by which 5-HT, 8-OH-DPAT and 5-CT reduce IOP is probably through increase in the drainage of aqueous humour when these drugs are injected by the I.C route.

The enzyme-immuno assay for cyclic AMP was performed to study the effects of adenosine, 5-HT and 8-OH-DPAT in bovine ciliary processes (CP) and trabecular meshwork (TM) homogenates. The aim of that was to make a correlation between the effects of these drugs on cAMP and their effects on IOP.

5-HT produced an increase in cAMP synthesis in CP and TM homogenates, although this effect was not dose-dependent. 5-HT produced changes in cAMP of CP homogenate which were too variable to give any conclusion. The conclusion reached is that 5-HT may reduce IOP through increases in cAMP synthesis in TM tissue, which may produce a rise in facility of outflow. The results for 8-OH-DPAT on cAMP in both TM and CP were too variable to draw any meaningful conclusion.

INTRODUCTION

(1.1) GROSS STRUCTURE OF THE EYE

The eyeball is spherical in shape. It is composed of three coats: (1) The outer protective layer is made up of sclera posteriorly and cornea anteriorly **(Berman, 1991).** The cornea is the first and the most powerful refractory surface of the optical system of the eye **(Davson, 1990).** (2) The middle coat is the most vascularized, pigmented part in the eye. It consists of the ciliary body, iris and choroid. The ciliary body is the site of production of aqueous humour. It also permits variation of the focus of the eye (accommodation) through its attachment to the lens by the ciliary zonules. The blood supply to the ciliary body is derived from the ciliary arteries **(Bill, 1981).** The iris is the most anterior portion of the vascular tunic of the eye. It contains circular muscle surrounding the pupil and controls its size, thus regulating the amount of light entering the eye. The choroid is the highly vascularized part of the middle coat. It extends from the ora serrata to the optic nerve. It conducts arteries and nerves to the anterior part of the eye. It also supplies nutrition to the outer retina by the choriocapillaries **(Kllntworth and Landers, 1976).** Blood vessels in the choroid are highly fenestrated and leaky, like the ciliary body. (3)The inner most coat, comprising the retina, contains the essential sensory elements responsible for vision (rods and cones) **(Cohen, 1981).** They respond to the visual stimuli and transduce the light into electrical signals transmitted along the nerve fibres to the optic nerve and finally to the brain **(Jackson & Finlay, 1991).** Within the three coats, are the aqueous humour, vitreous humour and the crystalline lens. Internally, the eye has two spaces, the larger filling the space between the lens and retina, termed the vitreous, and the smaller, between the internal surface of the cornea and lens

termed the aqueous humour **(Tripathi and Tripathi, 1984).** The aqueous chamber is itself divided into the anterior chamber (between the anterior surface of the iris and the internal surface of the cornea) and the posterior chamber (between the posterior surface of the iris and the anterior surface of the lens) **(Bowman and Rand, 1981).** The cornea and lens are avascular tissues, they obtain their nutrition from the continuous circulation of the aqueous humour containing the dissolved O_2 and nutrition, indirectly derived from various blood vessels **(Seidal, 1918; Coperland & Kinsey, 1950).**

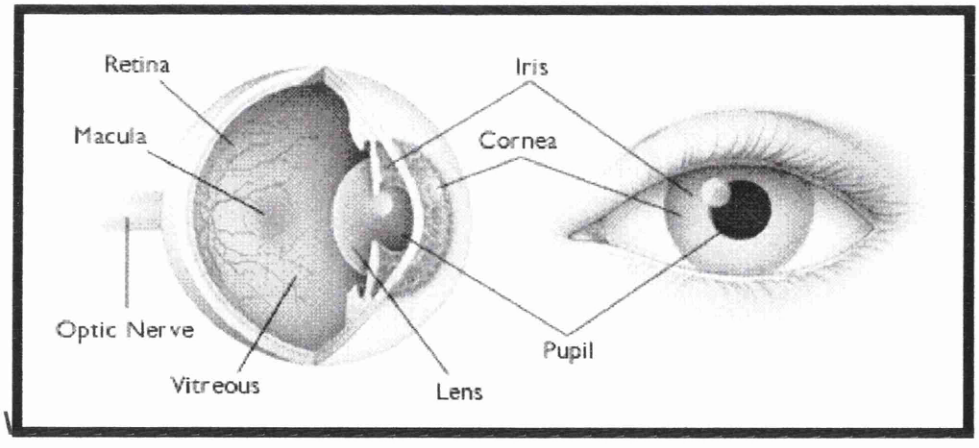


Figure1.1 Anatomy of the human eye. (Dalas.W.W.W).

(1.2) INTRAOCULAR PRESSURE

Intraocular pressure (IOP) of the eye is of interest to both the physiologist and the ophthalmic clinician. To the physiologist IOP is a manifestation of those biophysical and biochemical processes that produce and drain the intraocular fluids. These processes include passive osmotic pressure and active transport. To the ophthalmic clinician IOP is of deep concern because abnormally high IOP, a serious medical condition called glaucoma, can lead to irreversible blindness.

Pressure within the eye is the highest in the body except for the arterial system. The average IOP for the human is about 15mmHg and a range of 22-25mm.Hg is "suspiciously" high. All eyes with pressure of 25mm.Hg or greater, however, are not necessarily abnormal. The individual variation in responses of ocular tissues to elevated IOP is considerable. A patient may have continual increases in IOP but wit.h none of the other signs of glaucoma, such as cupping of the optic disc or change in the visual field. On the other hand, normal pressure may continually be recorded in a patient with progressive field changes **(Moses, 1981).**

At constant rates of aqueous humour production, IOP will rise until the resistance to outflow is exceeded. When outflow is exactly equal to the rate of aqueous humour production, IOP stabilizes at a level determined by these variables. Thus the IOP at any given moment is the result of a dynamic equilibrium exactly matching aqueous production and the rate of aqueous outflow **(Hart, 1992).**

IOP varies with time of day. It appears to be highest early in the morning and lowest in the evening. The variation is about 4mmHg. The patient with glaucoma may have a much higher diurnal variation (8 to 10mmHg) than

does the non-glaucomatous individual, and maximum pressure can occur at any time of the day or night.

(1.2.1) MEASUREMENT OF IOP

In the normal eye, intraocular pressure is governed by the relationship between the rate of aqueous humour formation and drainage. The rate of aqueous humour formation is equal to the rate of drainage and pressure is maintained by resistance in the outflow system. Any deviation from the normal relationship between inflow and drainage resistance will affect the IOP. IOP can be measured in three ways: Palpation, Tonometry and Manometry. (1) Digital palpation is the least reliable method and is useful only for estimating large deviation from normal. Its use should be restricted to eyes with external ocular infections and to uncooperative patients, such as infants, where there is contraindication to general anaesthesia. (2)Tonometric technique: is used almost exclusively in the clinic for estimating IOP. Schiotz tonometry, was previously one of the most commonly employed clinical means of estimating IOP. It measures corneal indentation by a known weight. The variable effects of scleral rigidity decreases the accuracy of this method in the presence of conditions such as extreme myopia and ocular inflammation. Sources of error from Schiotz tonometry include scleral rigidity, a poorly calibrated tonometer, or improper application of the tonometer to the cornea. Goldmann (1957) applanation tonometry, is a modified method of Schiotz tonometry. This method reduces the force needed to applanate or flatten the cornea. At this point the scleral rigidity factors are minimised hence increasing the accuracy of this method for measuring IOP. Applanation tonometry is widely used by ophthalmologists,

but the equipment is impractical for non ophthalmologists **(Leydhecker, 1985).**

All previously discussed tonometers require topical corneal anesthesia. The need for a tonometer that could be used without topical anesthetics led to the development of the Durham-Langham pneumatonometer(Leydhecker, 1958). This is also applanation tonometer because it causes flattening rather than indentation of the cornea. The volume of aqueous humour displaced by corneal flattening is so small that there is little or no effect on scleral rigidity. The manufacturer claims that this method will give true IOP readings when applied to the sclera as well as when applied to the cornea. A small downward correction must be made, however, when the sclera is used for measurment. The problem is that the sclera is covered with soft conjunctival and episcleral tissue so that proper applanation is less certain than on the cornea. The advantage of applying the tonometer to the sclera instead of to the cornea lies in the sclera's lack of sensitivity; the tonometer may then be used without topical anesthesia. It appears that Durham-Langham type of tonometers may give more accurate IOP measurements than does the Goldmann tonometer when used on diseased, scarred, and/or thickened corneas **(McMillan and Forster, 1975).**

(3) Manometry: this is the most accurate means of measuring IOP. This consists of inserting a needle into the anterior chamber and connecting the needle by tubing to a sensitive pressure transducer. This method has limited clinical use, but it is an extremely accurate procedure in the laboratory **(Davson, 1984).**

(1.3) AQUEOUS HUMOUR

Aqueous humour is a transparent fluid contained in the anterior and posterior chambers of the eye and is formed by the ciliary epithelium of the ciliary processes projecting from the ciliary body. As the aqueous is formed, it enters the posterior chamber where it flows in three directions: (1) most of the aqueous humour flows forward between the lens and iris and through the pupil into the anterior chamber **(Patrick and Trevor, 1986).** (2) a small portion flows into the vitreous to be absorbed in the posterior part of the eye and (3) some of the aqueous is apparently reabsorbed at the ciliary body. A part from supplying nutrition to the ocular tissues aqueous humour has other functions including: supplying the substrates required for normal metabolic function of the avascular ocular tissues ,drainage of the metabolic wastes into the venous system and maintaining an optimum pressure in the eye, which it is also important to give the rigidity necessary for optical alignment of cornea, lens and retina **(Sears, 1981).**

(1.3.1) FORMATION OF AQUEOUS HUMOUR

There are three basic mechanisms by which material may cross an epithelial barrier: active transport, diffusion and ultrafiltration. Approximately 80% of aqueous secretion is produced by the non-pigmented epithelium as a result of an active metabolic process. This process requires the input of energy usually in the form of ATP **(Abdel-latif, 1983)**. The active metabolic process depends on several enzymatic systems especially the Na^+/K^+ATPase pump which secretes Na^+ ions into the posterior chamber. This creates an osmotic

pressure difference across the epithelial layers and water movement follows passively along the chemical gradient **(Kanski et al, 1995)**. Among the ions that seem to be actively transported into the clefts between band in the epithelial cells are chloride and bicarbonate **(Bill, 1975)**. A very clear evidence was found for the transport of both sodium and chloride ions in rabbits in vitro, but sodium transport appeared to be the most important. In fact it does not matter which ion is transported as long as the transport causes a net flow of water into the posterior chamber and consequently the fluid entering the posterior chamber is almost isotonic with plasma **(Bill, 1975)**. The remaining 20% of aqueous is produced by ultrafiltration and diffusion which are dependent on the level of blood pressure in the ciliary capillaries, the plasma oncotic pressure and the level of IOP **(Kanski et al, 1995)**. The effect of hydrostatic and oncotic pressure differences across the ciliary epithelium thus is a pressure of about 13mmHg tending to move water in to the process from the posterior chamber **(Bill, 1975)**. Diffusion of solutes across cell membranes occurs from the side of greater concentration to the side of lesser concentration. Ultrafiltration occurs when the flux of a substance across the membrane is increased by a hydrostatic driving force. That transport is induced under the influence of increased hydrostatic pressure. Although all three processes may contribute to the formation of aqueous humour, it is likely that the greatest contribution comes from the active transport of solute (probably Na^+) followed by the osmotic flow of water into the posterior chamber **(Cole, 1984).** The reason for concluding that active secretion contributes to most of the aqueous production is that the concentration of electrolytes in the secreted aqueous is not equal to that of a

simple ultrafiltrate of the plasma. Also if the aqueous were simply ultrafiltrate of plasma, then the concentrations of positive ions such as sodium and potassium would be greater in the plasma than in the aqueous.

Morphological, biochemical and developmental approaches have been used to study the cellular mechanisms involved in the formation of aqueous humour. The effects of drugs commonly used in the treatment of glaucoma to suppress the rate of aqueous formation (for example, timolol and acetazolamide) have sparked additional interest in the study of the mechanisms of aqueous humour formation. Recent findings regarding the role of intracellular cyclic adenosine monophosphate (cAMP) levels in aqueous humour production and IOP have implicated the adenylate cyclase enzyme complex in the control of IOP .

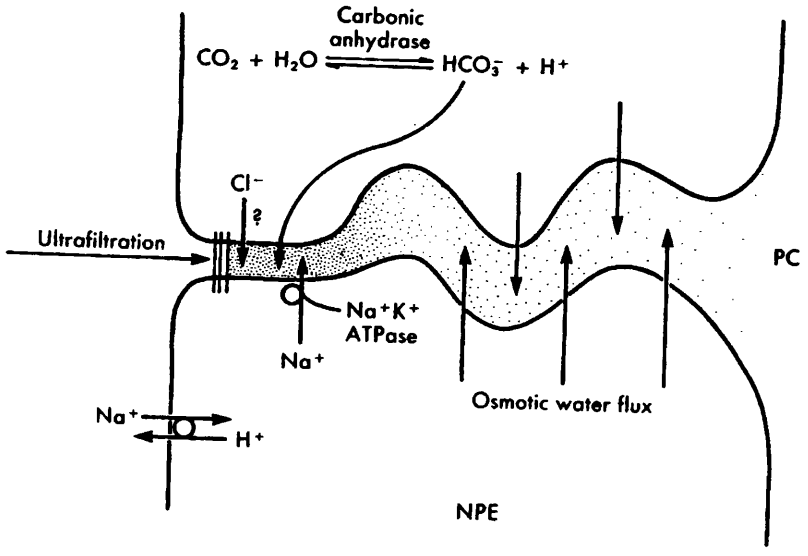


Figure1.2 Hypothetical program of aqueous production with standing gradient osmotic flow model. Na^{+}/K^{+} ATPase is located in highest concentration along lateral cellular interdigitations. Role of transport of Cl^- is still in question. NPE, nonpigmented epithelium, PC, posterior chamber. (Caprioli, 1984).

(1.3.2) CILIARY EPITHELIUM

The ciliary body is divided into two parts: an anterior pars plicata and a posterior pars plana. The pars plicata is composed of 70 radially oriented ciliary processes which project into the posterior chamber. The ciliary process consists of two layers of ciliary epithelium **(Kanski et al, 1995).**

The ciliary epithelium consists of two layers: the non–pigmented ciliary epithelium (NPE) and the pigmented epithelium (PE). The NPE lies adjacent to the posterior chamber and is in direct contact with the aqueous humour. The PE lies between the NPE and the stroma. These PE cells are cuboidal and contain numerous melanosomes but are relatively poor in intracellular organelles compared to the NPE. The NPE cells are columnar and contain numerous mitochondria and rough and smooth endoplasmic reticulum characteristic of a highly metabolically active cell. The cell membrane of the

NPE cells has numerous basal infoldings and multiple convoluted lateral interdigitation. Various types of intercellular junctions join the NPE and PE cells; gap junctions, puncta adherentia, and tight junctions are all present. The NPE is thought to be the key structure responsible for aqueous humour formation. A number of enzyme systems have been demonstrated in this layer. These include nucleotide phosphatases (especialy ATPase), adenylate cyclase and carbonic anhydrase. The role of the PE is still obscure **(Caprioli, 1984).**

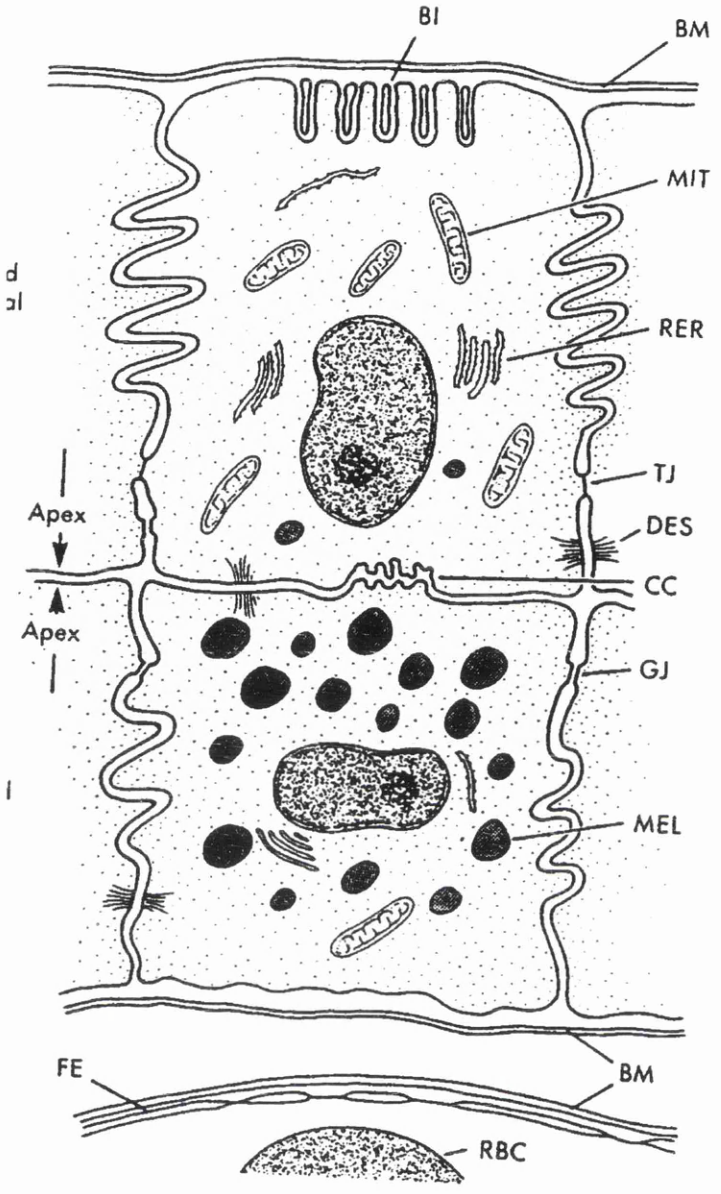


Figure1.3 Schematic diagram of nonpigmented and pigmented epithelial cells. Note apices of cells face each other. BI, basal infolding; BM, basement membrane; CC, ciliary channels; DES, desmosomes; FE, fenestrated capillary endothelial; GJ, gap junction; MEL, melanosome. ; MIT, microcondrion; RBC, red blood cell; RER, rough endoplasmic reticulum; TJ, tight junction. **(Caprioli, 1984).**

(1.3.3) COMPOSITION OF AQUEOUS HUMOUR

The aqueous humour is composed predominantly of electrolytes and low molecular weight compounds with some protein (see Table1) and there are significant differences from plasma in several of the components. Aqueous receives contributions from a variety of sources including the corneal endothelium, iris and lens, in addition to active secretion from the ciliary body. Several trace compounds are also present in aqueous humour, including steroid sex hormones, enzymes such as carbonic anhydrase, lysosomes and plasminogen activator. Low levels of catecholamines, prostaglandins and cyclic nucleotides are present in normal aqueous, but the source of these compounds is uncertain. The protein content of aqueous is very low (about 1/500) of plasma, and the major source is albumin **(Forrester et al, 1996).** It is not clear what happens to the proteins that passes out of the capillaries of the ciliary process. Part of it probably is transported with pinocytosis into the posterior chamber, part may be metabolized, some may return in to the capillaries with diffusion or pinocytosis, and some protein may leave the ciliary processes with a flow of the tissue fluid moving through the ciliary body into the supraciliary and suprachoroidal spaces **(Bill, 1975)**.

Some substances such as glucose and urea, are present in lower concentration in the aqueous than in the plasma. Utilization of these substances by the tissues of the eye or loss of the substances in the posterior portion of the globe may account for the differences in concentration. Other substances such as ascorbic acid and hyaluronic acid are present at much higher levels in the aqueous and are transported by active secretion 5-HT has been shown to exist in aqueous humour (martin,

1988). Chloride and bicarbonate concentrations vary among different species.

These differences in ionic concentrations have prevented the acceptance of a universal theory for the production of aqueous humour **(Harold and Daniel, 1977).**

Table1.1 Composition of aqueous humour compared with plasma. Concentrations expressed in mmol/kg of water, mg/dL, meq/dL in human. **(Forrester et al, 1996).**

Component	Aqueous	Plasma	Units
Glucose	7-3.9	5.6-6.4	
Lactate	4.5	0.5-0.8	mmol /kg
Ascorbate	1.1	0.04	
Albumin	5.5-6.5	3400	
transferrin	1.3-1.7		mg/dL
Fibronectin	0.25	29	
IgG	3.0	1270	
Na	142	130-145	
K	4	3.5-5.0	meq/dL
HCO_3	20	24-30	
Mg	1	0.7-1.1	
Ca	1.2	2.0-2.6	
Cl	131	92-125	

(1.3.4) DRAINAGE OF AQUEOUS HUMOUR

The outflow of aqueous from the eye is regulated at several different levels, including the trabecular meshwork, the uveoscleral system, and, outside the globe the episcleral vessels.

Aqueous flows from the posterior chamber into the anterior chamber through the pupil and is drained by the following two different routes **(Kanski, et al, 1995).**

(A) Schlemm's canal or trabecular (conventional) route accounts for approximately 90% of aqueous humour outflow in humans. The aqueous humour is drained through the tissues at the angle between the iris and the corneo-scleral junction the so-called filtration angle. It diffuses through the trabecular meshwork into a structure known as the canal of Schlemm. The aqueous humour is drained from the canal of Schlemm through collecting channels into the venous circulation (venules which are closely associated with it). This is a bulk flow, pressure-sensitive route so that increasing the pressure head will increase outflow. Trabecular outflow can be increased by drugs (parasympathomimetics, sympathomimetics), laser trabeculoplasty or trabeculotomy **(Kanski et al, 1995).**

(B) The uveoscleral (unconventional) route accounts for the remaining 10% of aqueous outflow .The aqueous flows from the iridocorneal angle (the filtration angle) into the interstitial tissue spaces between the muscle bundles of the ciliary muscle in the ciliary body. These spaces open in turn into the suprachoroidal space from which fluid can pass through the venous circulation in the ciliary body, choroid and sclera into the episcleral tissues. The pressure in the suprachoroid is lower than that in the anterior chamber; the difference being at least a few mm of Hg under normal conditions. In the uveoscleral outflow, a portion of the aqueous passing into the ciliary body is absorbed into the uveal blood vessels. Contraction of the ciliary muscle by parasympathomimetic drugs such as pilocarpine opens large spaces in the trabecular meshwork and increases the drainage through Schlemm's canal At the same time the uveoscleral drainage is almost stopped due to compression of the interstitial spaces within the ciliary muscle (**Kanski et al,**

1995). Atropine produces relaxation of the ciliary muscle which reduces the drainage through Schlemm's canal because of tight packing of the trabecular meshwork pores. On the other hand, the uveoscleral drainage increases due to increasing the spaces between the muscle bundles **(Bill, 1969).** The uveoscleral outflow drains some 0.2 to 0.5μl of aqueous/min at a steady rate not affected by IOP. The trabecular outflow carries the greater portion of aqueous from the eye in man, approximately 1.8 to2.5μl /min in man **(Bill and Maepea, 1989).**

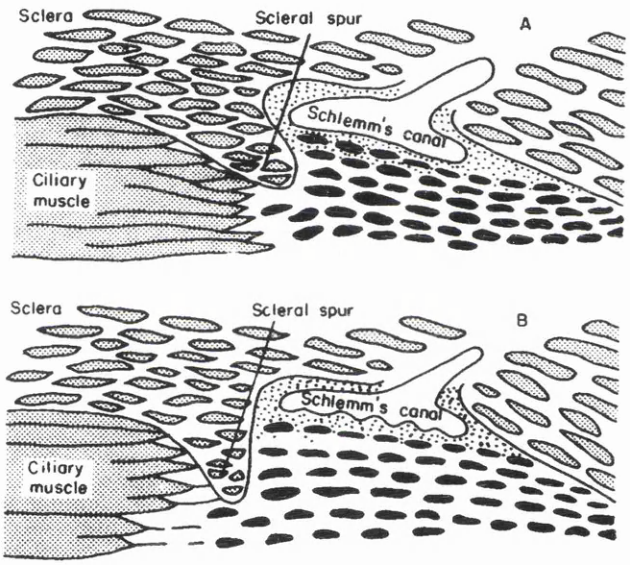


Figure1.4 Diagrams to illustrate the effects of pilocarpine-induced contraction of the ciliary muscle on the scleral spur and the trabecular tissues. A, untreated; B, treated (Davson, 1990).

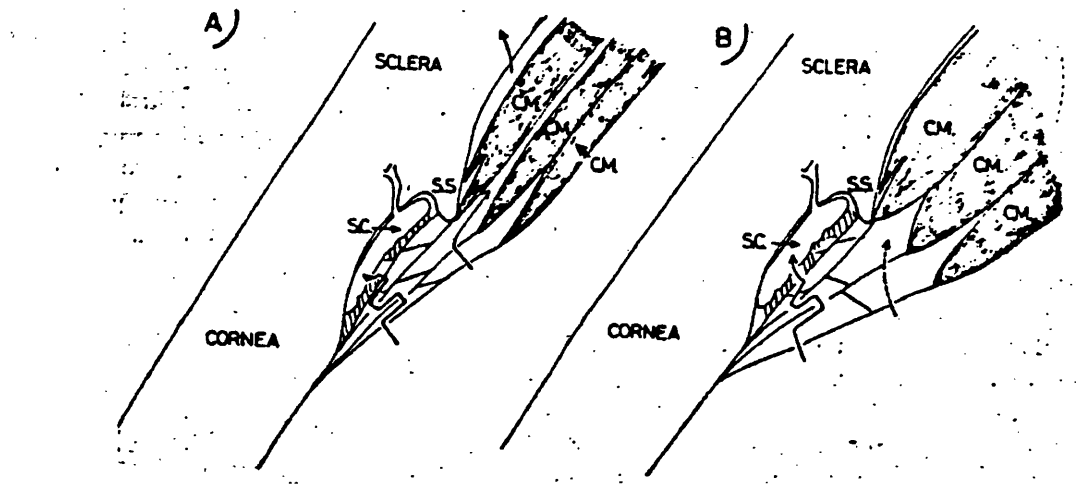


Figure1.5 A: ciliary muscle is relaxed, spaces between lamellae of uveal and coneoscleral meshwork are narrow, and interstitial spaces between muscle bundles of ciliary muscle are wide. Aqueous humour leaves the anterior chamber by routes via Shlemm canal and by routes via interstitial spaces of ciliary muscle. B: ciliary muscle is contracted, spaces in different parts of meshwork are relatively wide, and interstitial spaces between muscle bundles have almost disappeared Aqueous humour leaves the eye almost entirely by routes via Shlemm canal (Davson, 1990).

(1.4) CYCLIC AMP

Cyclic 3,'5'-adenosine monophosphate (cAMP) is an intracellular second messenger. The role of cAMP was revealed by the work of Sutherland and his colleagues in the late 1950s. This discovery demolished at a stroke the barriers that existed between biochemistry and pharmacology, to the great benefit of both disciplines **(Rang, Dale and Ritter 1995).**

CAMP is a nucleotide synthesized within the cell from ATP catalyzed by the action of adenylate cyclase. It is produced continually and inactivated by hydrolysis to 5'-AMP, by the action of a family of enzymes known as phosphodiesterases

CAMP has become especially important in pharmacology for several reasons. Firstly, because it led to the discovery of adenylate cyclase.

Secondly, because the stimulation of adenylate cyclase by hormones was the first receptor-mediated event that could be studied in a cell-free system. Moreover, continued study of this phenomenon has led to much of our understanding of what receptors are and how they act. Furthermore cAMP is important physiologically and clinically.

A large variety of drugs, hormones and neurotransmitters produce at least some of their effects by altering the intracellular level of cAMP by either stimulating or inhibiting the catalytic activity of adenylate cyclase, thereby leading to either an increase or a decrease in the level of cAMP intracellularly. **(Robinson, 1983).**

The regulatory effects of cAMP on cellular function are many and varied, including , for example, enzymes involved in cell metabolism, cell division and differentiation, ion transport and ion channel function, leading to changes in neuronal excitability. These varied effects are all brought about by a common mechanism, namely the activation of various protein kinases by cAMP.

(1.5) GLAUCOMA

The glaucomas are a group of potentially blinding ocular conditions. Because the pathophysiology, clinical presentation and treatment of the different types of glaucoma are so varied, there is no single definition that adequately encompasses all forms. Understanding this concept helps to explain, for example, why one patient with 'glaucoma' may have no symptoms whilst another experiences sudden pain and redness **(Kanski et al, 1995).**

The two main types of glaucoma are congenital (developmental) and acquired. Further subclassification into open–angle and angle–closure types is based on the mechanism by which aqueous outflow is impaired. The glaucoma may also be primary or secondary depending on the presence or absence of associated factors contributing to the pressure rise **(Crick, 1978).** In primary glaucoma's the elevation of IOP is not associated with any other ocular disorder whereas in secondary a recognisable ocular or non–ocular disorder alters aqueous outflow which, in turn, results in elevation of IOP **(Henson, 1993).**

(1.5.1)Primary open–angle glaucoma (POAG)

POAG, is generally bilateral, characterized by the following

- Adult onset
- An IOP>21mmHg at some point in the course of the disease
- Glaucomatous optic nerve head damage
- An open angle of normal apperance
- Visual field loss **(Kanski et al, 1995).**

Despite this definition it should be emphasized that approximately 16% of all patients with otherwise characteristic POAG will have IOP consistently<22mmHg and constitute a sub–group referred to as 'normal–tension glaucoma'.

POAG is the most prevalent of all glaucomas, affecting approximately 1 in 100 of the general population over the age of 40 years. The condition affects both sexes equally and is more severe in blacks than in whites. Age is one of the main risk factors in POAG. It is unusual for the diagnosis to be before the age of 40 years. Glaucoma can also affect younger people, and

measurement of eye pressure is an important of a routine eye examination **(Pavan, 1991).**

Elevation of IOP in patients with POAG is caused by increased resistance to aqueous outflow in the drainage channels. The development of visual field loss is related to progressive loss of axons in the optic nerve head. Several mechanisms have been postulated to be responsible for this damage, but no single mechanism can adequately explain the great variation in susceptibility to damage and the pattern of damage seen. Patients with POAG are usually asymptomatic until a significant loss of visual field has occurred **(Kanski et al, 1995).**

(1.5.2) Primary angle– closure glaucoma (PACG)

PACG is the condition in which elevation of IOP occurs as a result of obstruction of aqueous outflow by partial or complete closure of the angle by the peripheral iris. It is more common in females by a ratio of 4:1 **(Janski, 1992).** Unlike POAG the diagnosis depends to a great extent on examination of the anterior segment and careful gonioscopy. The presence of a normal optic nerve head and absence of visual field loss does not preclude the diagnosis of PACG. The disease occurs in anatomically predisposed eyes and is frequently bilateral, although presentation of the acute form is frequently asymmetrical. Age and family history are among the risk factors in PACG. The average age at presentation is about 60 years and the prevalence increases thereafter **(Kanski et al, 1995).**

(1.6) PHARMACOLOGY OF INTRAOCULAR PRESSURE

Parasympathomimetic drugs (those that cause the pupil to constrict) generally result in a lowered IOP. In addition to producing contraction of the iris sphincter muscle, these drugs also stimulate contraction of the ciliary muscle. The trabecular meshwork, being attached to the scleral spur, is in effect a tendon of origin for the ciliary muscle, traction on which will result in a significant change in the geometry of the trabecular meshwork. These drugs reduce uveoscleral outflow facility and produce a simultaneous increase in trabecular outflow facility. Since trabecular facility is approximately an order of magnitude greater than uveoscleral facility, the net effect is a marked elevation in the outflow facility with a concomitant decrease in IOP. This effect is particularly magnified in eyes that have abnormally high IOP (abnormally low outflow facility). The parasympathomimetics, include direct parasympathomimetic agonist drugs (which act directly on the ciliary muscle) such as pilocarpine and carbachol and indirect agonists (the choline esterase inhibitors) such as physostigmine **(Voulghan and Asbury, 1983).** Pilocarpine is widely used in glaucoma. Cholinesterase inhibitors are no longer used due to adverse effects on the eye.

Topically applied epinephrine (1% to 2%) is a valuable drug in the treatment of glaucoma. The drug increases the outflow through both routes of drainage (the trabecular meshwork and uveoscleral routes) and decreases the rate of production of aqueous humour, but its effect on the outflow is more predominant and long lasting. The decrease in production of aqueous humour is probably caused by stimulation of receptors in the ciliary

processes, but the reason for increasing outflow is still unclear **(Moses, 1981).**

Timolol is very widely used to treat glaucoma. Its mechanism of action has been controversial for many years; some evidence suggests that it acts through β-blockade while other results suggest a different mechanism.

Timolol is an antagonist at the β-adrenoceptor, but for a compound to be active as a pharmacological antagonist, there must tonic agonist-induced stimulation to block. Thus, the ability of timolol to reduce aqueous formation and IOP implies that endogenous adrenergic tone stimulating β-adrenoceptors exists in the eye.

The β-agonist isoprenaline stimulates cAMP synthesis and increases aqueous humour formation and IOP **(Nathanson, 1981a)**. Timolol as a potent non-selective β-adrenoceptor antagonist **(Scriabine et al., 1973)** blocks this effect and decreases aqueous secretion in the monkey eye suggesting the effect is mediated by β-adrenoceptors (**Miichi and Nagataki, 1983**).

The β_2-agonist, terbutaline, increases aqueous humour formation by about 100% in cynomolgus monkey and this effect was completely abolished by timolol **(Nilsson et al., 1990).**

It has been observed that the rate of aqueous humour flow in humans undergoes a circadian rhythm characterized by a higher flow during working hours (highest being in the morning) and lower flow during sleep **(Reiss et al., 1984)**. In contrast to day time, 0.5% timolol is devoid of any ocular hypotensive effect during sleep **(Topper and Brubaker, 1985).** In contrast

with the above, some data suggest that conventional β-adrenoceptors are not involved.

Topically applied timolol can block β-adrenoceptors in the eye as evidenced by the attenuation of the ability of topical isoprenaline to increase the production of cAMP in the aqueous humour of the rabbit **(Vareilles et al, 1977; Schmitt et al., 1981b).** However the dose of timolol required to block this response to isoprenaline is very much less than that required for ocular hypotensive activity, thus implying that different mechanisms are involved in both phenomena. This is supported by the observation that the effectiveness of various β-antagonists in lowering IOP in rabbit models of ocular hypertension did not correlate with their ability to block β-adrenoceptors **(Bonomi et al., 1979).** In the bovine perfused eye, it was concluded that there was little correlation between aqueous formation rate and the ciliary epithelial content of cyclic AMP **(Shahidullah, Wilson and Millar, 1995).** Thus the direct correlation between aqueous humour formation and ciliary cAMP production which many authors assume, is subject to considerable uncertainty.

It is possible that timolol reduces IOP by acting on more than one mechanism.

What might be the signalling action of cAMP in the ciliary epithelium to regulate flow?

One among several articles **(Crook et al, 1993)** implicating the activation of the cAMP pathway of the ciliary epithelium as regulatory for ion transport and aqueous production, proposes the upregulation of $Na^+/ K^+/ Cl^-$ cotransport by protein kinase A and downregulation by protein kinase C. This work was done in human fetal non-pigmented ciliary epithelium in short term culture. In

permeabilized rabbit ciliary epithelium, Na^+ K^+-ATPase is reported to be inhibited by activation of protein kinase A **(Delamere and King, 1992)**. Other in vitro findings **(Delamere and King, 1990)** appear to conflict with some in vivo research showing either increases or decreases of aqueous formation with accelerated cAMP production (which is increased by beta-adrenergic agonists and decreased by α_2 agonists or β-adrenergic blocking agents). Therefore aqueous formation in the eye is affected by the activation and the inhibition of several receptor subtypes. Further studies of other receptor interactions are required for better comprehension of the system **(Horio, Sears, Mead, Matsui and Bausher, 1996).**

Acetazolamide is a potent inhibitor of carbonic anhydrase enzyme, which catalyzes the reaction of $H_2O + CO_2$ to yield $HCO_3^- + H^+$. Systemic treatment with acetazolamide and other carbonic anhydrase inhibitors decreases IOP by reducing aqueous formation. There is a substantial evidence that acetazolamide directly affects the transport mechanisms of the ciliary epithelia. Inhibition of carbonic anhydrase lowers the aqueous Cl^- concentration in primates and the HCO_3^- in rabbits. Acetazolamide decreases the rate of Na^+ and HCO_3^- transport into the posterior chamber, suggesting a linkage of the accession of these two solutes into the posterior chamber. Knowledge of the mechanism by which carbonic anhydrase activity is coupled to Na^+ and HCO_3^- movement into the posterior chamber is still sought. Acetazolamide was the first such agent to be used in the treatment of glaucoma, but topically active carbonic anhydrase inhibitors, such as dorzolamide, are now available **(Hart, 1992).**

IOP may rapidly be lowered by increasing blood osmolarity; this may be accomplished by intravenous infusion of urea or mannitol or by oral ingestion

of glycerin. These agents are valuable in the treatment of acute angle closure glaucoma. Water loading, on the other hand lowers blood osmolarity so water will be drawn into the eye. If the facility of outflow is good, there will be little change of IOP , but if facility of outflow is reduced, ingestion of a quart of water may raise IOP by 8mmHg or more **(Moses, 1981).**

(1.7) OPTIC NERVE

The optic nerve is surrounded by the optic stalk through which arteries and veins enter the eye. The optic nerve is not a true nerve like peripheral nerve, but is actually a nerve fibre tract whose fibres are derived from the ganglion cells of the retina. It is composed of the axons and myelinated sheaths. Optic nerve fibres like all other nerves, degenerate if the connections with their nerve cell bodies are severed. The optic nerve is severely damaged under high IOP, but when this pressure is reduced so the damage to optic nerve ceases

(1.8) BLOOD SUPPLY TO THE EYE

98% of the blood to the eye passes through the uveal tract, of which 85% is through the choroid **(Forrester et al, 1996).** The blood supply to the eyeball is derived from the ophthalmic artery; which is derived from a branch of the internal carotid artery. This ophthalmic artery divides into 10-20 branches of ciliary arteries, two of them are long posterior ciliary arteries which run forward through the choroid to the ciliary body where they anastomose with the anterior ciliary arteries to form what has been incorrectly called the major circle of the iris (fig 1.6)., incorrectly, because the circle is actually in the ciliary body. From this circle, arteries run forward to supply the ciliary and iris

blood vessels and backward to supply the choroidal circulation. Venous blood from the uvea drains into the episcleral veins and from there into the four vortex veins, finally leaving the eye by way of the inferior and superior ophthalmic veins. The remaining blood entering the eyeball is carried by the central retinal artery **(Forrester et al, 1996).** This central retinal artery enters the eye through the optic nerve and divides into four main branches running within the retina to supply the inner retina (fig 1.6). The venous blood from the retina drains into the retinal veins and then into the ophthalmic veins. The outer retina has a lack of blood vessels, it derives its nutrient supply from the choriocapillaries **(Bill, 1981).**

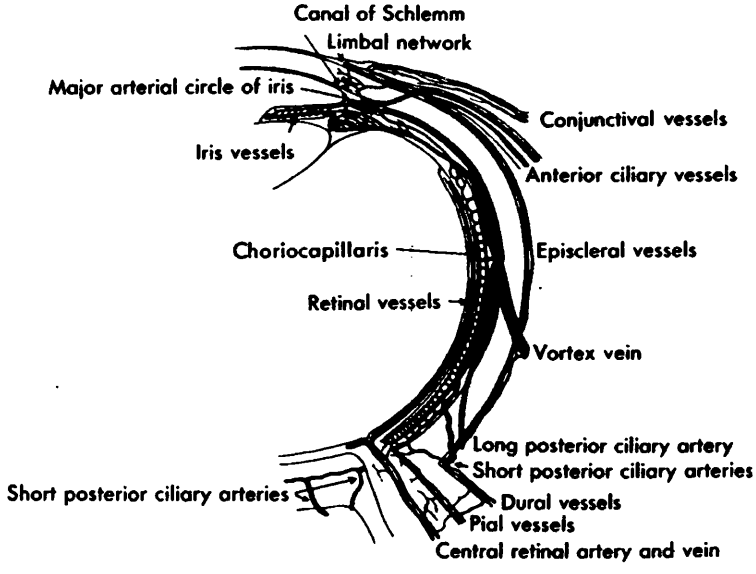


Figure1.6 Blood vessels of human eye. (Modified from Leber, 1903)

(1.9) BLOOD-AQUEOUS BARRIER

The blood-aqueous barrier means certain substances encounter difficulty in entering the ocular tissues. This barrier has two parts: the epithelial part is represented by the tight junctions between the non–pigmented cells of the ciliary epithelium in the ciliary body **(Davson, 1956).** The other part of the blood-aqueous barrier is the endothelial part, and is constituted by the non–fenestrated cells of the iris capillaries. The two parts of the barrier work together to exclude large molecular-weight substances such as proteins. They are effective with respect to the low molecular-weight substances such as sucrose and fluorescein due to the presence of a semi- porous membrane with a pore radius of about 10.4nm. This barrier excludes the proteins of the clotting cascade and maintains the clarity of the aqueous and the optical integrity of the eye. **(Vegge, 1971; Uusitalo, Palkama & Stjernschantz, 1973).**

(1.10) BREAKDOWN OF BLOOD- AQUEOUS BARRIER

Under certain experimental and pathological conditions such as trauma, injury or paracentesis (removal of aqueous humour from the anterior chamber), the blood- aqueous barrier breaks down **(Berman, 1991),** so that large molecular-weight substances such as proteins that are normally and almost completely excluded from penetration now appear in the aqueous humour in large amounts. The aqueous humour becomes cloudy (seen as flare in the slit-lamp microscope) due to leakage of plasma proteins into the posterior and anterior chambers. It may even become plasmoid owing to the

presence of fibrin and other proteins. Inflammatory cells are also likely to be present when the blood–aqueous barrier breaks down **(Forrester et al, 1996).** The breakdown of this barrier is usually but not always accompanied by a rise in IOP which may be spectacular i.e.up to 60-80mmHg **(Davson, 1990).** The vascular engorgement within the eye suggests that the prime action is on the blood vessels. A typical agent is the hydrochloride of nitrogen mustard. When this is applied topically to the eye the IOP rises rapidly **(Davson and Quilliam, 1947; Davson and Huber, 1950). Wessely (1900)** showed that the breakdown could be reduced by sympathetic stimulation or treatment of the eye with epinephrine, and **Cole (1961)** showed that the anti-inflammatory agent, polyphloretin, also prevents the breakdown of the blood aqueous barrier.

(1.11) ADENOSINE

Adenosine produces many physiological and pharmacological effects both in the periphery and in the central nervous system. There is evidence that it functions as a mediator in the central nervous system **(Phillis and Wu 1981, Stone 1991a).**

(1.11.1) Adenosine receptor agonists and actions

The effects of adenosine are mediated by two distinct receptors, A_1 and A_2, linked respectively, to the inhibition and stimulation of adenylate cyclase, thus reducing or increasing intracellular cAMP formation **(Stone 1991b, Collis & Hourani 1993).** The main effects of adenosine and its agonists are (1) vasodilatation, including coronary blood vessels (A_2), except for the kidney, where A_1 receptors produce vasoconstriction. Adenosine infusion causes a fall in blood pressure. (2) Inhibition of platelet aggregation (A_2). (3)

Bradycardia, block of atrioventricular conduction (A_1), and reduction of force of contraction of the heart. (4) Bronchoconstriction (A_1) **(Rang et al 1995).**

(1.12) ADENOSINE TRIPHOSPHATE (ATP)

Adenosine and adenosine triphosphate (ATP) are components of all cells, both are released from cells into the extracellular medium, and both have marked effects on peripheral tissues and the central nervous system. ATP is stored together with acetylcholine or noradrenaline in their respective synaptic terminals as a co-transmitter **(Stone, 1995).**

In the extracellular space, ATP is rapidly metabolized to adenosine by ATPase and nucleotidase enzymes. The effects of ATP are usually exerted in the postsynaptic cell, producing contraction or relaxation of smooth muscles. ATP also potentiates the effects of noradrenaline on some tissues **(Stone, 1995).**

ATP is one of the NANC (non-adrenergic non-cholinergic) transmitters which function as neurotransmitters at many peripheral effector junctions and also as a fast transmitter in the central nervous system. It also functions as an intracellular mediator, inhibiting the opening of membrane potassium channels.

ATP acts on several types of purinoceptors (P_2) They are distinguished on the basis of their agonist selectivity. One of these (P_{2X}) is a ligand-gated ion channel. Others, P_{2Y} P_{2z} and P_{2u} are all coupled to various second messengers.. Drugs acting selectively on ATP and ADP receptors have not yet been developed for clinical purposes (**Burnstock 1981, 1985, 1988, Evans et al. 1993).**

Burnstock and his colleagues have shown that ATP is released, in a calcium-dependent fashion, on nerve stimulation.

The fast transmitter function of ATP, including its vasoconstrictor function, is mediated by P_{2x}-receptors, which are blocked by suramin and a purine analogue, ANAAPP3 (**Burnstock, 1988**). The use of these blocking agents has helped to clarify the physiological role of ATP. Its other actions are linked to various second messenger systems, for which no specific antagonists are known.

In most cells, intracellular Ca^{2+} is contained mainly in the endoplasmic reticulum and in mitochondria. Many agents that contract smooth muscle do so by activating membrane-bound phospholipase C, thereby causing the intracellular formation of inositol tris-phosphate which causes Ca^{2+} to be released from the endoplasmic reticulum **(Berridge, 1993)**. This is the mechanism of ATP when it activates the P_{2u} receptor. This can occur without any movement of Ca^{2+} across the plasma membrane **(Shahidullah et al, 1995)**.

Secretory cells are also in general activated by an increase in intracellular Ca^{2+} and some secretory cells also have P_{2u} receptors **(Dubyak and EL-Moatassim, 1993).** The presence of these receptors has recently been confirmed in ciliary epithelial cells **(Shahidullah and Wilson, 1997)** where again they cause liberation of intracellular Ca^{2+}. Since ATP can cause such a fundamental change in function of ciliary epithelial cells, it would be of interest to know whether exogenous ATP can alter aqueous humour formation and hence, perhaps, IOP.

(1.13) 5-HYDROXYTRYPTAMINE (5-HT)

5-HT (5-hydroxytryptamine) was originally discovered in 1948 when the identity of a vasoconstrictor substance release from blood platelets was elucidated. It was originally called serotonin, a name that is still widely used. It was shown to have an important role as a neurotransmitter, as well as a local hormone in the central and peripheral vascular system, which is involved in the regulation of a variety of physiological functions and behaviours. In addition, 5-HT has been implicated in the pathophysiology of different neuropsychiatric disorders, such as anxiety, depression, schizophrenia, eating disorders and migraine **(Pasqualetti et al, 1996).** It comes from many sources other than blood, and has many effects in addition to causing vasoconstriction. 5-HT is present in the diet but most is metabolized before entering the blood. It originates from the precursor amino acid, tryptophan. Tryptophan is converted to 5-hydroxytryptophan in chromaffin cells and neurons by tryptophan hydroxylase enzyme. The 5-hydroxytryptophan is then decarboxylated to 5-hydroxytryptamine by the action of a decarboxylase enzyme. It is degraded by the action of mono amine oxidase enzyme to 5-hydroxy indole acetic acid **(Rang et al, 1995).**

(1.13.1) Pharmacological effects of 5-HT

(1) In the gastrointestinal tract it increases motility.

(2) On blood vessels (i) Constriction of some arteries and veins, mediated by 5-HT_{2A} receptors.

(ii) vasodilatation by several mechanisms, all operating through 5-HT_{1A} receptors.

(3) On platelets :- 5-HT causes platelet aggregation.

(4) On CNS : excites some neurons and inhibits others, but the normal effects are predominantly inhibitory. Four major classes of serotonin receptors termed $5HT_1$, $5HT_2$, $5HT_3$ and $5HT_4$ have been characterized **(Frazer, Maayani and Walfe, 1990).** The $5HT_1$ group can be further subdivided into $5HT_{1A}$, $5HT_{1B}$, and 5 HT_{1D} subtypes. They are differentiated by the kinetics of agonist and antagonist binding to the receptor, and by their regional distribution within the central nervous system **(Forrester et al, 1996).**

The 5-HT_{1A} subtype shows a high affinity for agonists such as 8-OH-DPAT and 5-CT **(Middlemiss and Fozard, 1983)** and is positively or negatively linked to the adenylate cyclase-cAMP cascade **(Frazer et al., 1990).**

These receptors are widely distributed throughout the central nervous system and some of the drugs which act on them have striking behavioural effects (**Stone, 1995).** The 5-HT_{1A} agonist 8-OH-DPAT facilitates male rat sexual behavior and produce an anxiolytic action in animals, but the role for 5-HT_{1A} binding sites is still in question. 8-OH-DPAT also produces a pronounced hypotensive effect in animals: this effect may be 5-HT_{1A}-mediated. In general, 5-HT_{1A} agonists produce a hypothermic response in rodents. The hypothermic effect of 8-OH-DPAT is due to its agonist action at presynaptic 5-HT receptors. 8-OH-DPAT also serves as a training drug in tests of discriminative stimulus control of behavior in cats **(Glennon, 1987).**

5-Carboxamidotryptamine (5-CT) is an agonist that acts on $5HT_1$-like receptors (5-HT_{1A}, 5-HT_{1B}, $5HT_{1D}$). It also causes elevation of cAMP by

interaction with a specific 5-HT receptor which is 5-HT_1-like **(Trevethick et al, 1986).**

(1.13.2) Pharmacological effects of 5-CT

(I) On CNS: It produces neuronal inhibition and behavioral effects (sleep, feeding, anxiety) mediated through 5-HT_1 receptors (5-HT_{1A}, 5HT_{1B}, 5HT_{1D}) and presynaptic inhibition produced via 5-HT $_{1D}$ receptor

(ii) On blood vessels: 5-CT produces a consistent, dose-related decrease in arterial (diastolic) blood pressure and carotid artery vascular resistance, due to its action on 5-HT_1-like receptors mediating relaxation of vascular smooth muscle**(Feniuk et al., 1981; 1984).** Vasodilatation was observed in several tissues, but the skin, ears and stomach responded most prominently **(Saxena et al, 1985).**

(iii) On the heart: 5-CT causes a dose-related tachycardia and is approximately fifty times more potent than 5-HT in this aspect **(Connor et al, 1986).**

Antagonists that block the effect of 5-CT on its receptors are spiperone (selective on 5-HT_{1A} receptors) methiothepin and ergotamine (on 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}) **(Zifa and Fillion, 1992).**

In 1981, **Moro and co-workers** found that intravitreal injection of 5,6-dihydroxtryptamine, a serotonergic neurotoxin, causes miosis implicating 5-HT in the control of pupil size. This has been substantiated by the finding that pupil size is also decreased by the intravenous injection of ketanserin, a specific 5-HT_2 receptor antagonist (**Krootila, Uusitalo and Palkama, 1987).** The presence of serotonergic neurones in the iris-ciliary body has been

described by **Tobin, Unger and Osborne (1988)** and functional attributes of the amine have been demonstrated by other studies.

There is now considerable evidence that serotonin is a transmitter in the retina **(Osborne and Barnett, 1990)**, but information on its role in the front of the eye is much less abundant. In the eye, the identification of 5-HT receptors, in human, is of particular importance in view of the known relationship between cAMP and the regulation of IOP **(Sears et al., 1984; Bartels et al., 1987).** Recent studies by **Martin et al. (1992)** showed that there is a correlation between the content of serotonin in the aqueous fluid and IOP in the human eye.

Previous workers suggest that the actual existence of endogenous serotonin neurons in either the trabecular region or the iris-ciliary body has not been demonstrated. However, when iris-ciliary body tissues are incubated in vitro with exogenous 5-HT, the amine can be shown to be taken up and localized in neurons by use of immunohistochemistry. It is also well known that immunohistochemical procedures have failed to localize endogenous serotonin in the mammalian retina, but when the retina is exposed to exogenous amine, it can be localized to certain amacrine cells **(Meyer-Bothling et al, 1993).**

It has been found that an intraocular injection of 5HT into rabbit anterior chamber causes an increase in both IOP and protein concentration in the aqueous **(Chidlow et al, 1995).**

5-HT_{1A} receptors exist in the rabbit iris-ciliary body (**Mallorga and Sugrue, 1987 ;Tobin and Osborne, 1988).** Therefore the effect of 5-HT on IOP in rabbits may be due to action on 5-HT_{1A} receptors. 5-HT_{1B} receptors can be distinguished from 5-HT_{1A} subtype by their lower affinity for spiperone and

lack of affinity for 8-OH-DPAT. They are found exclusively in rodents, are negatively linked to adenylate cyclase and are antagonized by propranolol **(Middlemiss and Hutson, 1990).** The 5-HT_{1C} subtype(now reclassified as 5-HT_2, which is found in high density in choroid plexus, is coupled to the hydrolysis of inositol phosphates **(Conn et al., 1986).** A 5-HT_{1D} receptor subtype has been characterized in bovine, pig, guinea pig, pigeon and human brain membranes but no reports indicate location in the eye. These sites display high affinity for 5-HT and related tryptamines but relatively low affinity for 8-OH-DPAT **(Peroutka, 1991).** These receptors are also negatively linked to cAMP production but can be distinguished from 5-HT_{1B} by their lower affinity for propranolol **(Hoyer et al., 1990; Shoeffter et al., 1988).** Three mechanisms can be envisaged by which 5-HT might alter IOP : action on outflow resistance, on the uveoscleral pathway and action on aqueous secretion. The action of serotonin in increasing IOP by any of these mechanisms can only be determined by experiment.

It has been found that topically applied ketanserin lowered IOP in rabbits, cats and monkeys **(Chang, Burke and Potter, 1985; Conway and Lewis, (1989).** The $5HT_{2A}$ antagonist, ketanserin has been evaluated on IOP in certain patients with ocular hypertension. It is also reported that orally administered ketanserin also significantly reduced IOP in glaucomatous patients **(Costagliola et al., 1989; 1990, 1991).** Both **Chang and co-workers (1985)** and **Conway and Lewis (1989)** suggest that ketanserin exerts its effect by suppressing aqueous humour formation and increasing outflow. Whether the marked increase in total outflow is due to increased trabecular outflow or increased uveoscleral drainage, or both, remains an open question. All these findings indicate that ketanserin could represent a

new anti-glaucoma drug. There is therefore potential for further work to analyze the mechanisms of these effects of 5-HT agonists and antagonists on aqueous humour dynamics(**Costagliola et al, 1991).**

A great need exists for a model in which aqueous humour dynamics and their pharmacological manipulation can be studied. *In vivo* work involves the influence of the central nervous system and hormonal and vascular systems, which often complicate the interpretation of results. Additionally, there is increasing widespread concern about the use of animals in experimental science **(Fox, 1984).** The benefits of its size and ready availability led us to investigate the bovine perfused eye further. We find that this preparation lends itself to the direct correlation of drug effects on IOP, aqueous humour formation, the uveal vasculature and the patency of the blood–aqueous barrier (**Wilson, Shahidullah and Millar, 1993).** The preparation permits also experimental manipulation of perfusion media and rapid sampling of the ocular tissues for biochemical analysis.

This is a good method for examining the action of drugs on IOP and aqueous humour formation and drainage.

(1.14) AIM OF PROJECT

A general aim of this project was to develop and improve the *in vitro* bovine perfused eye; also to show whether it is useful for experimental work since this model avoids the sacrificing of animals for research purposes. And its large size makes it straightforward to set up experimentally compared with eyes taken from other species.

An ultimate aim of such work is the development of safer and more effective drugs for the treatment of glaucoma.

It was proposed to study the effects of drugs related to adenosine and to 5-HT on IOP in the bovine perfused eye and to try to elucidate the mechanisms by which these drugs may alter IOP. A further intention was to ascertain the effect of various ocular hypotensive drugs that have been investigated and analyzed by other workers either to confirm or oppose their proposals and to see how the use of different species, techniques and routes of administration might alter the effect of these drugs upon IOP.

Drugs effective by the intra-arterial route are not most likely to influence IOP via an alteration in aqueous formation in the ciliary body, whereas drugs altering IOP by the intracameral route are more likely to do so by an effect on outflow. In seeking to further understand the mechanism of action of these drugs, a biochemical technique was employed.

We used an enzyme-immuno assay technique for measuring the rate of cAMP formation in the ciliary processes and trabecular meshwork of the bovine eye, in an attempt to find a correlation between the effects of these drugs on IOP and on cAMP synthesis in these tissues.

Since it is generally believed that these drugs probably mediate their effects through changes in tissue cAMP synthesis, we may be able to identify the presence of the relevant receptors by measuring changes in tissue cAMP synthesis in response to these drugs. This may provide good evidence of the site of action of these drugs on IOP.

MATERIALS AND METHODS

(2.1) *IN VITRO* METHOD FOR STUDYING INTRAOCULAR PRESSURE

Bovine eyes obtained from the abattoir were kept at ambient temperature and transported to the lab. Eyes were not placed on ice since the resulting solidity of the fatty tissues may hamper dissection and cannulation (**Wilson, Shahidullah and Millar, 1993).**

Within one hour of slaughter, one of the long posterior ciliary arteries was cleared of connective tissues and cannulated proximal to the heavily pigmented area which appears in the anterior wall before it enters the sclera. Care was taken not to damage the blood vessels running over the anterior surface of the globe; for this reason a few mm of each extraocular muscle were left attached to the globe. As shown in figure 2.1, the eye was placed in a jacketed holder maintained at 37°C and covered with a plastic cup to keep it warm and to prevent drying. The long posterior ciliary artery was perfused with Krebs' solution containing :(mM) NaCl, 118; KCl, 4.7; glucose, 11.5; $NaHCO_3$, 25; $MgSO_4.7H_2O$, 1.2; KH_2PO_4, 1.2; Ascorbic acid, 0.05 and $CaCl_2$, 2.5. The pH of this solution was adjusted to 7.4 by bubbling with O_2 containing 5% CO_2.

The experiments in the present study were conducted using a peristaltic pump to induce flow through the vasculature, during which time intravascular pressure was monitored via a pressure transducer (Isotec) and pen recorder (Linseis). The flow rate was commenced at 0.2ml.min^{-1} and increased in 5-10 increments to 2.25ml.min^{-1}). The four vortex veins were cut in order to allow flow of Krebs and successful perfusion was signalled by the appearance of small amounts of blood from the ends of the vortex veins. It was necessary to cut away some of the extraocular muscles to encourage visible flow. During

this process (25-40min), perfusion pressure often fluctuated, usually due to poor alignment of the cannula with the artery, but was not allowed to exceed 100mmHg. After approximately 50 minutes, when aqueous humour secretion had started (which was indicated by a firm globe), the anterior chamber was cannulated with a 23G needle connected via a rubber silicon tube to another needle that was connected to a water manometer. Observations of IOP from the water manometer were made at 5 minutes intervals.

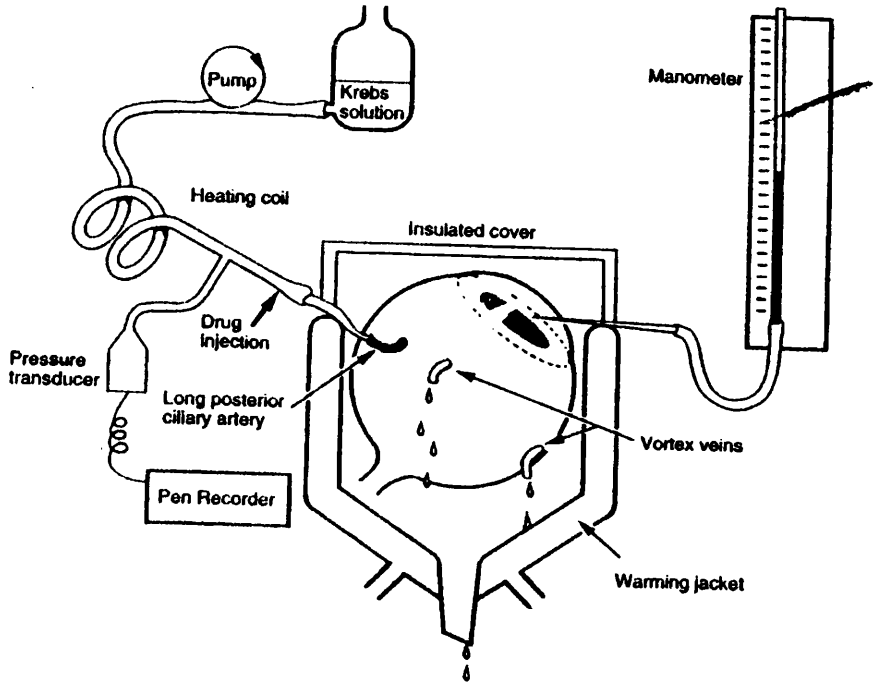


Figure 2.1 The bovine isolated eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under condition of constant flow rate. Perfusion pressure and IOP were both monitored as shown (Wilson and Shahidullah and Millar, 1993).

Once the eyes reached a stable IOP within the range of 95 to 165mm.H_2O (7-12mmHg) and a stable arterial pressure not exceeding 100mmHg (and usually much lower than this), a drug solution or vehicle control was injected as a bolus dose, proximal to the arterial cannula, and the effect upon both IOP and arterial perfusion pressure were measured and recorded as described by **Wilson et al, 1993.** Alternatively, drugs were administered by arterial perfusion (Art.per) or intracameral (I.C) injection. Art.per : the drug was added to the Krebs solution reservoir and after mixing, the drug reached the posterior ciliary artery after a delay of about 10 minutes as it moved through the tube between the reservoir and the eye. I.C injection:- 30-60 min after the stabilisation of IOP another 23G needle was inserted into the anterior chamber. This needle was already connected via a silicon rubber tube to a micro syringe, the whole assembly having been prefilled with the drug solution or vehicle control to be injected . When IOP again had stabilised, the drug or control solution in a volume of 2-10μL was injected into the anterior chamber and observations of IOP and perfusion pressure continued.

The data collected for a period of 90 minutes were analysed by calculating the regression lines for IOP versus time using a Minitab, and this yields values for the slopes of these lines. This way of expressing the change in IOP allows better statistical analysis than direct comparison of IOP before and after drug injection. Mean values and SEM of these slopes for control and test eyes were then compared by the Minitab statistical package and the statistical significance of differences between mean values were calculated using ANOVA and Student's t-test.

(2.2) *IN VITRO* STUDY OF DRUG EFFECTS ON CAMP FORMATION.

(2.2.1) Preparation of tissues

Bovine eye ciliary processes (CP) were exposed after dissecting the eye from the posterior pole, and removing the vitreous, lens and anterior lens capsule The remaining anterior segment was everted on a ball of plasticine (precooled to -70°c) and was then frozen by keeping at -70°C for 15-20 minutes. The frozen CP were then rapidly scraped from the eye by means of a sharp-edge spatula.

In order to harvest trabecular meshwork (TM) tissue, the eye was dissected as above then the ciliary body was gripped with forceps and torn free from the sclera.

This exposed the TM as a dense band of white tissue having a pigmented anterior edge, when it was stripped off by scraping with a small sharp spatula. Sometimes the TM was covered by a spongy layer of ciliary tissue which was easily removed to reveal the denser TM below. The identity of these tissues were confirmed histologically by Professor W Lee of the Department of Pathology, Western Infirmary , Glasgow.

(2.2.2) Assay procedure

Assay of adenylate cyclase activity was carried out by measuring the cAMP present in the incubation mixtures which resulted from the above incubation procedure.

The cAMP assay was done using an EIA Kit supplied by the Alexis Corporation (Nothingham, UK) and employing a 96-well plate.

This assay is based on the competition between free cAMP and a cAMP tracer (cAMP linked to an acetylcholinesterase molecule) for a limited number of cAMP-specific rabbit antiserum binding sites. The concentration of the cAMP tracer is held constant while the concentration of free cAMP (standard or sample) varies, thus, the amount of cAMP tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free cAMP in the well. This rabbit antiserum-cAMP (either free or tracer) complex binds to the mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to acetylcholinesterase) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of free cAMP present in the well during the incubation.

Definition of terms

Blank : background caused by Ellman's reagent.

Total Activity total enzymatic activity of the acetylcholinesterase linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-specific binding) : non-immunological binding of the tracer to the well. Even in the absence of a specific antiserum a very small amount of the tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding) : maximum amount of the tracer that the antiserum can bind in the absence of free cAMP.

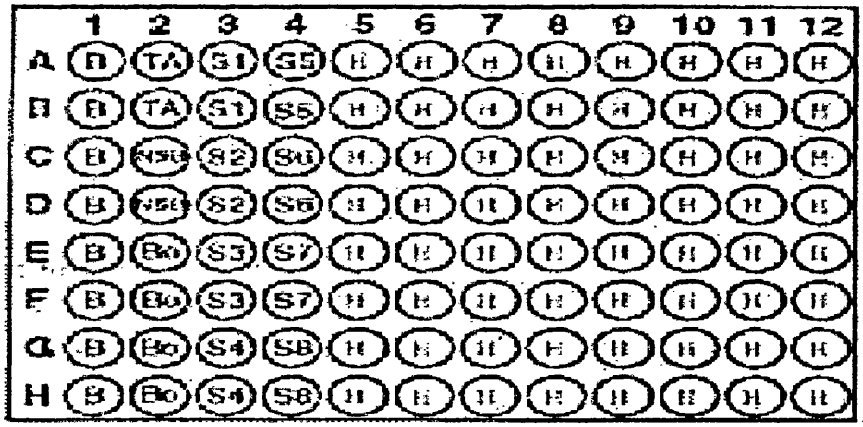
$\%B/B_0$ ($\%$bound/maximum bound): ratio of the absorbance of a sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the $\%B/B_0$ values versus concentrations of a series of wells containing various known amounts of cAMP.

Plate Set Up

Each sample was assayed in duplicate. At least two blanks, two maximum binding wells (B_0), and two non-specific binding wells (NSB) were included in the plate design.

Full Plate Format



B - Blank :

TA -Total Activity

NSB - Non - Specific Binding

B - Maximum Binding

S1 - S8 - Standards 1 - 8

H - Sample

Rinse the plates

The wells were rinsed and used once with wash buffer. All of the wash buffer was removed from each well before starting the assay. This was accomplished by inverting the plate and the last drops were shaken out.

50 μl from tube 0 was added to the NSB and B wells and 50μl of EIA buffer to the NSB wells

50 μl of tube 8 was added to both of the lowest standard wells (S8).

50 μl of tube 7 was added to each of the next two standards wells (S7).

This procedure was continued until all the standards were aliquoted.

The same pipette tip was used to aliquot all the standards.

50 μl of of tissue extract sample was now well was added. As was recommended, each sample was assayed in duplicate .

50 μl of cAMP Acetylcholinesterase Tracer (cAMP Tracer) was added to each well except TA and B wells.

50 μl of cAMP Antiserum was added to each well except TA, NSB and B wells.

The plate was covered with the a plastic cover and incubated for 18 hours at room temperature.

When the plate was ready to develop, one vial of Ellman's reagent was reconstituted with 20 ml of ultra pure water.. Reconstituted Ellman's reagent was unstable and was used the same day it was prepared. The Ellman's reagent was protected from light when not in use.

The wells were emptied and rinsed five times with wash buffer.

200 μl of Ellman's reagent was added to each well and 5 μl of the cAMP tracer to TA wells. The plate was covered with plastic film.

Optimum development was obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark. This assay typically developed in 60-90 minutes.

The plate was read at 412 nm using a plate reader.

Standard Curve Preparation

The cAMP standard was reconstituted with 1 ml of EIA buffer (This was labelled standard A). 10 μl of standard A (3000 pmol / ml) was aliquoted into 9.9 ml of phosphate buffer(This was labeled standard B). This standard was stored at 4°C , it will be stable for 4 weeks.

9 test tubes were obtained and numbered 0-8.

500 μl of phosphate buffer was added to tube 0 (this tube was to contain only buffer) and tube 2-8.

1 ml of standard B (3 pmol / ml) was added to tube 1.

500 μl of tube was aliquoted into tube 2 and mixed thoroughly.

500 μl of tube 2 (1.5 pmol / ml) was aliquoted into tube 3 and mixed thoroughly. This procedure was continued until reaching tube 8.

500 μl of solution in tube 8 was discarded so each tube contained 500μl.

A typical standard curve is shown in fig 2.2.1

Calculation of the results

The plate reader subtracted the absorbance readings of the blank wells from the absorbance reading of the rest of the plate. The results were then calculated manually as follows:

The absorbance readings from the NSB wells were averaged.

The absorbance readings from the B_0 wells were also averaged.

The NSB average was subtracted from the B_0 average. This was the corrected B_0 or corrected maximum binding.

The $\%B/B_0$ (% sample/ standard bound/ maximum bound) was now calculated for all the standard wells and for the test wells.

$\%B/B_0$ for standard S1-S8 was plotted against cAMP concentration (in pmol/ml) on semi log paper.

The concentration of each sample was calculated by identifying the $\%B/B_0$ on the standard curve and reading the corresponding value on the X-axis.

$\%B/B_0$ values greater than15% and less than 80% were regarded as acceptable since they generally fell on the standard curve.

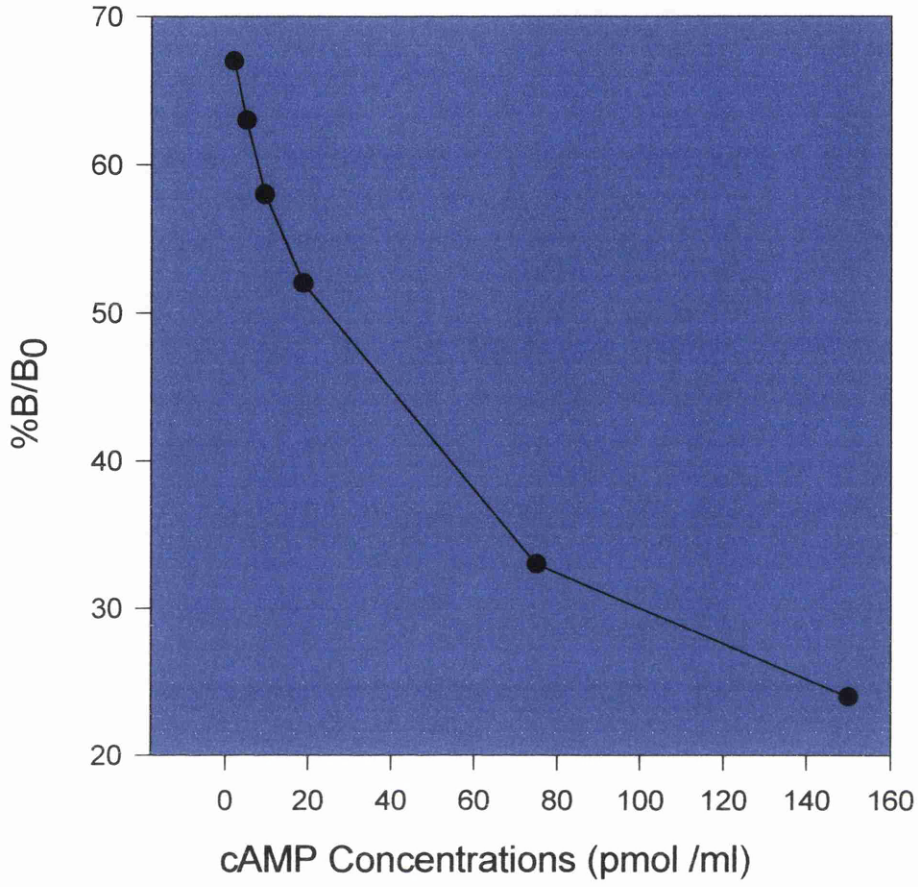


Figure 2.2.1 This standard curve shows $\%B/B_o$ for standards S1-S8 plotted against cAMP concentration (in pmol/ml).

During preliminary experiments great difficulty was experienced in achieving results for the cAMP concentration of incubation mixture which were proportional to the volume that was assayed. It appeared that some component of the tissue was interfering with the assay. This was confirmed by the following experiment:- About 20 μl of the extract (sample) was diluted with 480 μl of Kit phosphate buffer to make up the volume to 500 μl, so that each sample was diluted to (1 : 25). Then 100μl from each diluted sample was again diluted with 400 μl of the Kit phosphate buffer to give a final dilution of 1 ; 125.

Standard tubes numbered 0 to 8 were prepared using the non-acetylation procedure. The results obtained at the end of this experiment clearly showed that the absorbance values of the sample diluted 1 : 125 were in good gradation rather than the less diluted sample (1 : 25). For both dilutions, the proportionality between cAMP concentration and B/B_0 ratio was much closer to linear than had previously been found when undiluted homogenate was assayed.

This indicated that the more the sample was diluted , the less interference by tissue components could occur.

From this experiment we concluded that the acetylation procedure must be performed, which allowed assay of less than 10 pmol cAMP.

Before running the acetylation procedure, the samples were therefore diluted to 1 : 100 using EIA buffer, hence greatly reducing possible interference by components of the incubation mixture in the performance of the assay.

Acetylation procedure

All samples, as well as standard tubes 0-8, were acetylated so that each sample/standard acetylated individually. Care was taken to be consistent in acetylation technique as any difference in vortex time and/or delayed addition of KOH may result in irreproducible results.

100 µl of 4 M KOH was added and 25 µl Acetic Anhydride in quick succession to the first sample. vortexed for 15 seconds.

25 µl of 4 M KOH was added and then vortexed. This was repeated for all samples and standard tubes.

(2.2.3) Enzyme-immuno assay of tissue cAMP formation

The tissues from CP and TM were homogenized in a ground glass homogenizer using phosphate homogenization buffer (sodium phosphate 200 mM, maleic acid 2 mM, pH 7.4) using 750 µl at a tissue concentration of 240 mg wet weight ml $^{-1}$ for CP tissue and 500 µl at a tissue concentration of 160 mg/ ml $^{-1}$ for TM. After centrifugation (at 300 xg for 2 min) to remove large pieces of unhomogenized tissue the homogenate was either used immediately or stored at -25°C. In order to generate adenylate cyclase activity, the conditions for incubation were as described by **Tobin and Osborne (1993)**.

Forskolin was included to produce a high level of enzyme activity, in order that inhibition by 5-HT or other drugs might be observed.

10 µl of tissue homogenate was added to 85 µl of phosphate incubation buffer (sodium phosphate 200 mM at pH 7.4, EGTA,0.2 mM plus 10 µl GTP

(2.1mM), 10μl forskolin (105μM) and 50μl of phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX, 21mM) was applied and theadenylate cyclase activity initiated by adding 25 μl ATP (4.2 mM)

The final concentration of the reagents during incubation was ATP, 500 μM, IBMX, 1 mM, GTP, 100 μM and forskolin, 5 μM.

A number of experiments has been carried out to show forskolin stimulation and to prove time-dependence.

(A) <u>Forskolin stimulation</u>.

6 test tubes were incubated with different forskolin concentrations (0, 0.5, 1.5, 5.0, 15, 50 μM) at a constant time of incubation (5 min interval for each incubation) to observe only the effect of different concentrations of forskolin on cAMP production. A typical curve showing forskolin dependence appears as fig 2.2.2

This experiment proved that forskolin-stimulated cAMP production in a dose-dependent manner.

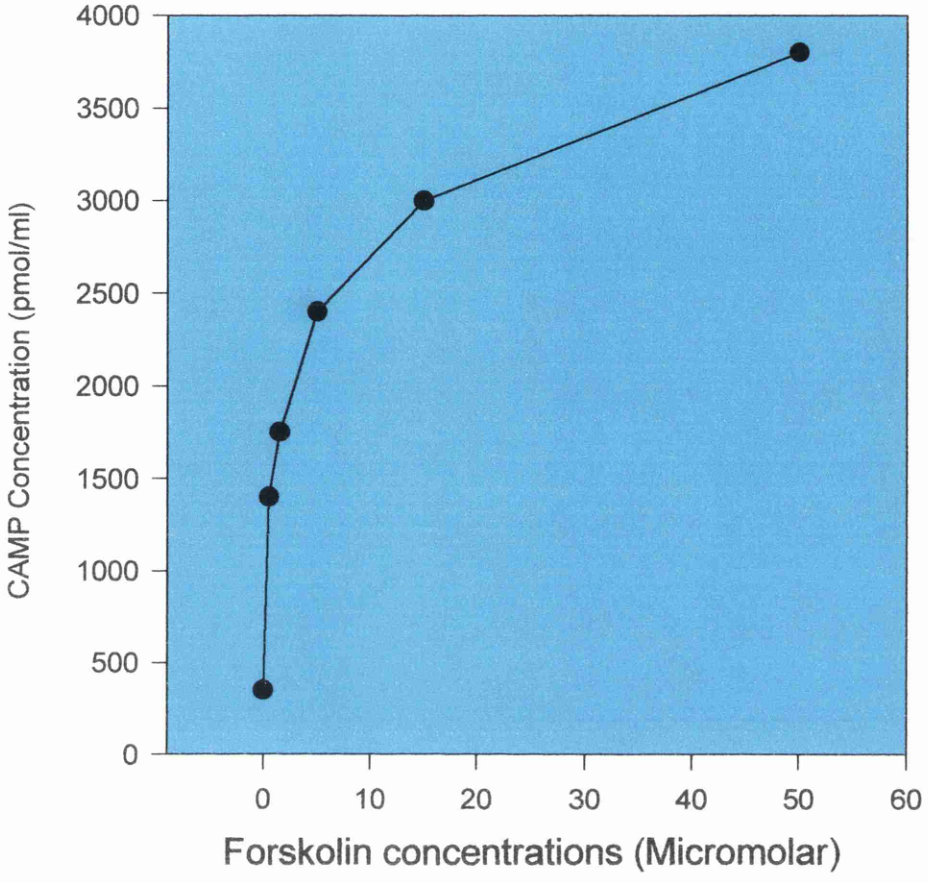


Figure 2.2.2 This curve shows a forskolin-dependent increase in cAMP synthesis at a constant time of incubation (5 min).

(B) Time dependence

6 test tubes were incubated with a constant concentration of forskolin (5 μM) to facilitate observing the effects of time dependence only. The tubes were incubated at different time intervals. This experiment proved that the level of cAMP increases with the time of incubation.

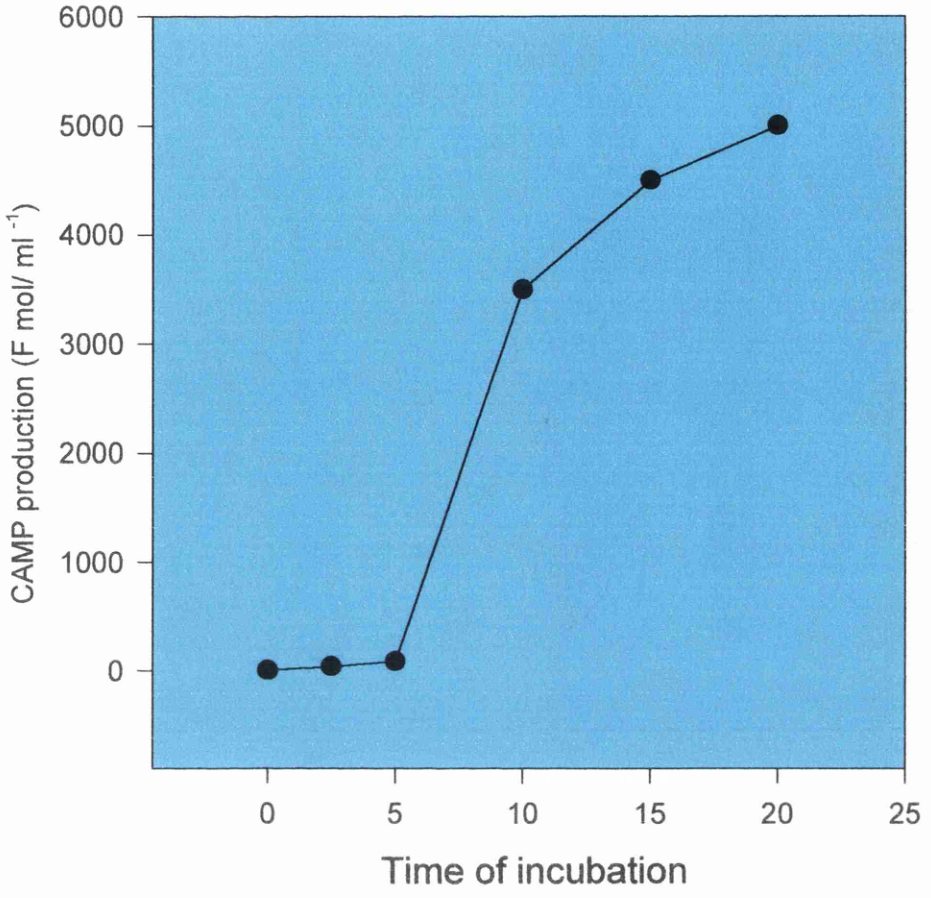


Figure 2.2.3 This curve shows a consistent increase in cAMP synthesis with the increase in the time of incubation when a constant concentration of forskolin was used (5μM). This curve is typical of four experiments demonstrating time dependence.

In order to determine their effects on the forskolin-stimulated synthesis of cAMP, 5-HT and 8-OH-DPAT were tested in three different final concentrations (500,50,5 μM) and incubated with both CP and TM.

Adenosine was incubated with CP only, because previously it was tested for its effect on IOP only by the intra-arterial route.

Control incubations from CP and TM were prepared and compared with these containing 5-HT and 8-OH-DPAT

The assay tubes were incubated in a gently shaking water bath at 37°C for 5 min. The reaction was stopped by adding 200 μl of 10% trichloroacetic acid (TCA).

The tubes then were centrifuged at 10,000 xg for 10 min, to encourage the protein to precipitate The supernatant was taken from each test tube (about 330 μl). TCA was removed by extracting 3 to 4 times with 3 volumes ether using the vortex mixer. The tubes were put in a 70°C water bath for 10 min to evaporate the remaining ether then stored at -25°C until assay for cAMP.

(2.3) DRUGS AND CHEMICALS

The following drugs were used in this study:

Adenosine, N^6-Cyclohexyladenosine (CHA)-(adenosine A_1 receptor agonist), N^6-(2-(3,5-Dimethoxyphenyl)-2(2-methylphenyl)-ethyl adenosine (DPMA)-(adenosine A2 receptor agonist), ATP, 5-Hydroxytryptamine (5-HT), 8-OH-DPAT(5-HT $_{A1}$ receptor agonist), 5-Carboxamidotryptamine (.5-CT)

Water was used as a solvent and as control during this research.

Composition of Krebs-Henseleit solution

(milliMoles per litre)

NaCl, 118

KCl, 4.7

Glucose,11.5

$NaHCO_3$, 25

$MgSO_4.7H_2O$, 1.2

$KH2PO_4$, 1.2

Ascorbic acid, 0.05

$CaCL_2$.

The pH of this solution was adjusted to 7.4 by bubbling with O_2 containing 5% CO_2.

(2.4) EIA CHEMICALS

The materials supplied in the EIA kit were reconstituted as follows Water : water used to prepare all reagents and buffers was of trace organic contaminants (ultra pure) produced by millipore.

(1) EIA buffer : the contents of this buffer is diluted with 90 ml of ultra pure water

(2) Wash buffer : diluted to total volume of 2 liters with ultra pure water, then 1 ml of Tween 20

(3) Phosphate buffer : diluted to 50 mM by adding ultrapure water to a final volume of 200 ml

(D) : Reagents :

(1) : Ellman's reagent was reconstituted with 20 ml of ultra pure water

(2) : KOH diluted to 4 M by diluting the solution with 7.5 ml ultra pure water

(3) : Acetic Anhydride .

(E) : Others:

(1) : cyclic AMP Tracer (cyclic AMP linked covalently to acetylcholinesterase)

The tracer was reconstituted with 6 ml of EIA buffer.

(2): cyclic AMP Antiserum

The antiserum was reconstituted with 6 ml of EIA buffer

(3) RESULTS

(3.1) CONTROL VALUES

The pre-injection IOP for all eyes tested by the arterial route (either arterial injection or perfusion) (n =127) was: 104.3 ± 9.0mmH_2O(mean ±SEM).

The pre-injection IOP value for all eyes tested by the I.C. route (n=98) was: 112.4± 4.9mmH_2O.

The pre-injection value for arterial perfusion pressure for all routes (n = 225) was 32.1± 7.0mmHg.

The mean IOP at the end of the experiments in which vehicle only was injected was not significantly different from the pre-injection value, whether the injection was intra-arterial or intra-cameral (see table 3.2, first line and table 3.4.2, first line). The mean perfusion pressure at the beginning of the control experiments was 25.7 ± 2.1 mmHg and at the end of the control experiments was 26 ± 4.3mmHg (n=15). There was no statistically significant difference between starting and finishing values. For statistical method see page 41

(3.2) ADENOSINE

In this procedure, single bolus doses of adenosine (Ad), CHA (an adenosine A_1 agonist) and DPMA (an adenosine A_2 agonist) were given in volumes of 1,3 or 10μl (ranging from 3–3000nmol). It was observed that adenosine and the other two agonists produce small increases in perfusion pressure only seconds after drug administration. This vasoconstrictor effect lasted for approximately 10 sec. The peak increase in perfusion pressure was between 5-10mmH_2O for the maximum dose 3000nmol. The effect of these drugs on IOP was not significant ($P>0.05$) when compared with control experiments in which water was injected in similar volumes intra-arterially **(see table 3.2)**.

Table 3.2 Effects on IOP of adenosine (Ad), CHA (selective adenosine A_1 receptor agonist) and DPMA (selective adenosine A_2 receptor agonist) given as bolus injections into the posterior ciliary artery.

Treat-ment	Dose(nmol)	n	Mean IOP(mmH_20)±SEM		Mean slope±SEM
			Before	After	
water		9	135.0±6.6	136±6.6	0.036±0.031
Ad	100	4	113±15.8	114±15.2	0.013±0.011
Ad	1000	6	138±5.9	137.8±5.9	-0.002±0.060
Ad	3000	12	121.7±6.1	124.3±6.5	0.041±0.025
CHA	10	9	123.3±8.0	120.5±8.2	-0.041±0.021
	30	10	138.1±6.0	135.3±6.0	-0.045±0.010
	300	9	122.1±6.4	120.2±6.5	-0.0296±0.0302
	1000	8	125.3±8.4	123.8±8.6	-0.026±0.011
DPMA	3	2	130±26.5	129±26.0	-0.005±0.005
	150	5	124.6±9.2	125.2±9.1	0.009±0.010
	500	6	106.3±8.6	106.5±8.6	0.002±0.002

The effects of bolus doses of adenosine, CHA and DPMA on IOP in the perfused eye, shown as the mean IOP values (in mmH_2O) before and 60 min after drug injection and as the mean slope of the regression lines on IOP vs time (60 min). Each value is a mean ± SEM of the number of experiments shown (n). None of the slopes differed significantly from the water control value (*$P>0.05$).

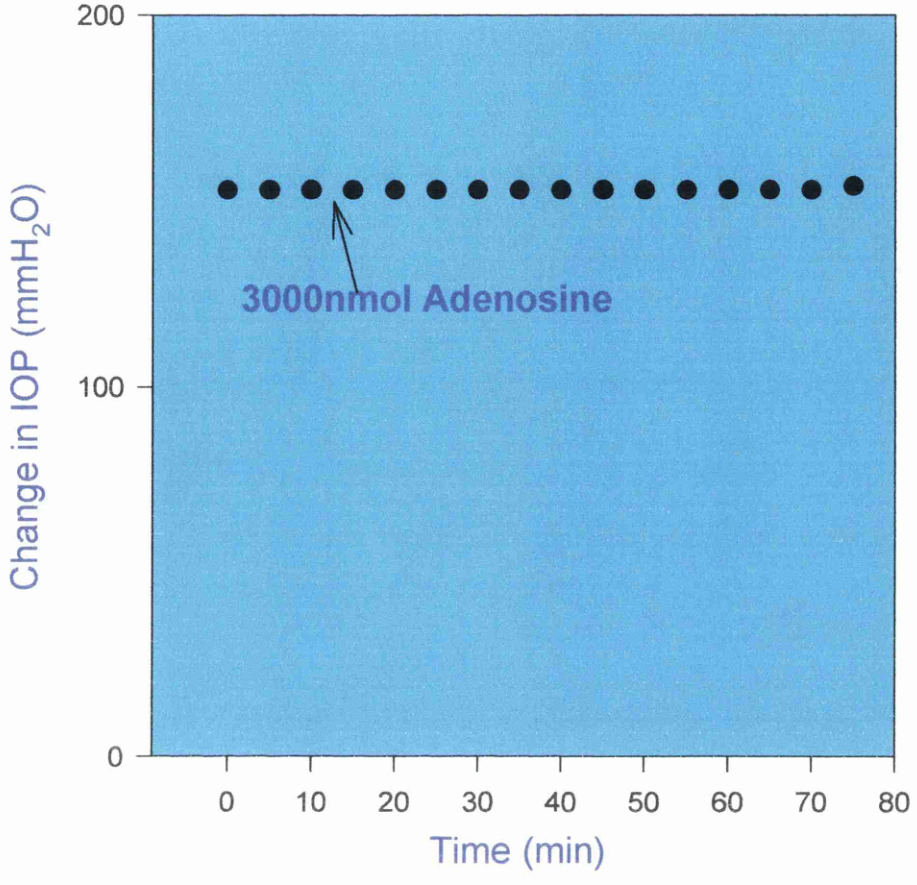


Figure 3.2 Effects of single dose of adenosine for one experiment via an arterial injection on the intraocular pressure of the bovine eye. The IOP values were stable prior to drug injection. Change in IOP was observed 15 min after injection. The change in IOP was insignificantly different from vehicle-control experiments. $P>0.05$, $n=6$.

The calculated slope following drug injection in this experiment was not significantly different from that in control experiments.

(3.3) ATP

Data presented in table 3.2 show that bolus arterial injection of exogenous ATP (300,1000, and 3000nmol) tested on the isolated bovine eye caused a significant effect on IOP in a dose-dependent manner during the 90 min after drug administration. The results also demonstrated that ATP produces an increase in perfusion pressure immediately after injection. This effect appeared within 5 to 10 sec of injection but only persisted for approximately 40 sec. The peak increase in perfusion pressure was between 60 and 80mmHg for the maximum dose of 3000nmol. The effect of this drug on IOP was significant ($P<0.05$) when the slope of IOP against time was compared with control experiments using the same route of administration **(see table 3 3)** although the magnitude of the decrease was relatively small.

Table 3.3 Effects on IOP of exogenous ATP given as bolus injection into the posterior ciliary artery.

Treat-ment	Dose (nmol)	n	Mean IOP(mmH_2O)±SEM		Mean Slope±SEM
			Before	After	
Water Control		9	135.0±6.6	136.4±6.6	0.036±0.031
ATP	300	8	114.6±3.4	112.6±3.9	-0.0178±0.1271
	1000	7	109.7±4.1	105.3±3.7	-0.0483±0.0141*
	3000	6	111.3±5.1	106.1±4.9	-0.0591±0.0152*

The effects of bolus doses of ATP in the isolated perfused eye, shown as the mean IOP values (in mmH_2O) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean±SEM of the number of experiments shown (n). Significance of difference from the water control values: *$P < 0.05$.

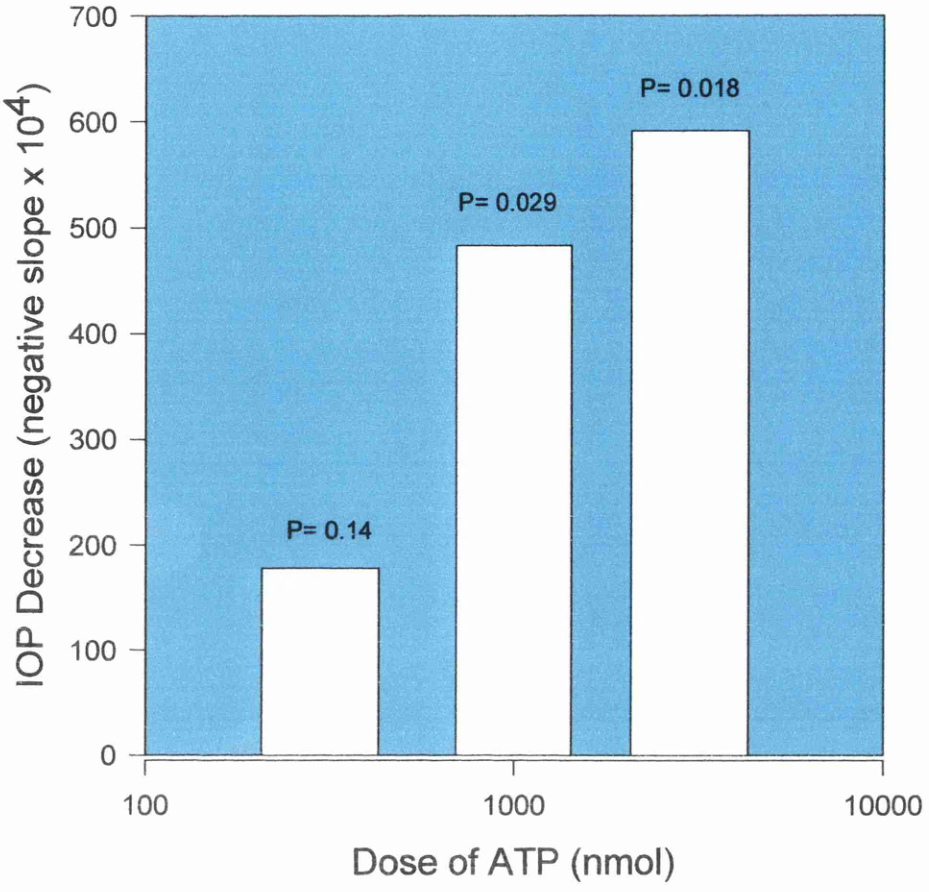


Figure 3.3 This histogram shows a graded dose-response relationship for the decrease in IOP when ATP was injected into the bovine posterior ciliary artery. Increases in IOP are expressed as the mean slope of the regression lines

on IOP vs time (90 min) Each value is a mean ± SEM of the number of experiments shown (n). Probability (P) refers to the difference in slope between each dose of the drug and the slope of the water control. For statistical method, see page 41.

(3.4) 5-HT

3.4.1 Intra-arterial injection and perfusion

Intra-arterial injection of 10nmol 5-HT into the posterior ciliary artery produced no effect on perfusion pressure

We also found that 5-HT did not have any significant effect on IOP (80 min after drug administration). For this reason we tested the effect of 5-HT by arterial perfusion may, accumulate to a higher concentration as the drug will stay longer in the ciliary body and might produce a significant effect on IOP. Different concentrations of 5-HT were added to the Krebs' solution (10^{-6}, $2x10^{-6}$ M). We found that 5-HT again produced no effect on perfusion pressure. When IOP values were recorded before and 90 min after drug administration, it was found that 5-HT does not produce a significant effect on IOP when compared with water control IOP values **(see table 3.4.1).**

Table 3.4.1 Effects on IOP of 5-HT , using arterial routes of administration.

Route	Treat-ment	Dose(nmol) or conc(Molar)	n	Mean IOP±SEM		Mean Slope±SEM
				Before	After	
IA.Inj	water		9	135±6.6	136±6.6	0.036±0.031
IA.Inj	5-HT	10nmol	3	115.7±7.	117.7±9.0	0.0164±.0.050
Art.Per	5-HT	10^{-6} M	5	104.2±2.0	104.0±2.0	-0.0019±0.024
Art.Per	5-HT	$2x10^{-6}$M	6	100.7±2.8	99.3 ±1.8	-0.0175± 0.012

The effect of the drug (administered by the routes indicated) on IOP in the perfused eye are shown as mean IOP values recorded (in mmH_20) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). Routes of administration: arterial injection (I A.inj); arterial perfusion (Art.per). None of the slopes differed significantly from the water control value.

3.4.2 Intracameral route

The above experiments with 5-HT using arterial routes, indicated that this drug has no effect in modulating bovine IOP. In contrast, a statistically significant decrease in IOP was observed when this drug was administered by the intracameral route.

A single bolus injection of 5-HT was given in volumes of 1, 3 or 10μl, ranging from 3-100nmol and tested on perfusion pressure and IOP of the bovine isolated perfused eye.

This drug produced small transient increases in arterial perfusion pressure in a dose- dependent manner.

The drug effects were compared with data from control perfusions in which water was injected in the same manner **(see table 3.4.2).**

A very small decrease in IOP occurred when bolus injection of 3nmol 5-HT was given but this was statistically insignificant ($P>0.05$). The doses of 10 and 30nmol 5-HT produced dose- dependent decreases of IOP ($P<0.05$). When bolus injection of 100nmol was administered, it was found that this dose appeared to lower IOP, though the effect was statistically insignificant ($P>0.05$).

Table 3.4.2 Effects on IOP of 5-HT given as bolus injection into the anterior chamber.

Treat-ment	Dose (nmol)	n	Mean IOP±SEM		Mean Slope±SEM
			Before	After	
Water Control		6	107.8±2.4	107.3±2.4	-0.0030±0.006
5-HT	3	9	113.1±6.2	111.0±5.9	-0.0258±0.011
	10	8	109.8±5.6	105.9±5.7	-0.0387±0.008*
	30	9	110.0±6.7	105.2±6.8	-0.0538±0.007*
	100	8	108.5±3.0	105.3±3.2	-0.0321±0.018

The effects of bolus doses of 5-HT in the bovine isolated eye, shown as the mean IOP values (mmH_2O) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean ±SEM of the number of experiments shown (n). Significance of difference from control: *$P<0.05$.

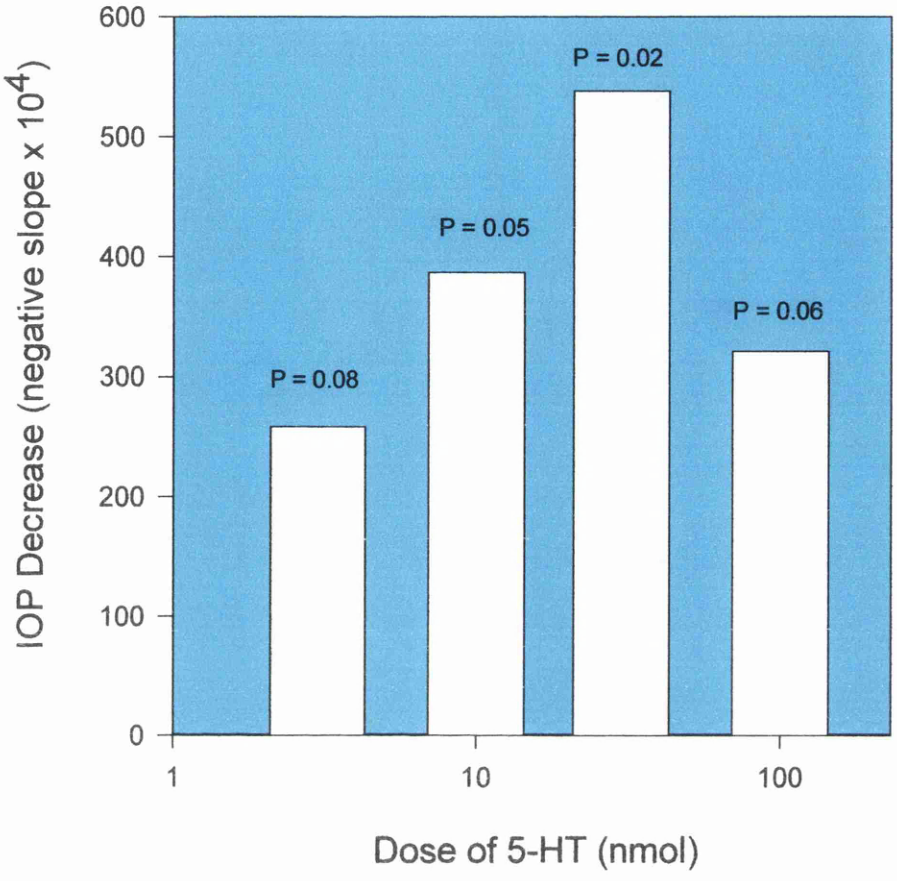


Figure 3.4 Histogram representation of 5-HT dose-dependence

This histogram shows the dose-dependent relationship for the decrease in IOP when 5-HT which was administered intra-camerally to the perfused bovine eye. It was clear from this histogram that these responses were dose-dependent up to the 30nmol level. But that the 100nmol dose was less effective.

(3.5) 8-OH-DPAT

3.5.1 Intra-arterial injection

The present work tested the effects of 8-OH-DPAT on the bovine IOP using different molar concentrations (10^{-6}, $2x10^{-6}$, $3x10^{-6}$ M) by arterial perfusion and it was found that the drug produces no effect on arterial perfusion pressure nor on IOP when compared with water control experiments using the same route of administration. This was true whether the comparison was done on IOP values before and after 90 min perfusion, or on the slopes of IOP against time.

Table 3.5.1 Effects on IOP of 8-OH-DPAT using arterial perfusion route of administration.

Treatment	conc(M)	n	Mean IOP±SEM(mmH_20)		Mean Slope±SEM
			Before	After	
Water Control		9	135.0±6.6	136±6.6	0.036±0.031
8-OH-DPAT	10^{-6}	4	103.5±2.3	100.3±3.2	-0.0357 ± 0.031
8-OH-DPAT	$2x10^{-6}$	2	106.0±6.0	106.5±6.5	0.0053 ± 0.010
8-OH-DPAT	$3x10^{-6}$	4	104.0±3.7	104.0±3.7	0.00023 ±0.331

The effect of the drug (administered by the route indicated) on IOP in the perfused eye are shown as mean IOP values recorded (in mmH_20) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). None of the slopes differed significantly from the water control values: *P>0.05.

3.5.2 Intracameral injection

Single bolus doses of 8-OH-DPAT given in volumes of 1, 3 or 10µl (ranging from 3-100nmol) using intracameral injection were tested on perfusion pressure and IOP and it was found that 8-OH-DPAT produces no effect on perfusion pressure, but produces a significant decrease in IOP when values were taken before and 80 min after drug administration are compared with water control IOP values.**(see table 3.5.2 and fig 3.5.)**

Table 3.5.2 Effects on IOP of 8-OH-DPAT using I.C route of administration.

Drug	Dose (nmol)	n	Mean IOP(mmH_2O)±SEM		Mean Slope±SEM
			Before	After	
Water		6	107.8±2.4	107.3±2.4	-0.0030±0.006
8-OH-DPAT	3nmol	8	118.4±5.4	112.0±5.4	-0.0907±0.014*
8-OH-DPAT	10nmol	10	105.2±4.4	100.0±4.0	-0.0670±0.023*
8-OH-DPAT	30 nmol	7	126.8±6.4	117.2±5.2	-0.1193±0.022***
8-OH-DPAT	100nmol	7	118.1±8.0	109.1±8.0	-0.1151±0.020**

The effect of 8-OH-DPAT (administered by the I C route) on IOP in the perfused eye are shown as the mean IOP values recorded (in mmH_20) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference from control : *P< 0.05, **P< 0.01, ***P< 0.001.

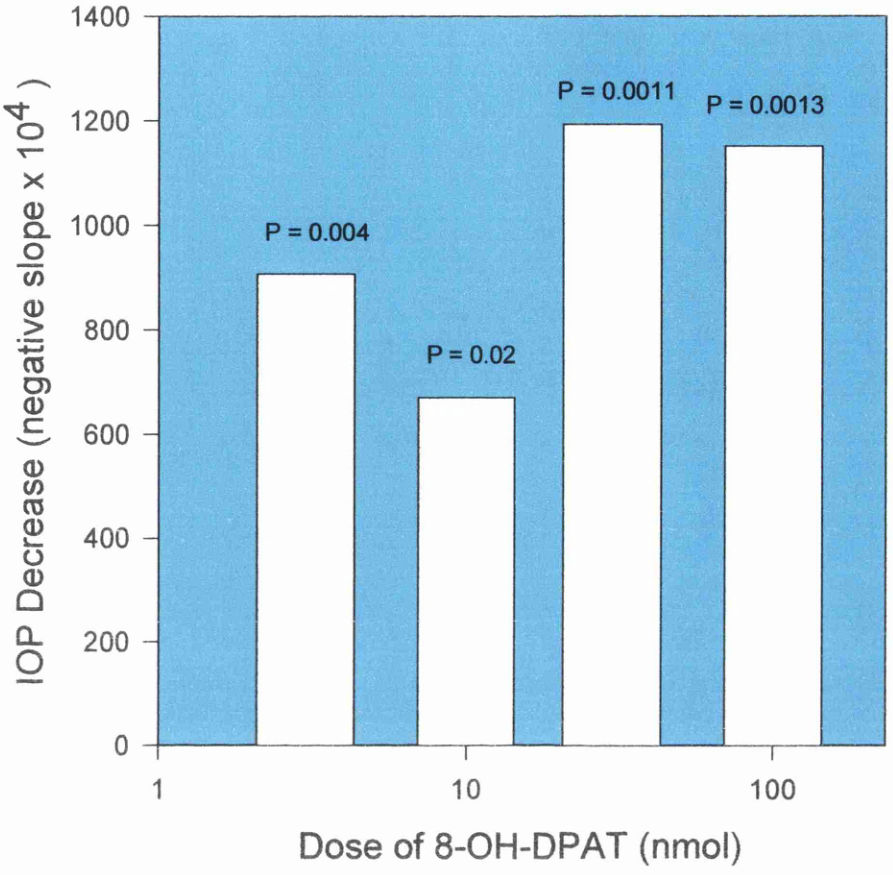


Figure 3.5. Histogram representation of 8-OH-DPAT dose-dependence

While all doses tested showed that 8-OH DPAT causes a decrease in IOP, there was little evidence of dose-dependence.

(3.6) 5-CT

In an in vitro model, the present study clarifies the role of 5-CT in the modulation of ocular function in the bovine eye.

Single bolus injections of 5-CT intracamerally were given in volumes of 3,6 and 20μL (20,60 and 200nmol) and tested on perfusion pressure and IOP of the bovine eye. Effect of 5-CT was observed on perfusion pressure. On the other hand 5-CT causes a statistically significant decrease in IOP in a dose-dependent manner compared with data from control experiments using water injected I.C. (**see Table 3.6).**

Table 3.6 Effects on IOP of exogenous 5-CT given as bolus injection into the anterior chamber.

Drug	Dose(nmol)	n	Mean IOP(mmH_2O)±SEM		Mean Slope±SEM
			Before	After	
Water		6	107.8±2.4	107.3±2.5	-0.0030±0.006
5-CT	20	9	108.3±4.7	104.8±4.5	-0.0407±0.008*
	60	9	113.8±3.5	109.8±4.2	-0.0556±0.021*
	200	8	117.8±7.0	105.1±6.9	-0.1405±0.017***

The effects of bolus doses of 5-CT in the isolated perfused eye, shown as the mean IOP values (in mmH_2O) before and 90 min after drug injection and as the mean slope of the regression lines on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference from the water control values: *$P< 0.05$; ***$P< 0.001$.

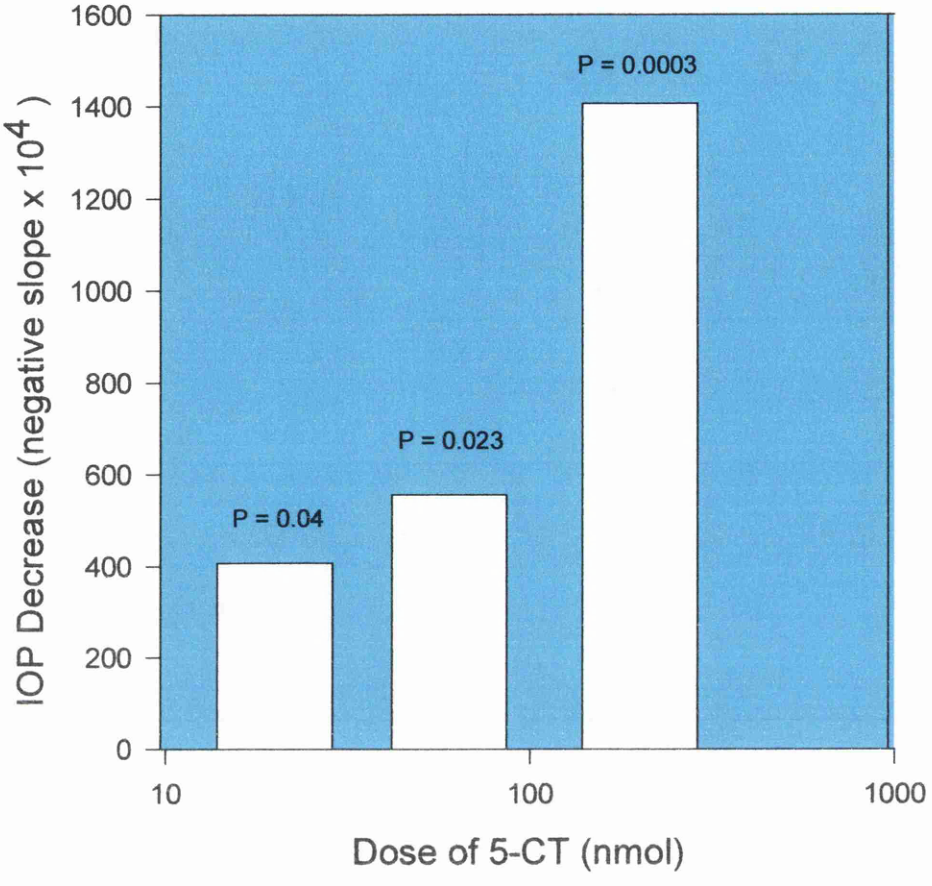


Figure 3.6 Histogram representation of 5-CT dose-dependence

This histogram showed a dose-dependent decrease of IOP when 5-CT was given I.C.

(3.7) Cyclic AMP

Forskolin is well established in its ability to stimulate cAMP synthesis without interacting with cell surface receptors. In the present work, this agent was added to stimulate cAMP production.

Preliminary experiments showed that forskolin increases cAMP production in a concentration-dependent and time-dependent manner **(see figs 2.2.2 and 2.2.3).**

For an incubation period of 5 min, maximum stimulation of 3.8 pmol cAMP / mg tissue is achieved by 50 μM forskolin. In subsequent experiments, a constant concentration of 5 μM forskolin was used, where small changes in forskolin-stimulated activity will manifest the greatest difference in cAMP production.

With a forskolin concentration of 5 μM to stimulate adenylate cyclase there is a linear relationship between incubation time and cAMP production up to 15 min. The production rate is greater than 3 pmol/ mg tissue/ 5min.

The level of cAMP synthesis increases over 15 min incubation period, but at a much slower rate. As the relationship is linear, the time of 5 min forskolin stimulation in each incubation was chosen, because it produces cAMP amounts which were within the range and accurately determined by the enzyme-immuno assay. Control levels of forskolin-stimulated cAMP production in TM (around 20 pmol/mg prot/5 min) were considerably higher than in CP (approximately 5 pmol/mg prot/5 min).

Adenosine (500, 50 μM) failed to produce change in cAMP of the CP homogenates, as shown in **table 3.7.1.**

The effects of multiple concentrations of 5-HT or 8-OH-DPAT (500, 50, 5 μM) were studied on forskolin-stimulated cAMP production in the bovine CP and TM homogenates. Both 5-HT and 8-OH-DPAT caused a non-significant increase in cAMP level compared to the control values, as shown in **tables 3.7.2 and 3.7.3.**

Table 3.7.1 Effects of Adenosine on forskolin (5μM) stimulated cAMP production in homogenates of ciliary processes of the bovine eye. The incubations were each conducted twice (homogenates A and B). Each incubation mixture was assayed in duplicate or triplicate.

Treatment	Concentration (μM)	Tissue	cAMP production pmol /mg protein
Control		CP(A)	3.3
Ad	500	CP(A)	6.6
	50	CP(A)	2.15
Control		CP (B)	6.72
Ad	500	CP (B)	8.13
	50	CP (B)	10.17

Table 3.7.2 Effects of 5-HT on forskolin (5μM) stimulated cAMP production in homogenates of trabecular meshwork (TM) and ciliary processes(CP) of the bovine eye. The incubations were each conducted twice (homogenates A and B). Each incubation mixture was assayed in duplicate or triplicate.

Treatment	5-HT Conc (μM)	Tissue	cAMP production pmol /mg protein
Control		TM (A)	18.3
5-HT	500	TM(A)	22.5
	50	TM(A)	308
	5	TM(A)	25
Control		TM (B)	13.7
5-HT	500	TM (B)	26.3
	50	TM (B)	23.2
	5	TM (B)	38
Control		CP (A)	3.3
5-HT	500	CP (A)	5.7
	50	CP (A)	4.6
	5	CP (A)	5.88
Control		CP (B)	6.72
5-HT	500	CP (B)	5.2
	50	CP (B)	6.72
	5	CP (B)	8.21

Table 3.7.3 Effects of 8-OH-DPAT on forskolin (5μM) stimulated cAMP production in homogenates trabecular meshwork and ciliary processes of the bovine eye. The incubations were each conducted twice (homogenates A and B). Each incubation mixture was assayed in duplicate or triplicate

Treatment	Concentration (μM)	Tissue	cAMP production pmol /mg protein
Control		TM (A)	18.3
8-0H-DPAT	500	TM (A)	16.7
	50	TM (A)	14.8
	5	TM (A)	11.5
Control		TM (B)	13.7
8-0H-DPAT	500	TM (B)	20
	50	TM (B)	31.5
	5	TM (B)	26.3
Control		CP (A)	3.3
8-0H-DPAT	500	CP (A)	5.0
	50	CP (A)	4.6
	5	CP (A)	2.15
Control		CP(B)	6.72
8-OH-DPAT	500	CP (B)	8.21
	50	CP(B)	8.21
	5	CP (B)	0.04

(4) DISCUSSION

(4.1) INTRODUCTION

The bovine perfused eye offers a useful method for studying the mechanisms of action of drugs on IOP and aqueous humour formation without the necessity to kill animals for experimental purposes. In addition to that it is readily available and cheap. Although **Kishida et al, (1985)** expressed doubts about its potential usefulness, this model has allowed considerable success in demonstrating drug effects on IOP and aqueous humour dynamics *in vitro* **(Wilson et al, 1995).**

Control values for IOP and perfusion pressure in the present work are similar to previously published data from this preparation **(Wilson et al, 1993).**

(4.2) ADENOSINE

This is the first study to test the effect of adenosine and its receptor agonists (CHA and DPMA) on the IOP of the bovine eye.

Adenosine and its receptor agonists have been shown to modulate a variety of physiological functions, however little is known about the role of these receptors in the modulation of ocular function .

The present study revealed that neither adenosine nor CHA nor DPMA was effective in altering IOP when given by the intra-arterial route. They produced small transient increases in the perfusion pressure. This was probably because of a vasoconstrictor effect on the uveal vasculature. The lack of the effect on IOP in contrast with the previous study by **Crosson and Gray (1994)**, who investigated and evaluated the potential role of adenosine and some of its analogues in modulating IOP in rabbit. The compounds tested were: the A_1 agonist, N^6-cyclopentyl adenosine (CPA), the non- selective adenosine agonist,

5'-N-ethyl carboxamide adenosine (NECA) and the A_2 agonist, 8-phenyl amino adenosine (CV-1808). They observed that topical administration of NECA produced a dose- related reduction in IOP. However, an initial ocular hypertension of 1 to 2 hours was also observed in rabbits treated with NECA. The administration of the A_2 agonist produced only initial ocular hypertension. These results indicate that selected adenosine agonists can modulate IOP at least in the case of the rabbit. Adenosine A_1 receptors have been shown to be negatively coupled to adenylate cyclase in several systems and CPA was found to suppress cAMP formation in the isolated iris and ciliary body

The ocular hypotension induced by these agonists is consistent with the activation of adenosine A_1 receptors and may involve the reduction of cAMP levels in the iris/ciliary body, **(Crosson and Gray, 1994)**.

Tian, Gabelt, Crosson and Kaufman (1997) investigated the effects of single or multiple topical doses of the relatively selective A_1 receptor agonists (R) - phenyl isopropyl adenosine (R-PIA) and (CHA) on IOP, aqueous humour flow and outflow facility in the ocular normotensive cynomolgus monkeys. IOP, aqueous flow and outflow facility were determined under specific experimental conditions. They found that a single unilateral topical application of R-PIA or CHA produced ocular hypertension (maximum rise 4.9 or 3.5 mmHg) within 30 min, followed by ocular hypotension (maximum fall = 2.1 or 3.6 mmHg) from 2-6 hr Neither R-PIA nor CHA clearly altered aqueous flow. Total outflow facility was increased by 71% after R-PIA topical application.

In conclusion, the early ocular hypertension produced by topical adenosine agonists in cynomolgus monkeys is associated with the activation of adenosine

A_2 receptors, while the subsequent hypotension appears to be mediated by adenosine A_1 receptors and resulted primarily from increased outflow facility **(Tian, Gabelt, Crosson and Kaufman,1997).**

The difference in results between the present work and those reported by these other workers probably because of using different species (bovine in contrast with rabbit and monkey) and perhaps also of the influence of different routes of administration on the effect of the drugs on ocular function. The use of different routes would lead to different absorption in the eye and therefore different access to sites of action, which may affect the drug effects on IOP. This would be particularly true if a drug affected IOP by an action on the drainage meshwork. This tissue is readily accessible to topically administered drugs, but, since it is avascular, it is relatively inaccessible to drugs administered by the intra-arterial route.

(4.3) ATP

The increase in perfusion pressure produced by exogenous ATP was probably due to its strong constriction of the ciliary blood vessels. This action was very short and lasted only for 1-2 minutes (because of its rapid destruction by the ATPase enzyme). For this reason it is unlikely to have any lasting effect on IOP. Small doses of ATP produced an insignificant lowering effect on IOP and a significant effect was observed only when a large dose of ATP was used.

It has been shown that ATP and UTP cause an increase in intracellular Ca^{2+} in cultured bovine ciliary epithelium (**Wilson and Shahidullah, 1995**). Increase in cytosolic Ca^{2+} has also been reported for noradrenaline and carbachol in rabbit ciliary epithelium (**Yoshimura et al, 1995**) and histamine, thrombin and

neuropeptides in human non-pigmented ciliary epithelium (**Crook and Polansky, 1992**), but no physiological significance of these observations has been put forward. There may be great significance in the recent observations (Wilson and Shahidullah, personal communication): (i) that terbutaline (which suppresses aqueous humour formation in the bovine eye) decreases the peak value of the Ca^{2+} transient and elevates and prolongs the plateau phase of the Ca^{2+} response to ATP and (ii) that sodium azide or atriopeptin (both of which have been found to suppress aqueous humour formation) also inhibit this Ca^{2+} response to ATP, although in a subtly different manner from terbutaline. Secretion in the ciliary epithelium may be regulated by the combined effect/interaction of multiple receptors (**Wax, 1992**). Thus it can be postulated that cAMP, cGMP and Ca^{2+} may all play a part in modulating aqueous humour formation.

The major finding of the experiments in the present work is that exogenous ATP by arterial injection has lowered IOP in the bovine eye . Among the possible mechanisms by which ATP has lowered the IOP, is an effect on aqueous formation in the ciliary epithelium which may be connected with its effects on intracellular Ca^{2+} described above).

The mechanism of this effect on ATP seems to be that it is acting on $P2Y_2$ receptors in the ciliary epithelium (**Shahidullah and Wilson, 1997**), thereby releasing Ca^{2+} from intracellular stores. Much work remains to be done to show links between intracellular Ca^{2+} and the ion pumps and channels which are responsible for aqueous humour formation. Further study of this action may help towards discovering these links.

(4.4) 5-HYDROXYTRYPTAMINE

Meyer-Bothling, (1993) found that topical application of exogenous 5-HT or the 5-HT_1-like agonist, 5-CT, increases IOP in rabbit. They also found that the 5-HT_{1A} receptor agonist, 8-OH-DPAT, causes a decrease in rabbit IOP when applied topically. But they couldn't provide a better understanding of how 5-HT_{1A} receptors are involved in the regulation of IOP. From these results we may conclude that the 5-HT_{1A} receptor in the rabbit is responsible for decreased IOP, whereas some other 5-HT receptor(s) appear to mediate increased IOP. **Osborne et al. (1987)** suggested that the increases in rabbit IOP may be caused partly or entirely by an increase in aqueous secretion mediated by 5-HT_1-like receptors. However no evidence was adduced to support his proposal. In the case of these opposing drug effects , we have little clue as to whether they involve an effect on formation or drainage of aqueous humour (or perhaps both).

The present work shows that exogenous 5-HT or 8-OH-DPAT by the arterial route (either bolus or continual perfusion) have no effect on IOP in the bovine isolated eye, while both these drugs have a lowering effect on bovine IOP when given by I.C injection. These results could lead to the conclusion that the route of administration has a significant influence on the effect of 8-OH-DPAT and 5-HT in modulating IOP. The fact that the I.C. route is effective while the arterial route is not, may indicate that the site of action of both drugs may be the trabecular meshwork, rather than the ciliary body.

Intracameral injection delivers the drug directly to the anterior chamber and, after a short delay for mixing in the aqueous, it will be carried into the meshwork

by flow of aqueous and there may cause an increase outflow facility. Arterial perfusion, although it rapidly reaches the ciliary body, does not deliver a drug quickly to the trabecular meshwork, since it is avascular. The drug can only reach the meshwork by a circuitous route, diffusing through the iris / ciliary body or by entering the posterior chamber and travelling with the flow of aqueous humour to the anterior chamber and then the drainage pathway. The observed decrease in IOP caused by I. C. 5HT or 8-OH-DPAT may therefore indicate that they increase the facility of outflow. Moreover it appears from the results obtained in the present work and those of **Osborne et al,(1996),** that topical and I.C. injection of 8-OH-DPAT have similar effects on IOP, despite the different species involved (bovine and rabbit). This similarity supports the idea that 8-OH-DPAT is affecting an anterior rather than a posterior chamber structure, i.e. perhaps the trabecular meshwork. From this we may also conclude that topical application and I.C. injection are similar to each other . This similarity allows us to suggest that there may be no species variation between bovine and rabbit at least in relation to 5-HT_{1A} receptor-mediated effects on IOP.

Other workers (**Krootila, Uusitalo and Palkama, 1987**) have reported that topical 5-HT application of 2% 5-HT caused a decrease in IOP in rabbits, whereas an I.C. injection of the drug caused a rise in rabbit IOP. They suggested that the differences between the responses to topical and I.C. 5-HT could be from variations in the concentration of the amine at different sites of action. I.C. 5-HT might, for example, be of a sufficient concentration to cause an inflammatory response, whereas topically applied amine reaches the level where it may have an action on adrenergic nerves (i.e increasing the outflow of

aqueous humour through increasing the release of noradrenaline from the adrenergic neurons). This was similar to the report of **Meyer-Bothling, Bron and Osborne, 1993).**

Receptor binding experiments have shown that 5-CT and 8-OH-DPAT were the compounds showing highest affinity for the 5-HT_{1A} receptors in the rabbit iris-ciliary processes **(Chidlow et al, 1995)**, thus since they are both agonists it is surprising that they appear to have opposing effects on IOP. It is possible that the two compounds have different rates of penetration through the cornea or different routes of entry into the eye and consequently separate sites of action. More likely, the explanation is that 5-CT is known to have affinity for receptors other than 5-HT_{1A} **(Alexander and Peters, 1998)** and activation of these other receptor types (5-HT_5, 5-HT_6, 5-HT_7) might produce ocular hypertensive effects which could override the effects on 5-HT_{1A} receptors.

5-Hydroxytryptamine by I.C route

Chiang et al., (1974) found a decrease in the IOP of dogs and rabbits after intravenous injection of 5-HT. In contrast, **Moro et al, (1981)** reported no change in IOP after treatment with 5-HT by intravitreous injection. From his pharmacological point of view, Moro suggests that the absence of 5-HT effect that he found in the eye could be correlated with a simple depletion of catecholamine (i.e., to a lysis of the sympathetic ocular tone) Similarly, neither did **Chiou et al, (1985)** find a change in the IOP after topically or intracamerally administered 5-HT in rabbit.

However, **Krootila et al, (1987)** reported a decrease in the IOP (measured with pneumatonometer) after topical application of 5-HT and an increase in the IOP (measured electromanometrically with an intracameral needle) after pentobarbital anesthesia following I.C. injection of 5-HT.

In summary then, the literature on this subject is full of controversy.

It is worth noting in this context, however, that pentobarbital anesthesia itself has an effect on IOP (**Meyer-Bothling et al, 1993)**. This could influence some of the data reported because some of the experiments on I.C administration of 5-HT were carried out in animals anaesthetized with pentobarbital.

Topical application of different concentrations of 5-HT to rabbit eyes revealed a dose-dependent, statistically significant increase in the IOP over at least a 2 hours period **(Meyer-Bothling et al., 1993).**

Moreover, recent studies by **Chidlow and coworkers (1995)** have shown that topical application of 5-HT increases IOP in rabbit. Although topical and I.C. routes would seem to deliver the drug to the same site of action, it is surprising that both of the drugs have different effects on IOP.

The present work has shown administration of 5-HT by arterial routes has no effect on the bovine IOP. This route is convenient in vitro, but very difficult in *vivo*. The pattern of drug distribution clearly differs from that resulting from topical administration or I.C. injection. To determine whether 5-HT could modulate IOP when delivered primarily to the anterior chamber, the present study was extended to include injection of 5-HT using the I.C. route. Here it was found that 5-HT caused a significant reduction of IOP in a largely dose-dependent manner.

The results that were obtained in the present work have confirmed the previous proposal that the mechanism by which both 8-OH-DPAT and 5-HT could modulate the bovine IOP is more likely to be through their effect on the outflow facility, as the I.C. route delivers the drug directly to the trabecular meshwork but does not deliver the drug to the ciliary body (at least, not nearly so efficiently as by arterial injection or perfusion).

It is not clear if the difference in the results obtained by the present work and these reported by **Chiang et al. (1974)**, **Moro et al. (1981)**, **Chiou et al (1985)**, **Krootila et al (1987)**, **Meyer-Bothling et al (1993)** and **Chidlow et al (1995)** relate to the dissimilar experimental conditions, different drug concentration, species variations, *in vivo and in vitro* situation or to some other factors.

For any set of conditions decided upon there is some variability in the results, so there is a need to carry out many experiments under controlled conditions to obtain a consensus.

(4.5) 5-CARBOXAMIDOTRYPTAMINE

Previous workers demonstrated that intravitreal injection of the 5-HT_1-agonist, 5-CT (**Tormay, Severin and Mittag, 1985**), or topically applied 5-CT (**Meyer-Bothling et al., 1992: Meyer-Bothling, Bron and Osborne, 1992**) increases IOP in rabbits, thus suggesting that 5-HT_1-like receptors exist on the ciliary processes and or/trabecular cells. Moreover, in their studies **Meyer-Bothling et al. (1993)** and **Chidlow et al. (1995)** reported that topical application of 5-CT to the rabbit eye caused an increase in IOP with time course similar to 5-HT but with greater efficacy.

This demonstrates that 5-CT penetrates the cornea and possibly activates 5-HT_1 receptors more effectively than 5-HT itself. This is the first reported evidence of a 5-HT-agonist influencing IOP **(Meyer-Bothling et al, 1993)**. Their evidence suggests that the rise in IOP was not caused by a breakdown of the blood-aqueous barrier, but may have been caused by an increase in aqueous formation mediated through 5-HT_1-like receptors **(Chidlow et al, 1995)**.

In contrast to the results obtained by the previous workers; the present work demonstrates significant decrease in IOP of the bovine eye when 5-CT is used by the intracameral route. The mechanism of action of 5-CT in lowering the bovine IOP is probably through the activation of 5-HT_1-like receptors but clearly differs from that which occurs in the rabbit eye.

In this study, the present work addressed and sequenced a number of important points concerning factors that might influence the effect of 5-CT in the modulation of ocular function.

(i) The route of administration

The similarity in the results obtained by **Tormay et al., 1985** and **Meyer-Bothling et al., 1993**, indicate that the use of different routes of administration has no influence on the effect of 5-CT on the regulation of IOP at least in the case of rabbit eyes.

The present data suggested that 5-HT and 8-OH-DPAT have no effect on IOP in the bovine eye when used by arterial perfusion and injection. For this reason 5-CT has not been tested by the arterial routes and was only used by the I.C. injection

(ii)The dose of the drug

The use of different doses of 5-CT might give a clue to the dissimilar results obtained by the present work and the other workers. The use of different doses of the amine favours to different sites of action. The present work used lower doses of 5-CT (20, 60, 200nmol), which may be enough to activate 5-HT_{1A} receptors. Other workers (**Meyer-Bothling et al., 1993)** used higher doses of 5-CT (300, 600, 1200nmol) that may activate other 5-HT_1 receptor subtypes responsible for the ocular hypertensive effects of this drug and that could over ride its effect on 5-HT_{1A} receptors.

(iii) Species variation and experimental variation

The data suggested that both species differences and experimental differences may influence the effect of the amine on the regulation of IOP and might explain and clarify the dissimilarity of the results obtained by different workers. This is because *in vivo* work involves the influence of the central nervous system and hormonal and vascular systems, which often complicate the interpretation of results.

(4.6) CAMP

This section of the project attempted to make a correlation between the effects of the drugs under study on IOP and their effect on cAMP production in tissue homogenates of the bovine eye. This has been done by measuring the cAMP formation rates in CP and TM in the present of 5-HT and related drugs, using the recently developed enzyme-immuno assay technique.

The diversity of assays available for adenosine 3',5'-cyclic monophosphate are impressive and highly desirable. Analytical difficulties are imposed by extremely low tissue levels of the cAMP and considerably higher levels of potentially interfering compounds. The existence of several good techniques for estimation of quantities of cAMP in biological material ensures the fact that different laboratories will be subject to different sets of limitations and possible errors. The ultimate result should thus be beneficial **(Gilman, 1972).**

The main advantage of EIA assay is sensitivity, especially when it is used with acetylation procedure. Only by using acetylation was it possible to measure the very small amounts of formed in CP and TM homogenates.

We could not compare the control values of the present work with other worker's control because of lack of literature.

The cAMP assay was clearly sensitive to interference by substances present in the tissue extracts and hence we had to use high dilutions of the tissue homogenates, assayed using the acetylation technique.

The assay results show rather a high variability, and so much repetition of these experiments would be necessary before reliable results could be obtained. Unfortunately, the assay Kit was too expensive to permit more than two

measurements of each drug concentration on each tissue. despite this limitation, some interesting trends in the results can be seen.

Even allowing for the variability of the data, cAMP production in TM seems to be more than three times greater than in CP.

Results from the present showed no effect of adenosine on cAMP formation rate. This finding is compatible with our failure to observe any change in IOP in the bovine eye.

In CP homogenate, 5-HT produced inconsistent results **(Table 3.7.2)** with some apparent increases occurring, but with no sign of concentration-dependent changes. This again is at least compatible with the IOP results for 5-HT by arterial routes **(Table 3.4.1)** where the drug concentration is likely to be much higher in the ciliary body than in the drainage meshwork.

The most convincing result among the cAMP data is that in which the formation rate in TM is increased by 5-HT in every case (i.e. in tissues samples A and B, and at every concentration of 5-HT). This implies that 5-HT is activating adenylate cyclase (although it has to be admitted that the effect is not concentration dependent). Such an increase in cAMP formation implicates not 5-HT_1 receptors (which are linked to G_i protein and *inhibition* of adenylate cyclase) but rather perhaps 5-HT_5, 5-HT_6 or $5HT_7$ receptors (which are linked to G_s and *stimulation* of adenylate cyclase). Some support exists from receptor binding experiments that $5HT_7$ receptors are located in the rabbit anterior eye **(Chidlow et al, 1995).**

β-adrenoceptors also act by stimulating adenylate cyclase. Activation of these receptors in the outflow apparatus is thought to be the mechanism by which

adrenaline lowers IOP both in the rabbit **(Anderson and Wilson, 1990)** and in man **(Erickson-Lamy and Nathanson, 1992).** It is this very likely that adrenaline lowers IOP by increasing cAMP formation in the TM.

By analogy with adrenaline, 5-HT by the I.C route in the bovine eye may lower IOP by activating 5-HT receptors linked to G_s, which would stimulate cAMP formation leading to an increase in the facility of outflow of aqueous humour and a decrease in IOP.

The results for 8-OH-DPAT effects on cAMP showed some consistency, since in both CP homogenates (A and B), this drug appeared to inhibit cAMP synthesis at the lowest concentration (5μM) while increasing cAMP at higher concentrations (50 and 500 μM).

Such a biphasic pattern of effect is not necessarily impossible, since we know that some 5-HT receptors are inhibitory while others stimulate adenylate cyclase. It is possible, therefore, that 8-OH-DPAT at higher concentrations, might activate another receptor, e.g. 5-HT_5, 5-HT_6 or 5-HT_7, all of which are linked to G_S protein. Any of these receptors might lead to an increased cAMP production.

8-OH-DPAT appeared to produced different effects on both TM homogenates (A and B). In the case of TM homogenate (A), 8-OH-DPAT decreased cAMP production with apparent concentration-dependence. However, in TM (B) 8-OH-DPAT produced an opposite effect with no-concentration dependency. No conclusion can therefore be drawn from these results.

Previous studies by **Tobin and Osborne (1993)** showed that 5-HT and 8-OH-DPAT reduced the forskolin-stimulated cAMP formation in the rabbit iris-ciliary body, suggesting the presence of 5-HT_1-like receptors.

Although an inhibition or stimulation of basal cAMP levels was seen in tissue homogenates following exposure to serotonin. These authors observed that it is of significance that the inhibitory effects of 5-HT_{1A} receptor agonist, 8-OH-DPAT on adenylate cyclase

activity are greater in the ciliary process than in the iris muscle.

The ciliary processes are associated with the production of aqueous humour and this process is cAMP dependent **(Sears et al, 1984; Bartels, Lee and Neufeld, 1987)**. The possibility of 5-HT_{1A} receptor being involved in the formation of aqueous humour and therefore the regulation of IOP must be considered.

In view of the different patterns of results in the tissues of various species, it would appear that the effects of 5-HT and 8-OH-DPAT on IOP and cAMP level may vary from one species to another. This could explain the dissimilar results obtained by different workers. Some of the conflict in the results may also vary because of the use of different routs of administration and other experimentable variables, including drug dosage. The present work has demonstrated an ocular hypothensive effect of ATP for the first time and has added to the existing evidence that some 5-HT analogues can lower IOP. The evidence that the later effects seem to be mediated via an effect on outflow requires confirmation by direct measurement.

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