PHARMACOLOGY OF AQUEOUS HUMOUR FORMATION

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Abstract

This study is a continuation of ongoing research in our laboratories, aiming to investigate the mechanism by which BK acts in the eye, which started by investigating the effect of BK on the ciliary artery (McNeish et al., 2003). The present work extends this study to the ciliary body, by examining BK actions on aqueous humour formation in the bovine arterially perfused eye and on intracellular calcium movements in cultured ciliary epithelium. Aqueous humour was estimated using a fluorescein dilution technique. The objective of this research was to reveal the possible pathway(s) and mechanism(s) by which BK affects the aqueous humour formation. The effect of BK on aqueous humour formation was concentration-dependent over the range 10^{-9} to 10^{-7} M and was examined in the presence of various drugs which are known to interfere with BK's actions in other tissues including L-NAME, flurbiprofen and clotrimazole, which inhibit nitric oxide synthase, cycloxygenase and endothelium-dependent hyperpolarising factor(EDHF) respectivcly.

The study revealed that of these inhibitors, only L-NAME did block the effect of BK, therefore it can be concluded that the pathway of BK in causing reduction in aqueous humour formation in bovine eye is through nitric oxide, and not through cycloxygenase or EDHF.

The involvement of cGMP in the effect of BK on aqueous humour formation was also examined. The analogue of cGMP, 8-Br- cGMP, was tested and produced

significant suppression of aqueous humour formation in a manner that was clearly concentration-dependent. It appears that we may be observing a truly physiological mechanism for cGMP. ODQ, a soluble guanylate cyclase inhibitor, was used to differentiate cGMP-mediated effects of NO from cGMP-independent effects. When used in conjunction with BK, ODQ did block the effect of BK. Therefore it can be concluded that the mechanistic pathway of BK causing reduction in aqueous humour formation is through cGMP.

For further confirmation of the participation of cGMP in the BK pathway in aqueous humour formation, the effects of KT-5823 an inhibitor of proten kinase G were examined. KT-5823 alone did not have any effect on aqueous humour formation suggesting that there is no background influence of endogenous cGMP on the basal rate of aqueous humour formation. However, when KT-5823 was used with BK, it completely suppressed the BK effect on aqueous humour formation. This blockade of BK-induced suppression of aqueous humour formation strongly suggests that an increase in cGMP production by BK is the most likely mechanism of its effects on aqueous humour formation.

The influence of endogenous cGMP and also its participation in the BK pathway was tested using UK-114,542 (a specific inhibitor of phosphodiesterase type V). UK-114,542 alone had a small but significant effect on aqueous humour formation. This suggests that there is some baseline production of cGMP in the bovine ciliary body and that PDE inhibition enhances cGMP levels to a level at which aqueous humour formation is affected. When used with BK, UK-114,542 appeared to have enhanced

the effect of BK, consistent with the result for UK-114542 alone and also strengthening further the argument that cGMP is the mediator of its effects on aqueous humour formation.

Two other minor questions were answered during this part of the study. It was shown that the absence of ascorbate from the arterial perfusate had on effect on the reduction of on aqueous humour formation by 8-Bro-cGMP and BK. The results of the present study also suggested that when a high concentration of BK (10^{-7} M) was included in the perfusate there was no significant association between protein accumulation in aqueous humour and a reported rise in aqueous humour formation. Instead, the effect of this concentration of BK was to suppress aqueous humour formation by 41%, a slightly greater inhibition than was exerted by any of the lower concentrations of bradykinin tested. The method used Fura-2 as a calcium-sensitive fluorescent marker to monitor free intracellular calcium in individual cells.

The objective of the next section was to investigate the effect of BK administration on calcium release in non-pigmented ciliary epithelial cells and ultimately reveal the possible pathway(s) and mechanism(s) by which BK affects the calcium release. Bradykinin (10^{-9} to $3x10^{-8}$) was found to have a concentration-dependent effect on calcium release in these cells. The effects on BK-induced calcium release in the presence of inhibitors such as L-NAME, flurbiprofen and of clotrimazole were examined.

The study revealed that L-NAME in conjunction with BK (3×10^{-8}) it inhibited calcium release while with low concentration of BK (3×10^{-9}) it increased calcium release. Therefore it can be concluded that the pathway of BK in causing calcium release in non-pigmented ciliary epithelium somhow involve nitric oxide.

ODQ, a soluble guanylate cyclase inhibitor, was used to differentiate cGMP-mediated effects of NO from cGMP-independent effects and was found to have no effect on calcium release, when used on its own. When used in conjunction with a high concentration of BK (3×10^{-8} M) ODQ did block the effect of BK. On the other hand, as was observed with L-NAME, BK at low concentration (3×10^{-9} M) it increased calcium release. Therefore it can be concluded that the mechanistic pathway of BK causing calcium release in non-pigmented ciliary epithelial is influenced by cGMP. This would suggest that NO and cGMP play a part in the release of calcium induced by BK.

The involvement of cGMP in the effect of BK on calcium release was also examined. cGMP (10^{-8} to 10^{-11} M) with BK was tested and produced significant suppression of calcium release in a manner that was clearly concentration-dependent. For further confirmation of the participation of cGMP in the bradykinin pathway in calcium release, the effects of KT-5823 were examined. KT-5823 alone did not have any effect on calcium release. However, when KT-5823 was used with BK and cGMP, it completely suppressed the ability of cGMP to inhibit the BK effect on calcium release. This suggests that an increase in cGMP production by BK acts as a negative feedback on its release of calcium.

The influence of endogenous cGMP and also its participation in the bradykinin pathway was tested using UK-114,542 (a specific inhibitor of phosphodiesterase V). UK-114,542 alone had no significant effect on calcium release. This suggests that there is not sufficient baseline production of cGMP in the cultured ciliary epithelial cells such that inhibition could enhance cGMP to a level at which calcium release is affected. When used with BK, UK-114,542 appeared to have enhanced the effect of bradykinin, consistent with the result for KT-5823 and strengthening further the argument that cGMP is produced as a result of BK action and that cGMP then inhibits further release of calcium.

A possible role of EDHF in bradykinin-induced calcium release was assessed using clotrimazole. The calcium release was unaffected by administration of clotrimazole on its own, nor when clotrimazole was combined with BK. It therefore appears that EDHF is not involved in the action of BK on calcium release in ciliary epithelial cells.

The other option, which BK-induced calcium release in bovine eye was due to a product of cyclo-oxygenase was challenged using flurbiprofen. The calcium release was unaffected by administration of flurbiprofen on its own, noe when flurbiprofen was combined with BK. It therefore appears that cyclo-oxygenase is not involved in the action of BK on calcium release.

The results suggest that in the bovine ciliary body, similarly low concentrations of BK are required to inhibit aqumous humour formation and to stimulate intracellular calcium release. It is argued that these two effects are connected and that cGMP is the mediator of the BK-induced suppression of aqumous humour formation.

The results of the calcium experiment support the idea that BK raises endogenous cGMP levels via its ability to release intracellular calcium. This strengthens the status of calcium as a primary mediator of intracellular events. A further implication of the low concentration of BK required to elicit these effects is that endogenous BK may modulate aqueous production in vivo.

Table of contents

List of Figures

List of Tables

Acknowledgements

All praise be to ALLAH alone, and many peace and blessings be upon him the final messenger. Abundant blessed praise be to ALLAH as he loves and is pleased with and thanks be to him for the favours he bestwed upon me. Praise him and glory be to him for his grace and making many things easy for me. So praise be to you as the greatness of your kigdom and excellency of your power deserve it. Whoosoever dose not thank people will not thank ALLAH. Hence I thank my beloved prophet muhammed peace and blessing of ALLAH be upon him, the one whome ALLAH sent him as a mercy for all the worlds. O Allah grant me the love your prophet.

I would like to express my sincere thankfulness to my supervisor Dr.wilson for his guidance, support and motivation during my PhD.

I show thanks to my parent as they rightfully deserve. ALLAL say in holy quran (And we have enjoined on man to be dutiful and kind to his parents. His mother bears him with hardship and the she bearing of him and the waening of him is thirty months, till when he attains full strength and reaches forty years, he say: my lord grant me the power and ability that I may be grateful for your favour which you have bestowed upon me and upon my parents, and that I may do righteous good deeds, such as please you and make my offspring good. Truly, I have turned to you in repentance and truly I am one of the muslims(submitting to your will).(surah al-ahqaf., part 26).

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Sincere thanks to my wife , sisters and kids for your love and support.

Declaration

I hereby declare that this thesis has been composed by me and that it embodies the results of my own research, carried out in the Ocular Pharmacology Laboratory within the Institute of Biomedical and Life Sciences, the University of Glasgow. No part of this thesis includes work that has previously been presented for any degree either at this or any other university.

Chapter 1: Introduction

1 Introduction

1.1 General eye anatomy

The eye is an optical instrument, which receives images in the form of light waves from an object, enters the eye first through the cornea. The light then progresses through the pupil, the circular opening in the centre of the coloured iris. Next, the light passes through the crystalline lens. Initially, the light waves are bent or converged first by the cornea, and then further by the crystalline lens.

The light continues through the vitreous humour, the clear gel that makes up about 80% of the eye's volume, and then, ideally, back to a clear focus on the retina behind the vitreous. The small central area of the retina is the macula, which provides the best vision of any location in the retina. Within the layers of the retina, light impulses are changed into electrical signals and then sent through the optic nerve, along the visual pathway, to the occipital cortex at the posterior or back of the brain. Here, the electrical signals are interpreted or "seen" by the brain as a visual image.

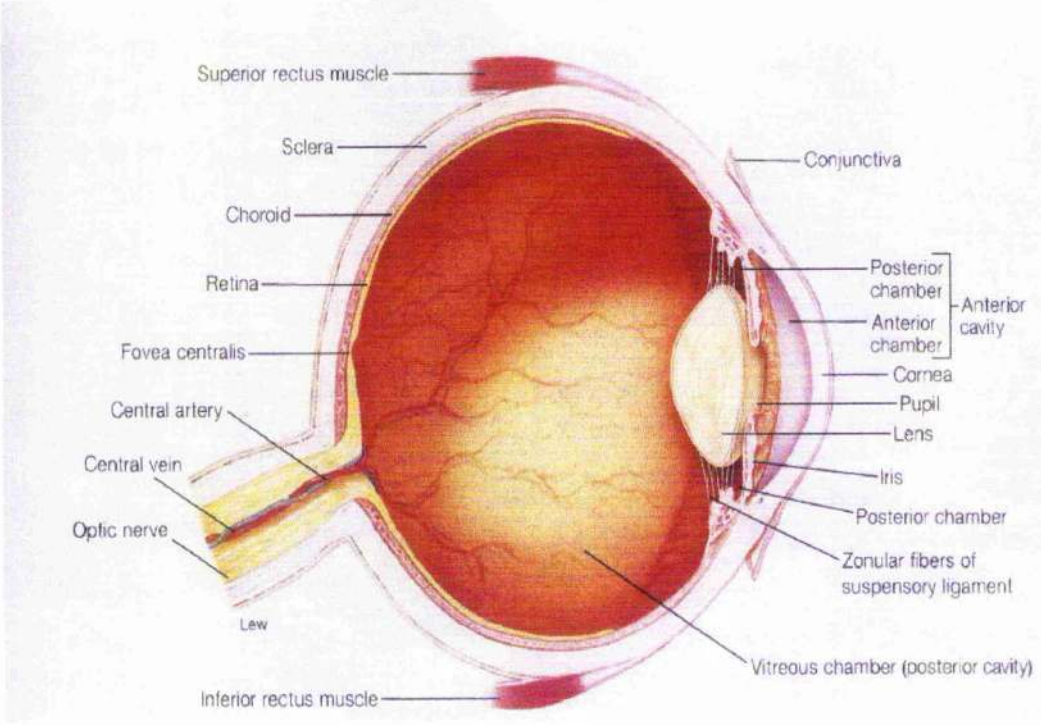

Figure 1.1: Principal eye structure (Fox, 2006).

1.1.1 The Eye and sense of vision

The eyeball is located in a bony socket called the orbit, where it is suspended and surrounded by fat and blood vessels together with motor and sensory nerves, including the optic nerve. The eye has three main layers: the outer fibrous layer, middle vascular layer and inner nervous layer (Figure 1.1).

1.1.2 Outer layer

The fibrous tunic is the outermost covering of the eye and is composed primarily of collagen proteins that form a dense fibrous connective tissue. This connective tissue

is similar in structure to a tendon and makes the eye a very rigid or fixed shape container. The anterior portion of the fibrous tunic is called the cornea and the posterior region is the sclera.

1.1.2.a The sclera or white of the eye is vascularized. The sclera is divided into three layers: episclera, stroma and lamina fusca (Geroski and Edelhauser, 2001), and only a limited number of blood vessels are found. (Maurice and Mishima, 1984)

1.1.2.b The cornea is transparent because the collagen fibres in this region are more regularly arranged and do not reflect light. The cornea lacks blood vessels and receives its nutrients from the aqueous humour and most of its oxygen from its surface. The cornea functions to aid the bending of light and in part, contributes to the formation of a clear image. If the cornea is not shaped properly or if its transparency is lost, the image will not be focused on the retina and blurred vision will result (Robinson, 1993).

1.1.3 Middle layer

The middle layer is highly vascularized and is composed of three structures: the choroid, ciliary body and the iris.

1.1.3.a The choroid is a thin, darkly pigmented membrane rich in blood vessels. The pigment is melanin, a blackish or brown pigment that absorbs light so it does not reflect within the eyeball.

1.1.3.b The ciliary body is composed of ciliary processes and the ciliary muscle. The tiny ciliary processes secrete aqueous humour. Aqueous humour supplies nutrients to the lens and the cornea of the eye and then drains back into the cardiovascular system via small canals. The ciliary muscle is made of smooth muscle cells in a ring or sphincter around the lens. This muscle is controlled by the autonomic nervous system. The ciliary muscle is connected to the suspensory ligaments that are directly attached to the lens.

The lens is a biconvex, transparent, avascular structure made of epithelial cells. These cells are highly organized so that light passes through it, and the cells contain highly elastic proteins so its shape can be altered. When the lens is flat or thin, it bends light less than when it has a thicker or wider shape.

1.1.3.c The iris is thinner than the ciliary body and partly overlaps the front of the lens. It is composed of circular and radial smooth muscle fibres. In the centre of the iris is an opening called the pupil. Light passes through this opening to reach the lens and the interior of the eye. Through contraction or relaxation of its smooth muscle cells, the diameter of the pupil can be finely regulated. Responses of the iris are also controlled by both divisions of the autonomic nervous system. The space behind the lens is filled with a clear, non-replaceable, jelly-like substance called vitreous humour.

1.1.4 Inner layer

The innermost area of the three eye tunics is the retina. The retina is the primary tunic that regulates sight. The retina consists of millions of receptor cells known as rods and cones. Rods and cones are also known as photoreceptors, due to the fact that they primarily react to light. Electrical signals travel through the two photoreceptors via a two-neuron chain. From the neuron chain the signal travels to bipolar cells then to the ganglion. From the retina, nerve impulses are sent to the optic cortex.

1.1.5 Ocular blood supply

The eye's major blood supply comes from the ophthalmic artery. It arises from the internal carotid artery, as the internal carotid artery is emerging from the cavernous sinus, on the medial side of the anterior clinoid process. It enters the orbital cavity through the optic foramen, below and lateral to the optic nerve. The ophthalmic artery passes over the nerve (in 85% of cases) to reach the medial wall of the orbit. Then, the artery proceeds forward horizontally, beneath the lower border of the superior oblique muscle, and divides into two terminal branches, frontal and dorsal nasal.

As the artery crosses the optic nerve, it is accompanied by the nasociliary nerve and is separated from the frontal nerve by the superior rectus muscle and the superior levator palpebral muscle. Most branches of the ophthalmic artery arise in the

posterior one third of the orbit and pass interiorly. The ophthalmic artery is divided into an orbital group, distributing vessels to the orbit and surrounding parts, and an ocular group, distributing vessels to the muscles and bulb of the eye (Forrester et al, 1996, Bill, 1981).

1.1.6 Special characteristic of ocular blood supply in bovine eye

The comparison of the human eye and bovine eye will lead to better understanding of the anatomical arrangement of the bovine eye. It is important to note that there exist some main differences (Table 1.1). The bovine eye receives most of its blood via the ciliary arteries (Prince et al., 1960) (Figure 1.2) that are branches of the external ophthalmic artery. The ciliary artery starts from the external rete then divides into large medial and smaller lateral ciliary arteries. They travel parallel to each other. Both of them divide before arriving at the globe and then form many small short posterior ciliary arteries. One of the short posterior ciliary arteries branches to form the retinal artery (Prince et al. 1960). The large medial ciliary artery is divided into two long posterior ciliary arteries, which penetrate the globe at the equator, one along the medial side and the other along the lateral side. Both of these arteries pass through the choroid and anastomase with anterior ciliary arteries and form the major arterial circle of the iris, which is well developed in bovine eyes (Prince et al. 1960). Blood returns from the choroid, iris and ciliary processes to drain via the vortex veins. In the bovine eye there are four vortex veins that leave the eye in the region of the equator (Prince et al., 1960).

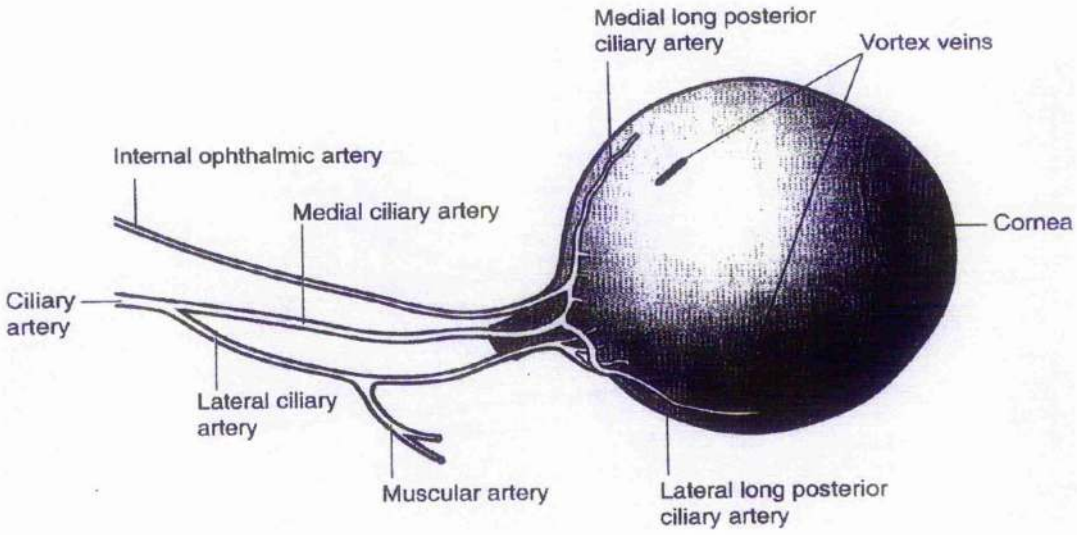

Figure 1.2: Schematic diagram of the vascular blood supply to the bovine eye (Prince et al., 1960)

Table 1.1: Anatomical differences between the human and the bovine eye adapted from Cole (1970), Doughty (1995, 1997), Forrester et al. (1996), Prince et al. (1960), Shahidullah (1994), Tripathi (1984), Williams and Warwick (1980) and McNeish (2001).

AnatomicalCharacteristic	**Human eye**	**Bovine eye**
Anterior chamber volume	0.35 mL	1.7 mL
Canal of Schlemm	Present	Absent
Ciliary muscle	Well developed	Poorly developed
Ciliary processes	70	90-110
Corneal dimensions	11.7 mm (horizontal) 10.6 mm (vertical) 1 mm (thickness)	27-34.5 mm (horizontal) 20-30.5 mm (vertical) 0.7-1.5 mm (thickness)
Ending point of retina / beginning of ciliary body	Ora serrata	Ora ciliaris retinae
Lens volume	0.2 mL	2.2 mL
Main arterial blood supply	Internal ophthalmic artery	External through ophthalmic artery through Ciliary artery
Globe dimensions	21-26mm Anterior-posterior 23-25 mm Diameter	28-30mm Antero-posterior 30 mm Diameter

1.1.7 Blood-aqueous barrier

Passage of many solutes from the blood vessels of the ciliary stroma into the aqueous humour is restricted. This limitation is known as the blood-aqueous barrier. The blood-ocular barriers system is formed by two main barriers: the blood-aqueous barrier and the blood-retinal barrier. They combine to maintain the eye as a privileged site and are essential for normal visual function. The membrane of the capillary bed of the ciliary body of the eye permits two-way transfer of fluids between the ciliary stroma and the blood (Smith, 1971, Cunha-Vaz, 1979). Tight junctions of non-pigmented epithelial cells and endothelial cells of iridial vessels form the blood-aqueous barrier; whereas, the tight junctions of retinal pigment epithelium and the endothelium of retinal vasculature form the blood-retinal barrier. Blood-aqueous and -retinal barriers play important roles not only in restricting molecular movement into the anterior and posterior compartments but also in their elimination of certain types of chemical from the ocular compartments (Knudsen, 2002).

The blood-aqueous barrier is a major restraint to the free passage of many solutes from the blood vessels of the ciliary stroma into the aqueous humour (Millar & Kaufman, 1995). The blood-aqueous barrier is formed by two components. One is an epithelial barrier localized in the ciliary and iridial epithelia which protects the posterior and anterior chambers from circulating macromolecules (Raviola, 1977). This is represented by the tight junctions between the non-pigmented cells of the ciliary epithelium in the ciliary body. The second is an endothelial barrier which

prevents movement of macromolecules from the lumen of the vessels of the iris into the iridial stroma and then into the anterior chamber (Raviola, 1977). This is constituted by the non-fenestrated cells of the iris capillaries. Both of them work to exclude large molecular weight substances such as proteins.

There is a difference in permeability of epithelia of the ciliary body, iris and vascular endothelium. This has been shown experimentally by injection of horseradish peroxidase. It has been seen that horseradish peroxidase diffuses through the walls of the vessels of the ciliary stroma and into the intercellular clefts of the pigmented epithelium. However horseradish peroxidase could not pass the tight junction between the non-pigmented cells (Raviola, 1974). These tight junctions are also impermeable to materials that diffuse from the intercellular clefts located on the posterior chamber side of the non-pigmented epithelium (Raviola, 1977).

In certain experimental and pathological conditions such as injury (chemical or physical) and trauma the blood-aqueous barrier breaks down (Berman, 1991). This will lead to leakage of the large molecular weight substances into the aqueous humour. After breakdown of the blood-aqueous barrier the resultant aqueous produced is known as secondary or plasmoid aqueous (Millar and Kaufman, 1995). In such cases the aqueous humour becomes cloudy and fibrin clots and inflammatory cells are also likely to be present when the blood-aqueous barrier breaks down (Forrester et al., 1996).

1.1.8 Ciliary body

The ciliary body is an anterior extension and expansion of the uveal tunic, located in the angle between the margin of the cornea and the lens. Most of the ciliary body is composed of smooth muscle whose contractions work the lens accommodation mechanism via the tension imposed on the zonule fibres. The ciliary body runs radially around the anterior part of the eye (Millar and Kaufman, 1995).

In addition to its role in the mechanism of accommodation, the ciliary body is a secretory structure. The ciliary body is lined on its inner surface with a double layer of cells, the ciliary epithelium. The outer of these two layers is heavily pigmented, the inner one is not. The inner portion of the ciliary body is divided into two segments: pars plicata and pars plana. The ciliary body which is a well vascularized tissue with a high rate of blood flow extends from the iris to the posterior edge of the ora serrata (Millar & Kaufman, 1995). This arrangement of the human ciliary body is similar to that of the bovine eye where it extends from the ora ciliaris retina to the iris root (Prince et al., 1960). It is divided into two parts, uveal and epithelial portions. The uveal portion consists of suprachoroid, the ciliary muscles, the vessels layer, the connective tissue and lamina basalis choroideae. The epithelial portion or ciliary process consists of interiorly the pars plicata and posteriorly the pars plana (Kanski et al., 2004, Newel, 1996).

The largest component of the ciliary body is smooth muscle and is the most prominent structure in the uveal portion of the ciliary body (Newel, 1996). The

smooth muscle is arranged in three bundles longitudinal, reticular and circular. In the bovine eye the circular and radial muscle fibres do not seem to have any particular function (Prince et al., 1960). In the human eye the ciliary processes number approximately 70 villus-like structures (Millar and Kaufman, 1995) on the other hand in bovine eye, the ciliary processes are about 90-110 in number and are large, ranging between 3-5 mm in length (Prince et al., 1960). The ciliary processes have a rich blood supply and project inward from the pars plicata. The processes have a convoluted shape, consisting of capillaries surrounded by connective tissue (the stroma) and are covered by a double epithelial layer, the ciliary epithelium (Millar & Kaufman, 1995). The ciliary epithelium separates the stroma of the ciliary processes from the aqueous humour and vitreous humour and consists of two layers of cells, non-pigmented and pigmented ciliary epithelial cells. Figure 1.4 and Table 1.2 show a list of the differences between the non-pigmented and pigmented ciliary epithelial cells.

Table 1.2: List of the differences between the non-pigmented and pigmented ciliary epithelial cells (To et al. 2002).

	NPE	**PE**
Location	face the posterior chamber	face the ciliary stroma
Shape	Columnar	Cuboidal
Content	mitochondria and rough and smooth endoplasmic reticulum, few melanosomes	numerous melanosomes, but have few other intracellular organelles
Infoldings of basal surface	many infoldings face the lateral surface provide large surface area	many infoldings face the lateral surface provide large surface area

The ciliary body epithelium secretes the aqueous humour into the posterior chamber of the eye. The two layers are so intimately connected, and their interaction so complete, however, that they're always considered to be part of the ciliary body--which is part of the uvea.

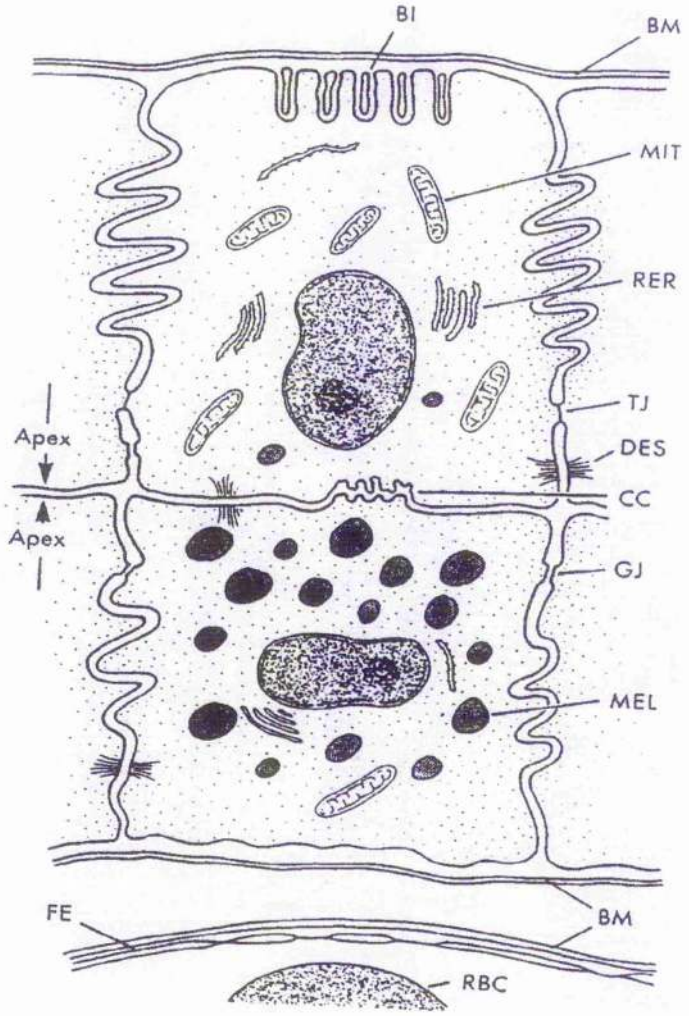

Figure 1.3: Schematic diagram of nonpigmented and pigmented epithelial cells. BI, basal infoldings; BM, basement membrane; CC, ciliary channels; DES, desmosomes; FE, fenestrated capillary endothelium; GJ, gap junction; MEL, melanosome; MIT, mitochondrion; RBC, red blood cell; RER, rough endoplasmic reticulum; TJ, tight junction (Caprioli, 1992).

The ciliary epithelium is densely populated by a variety of specialised intercellular junctions. Zonula occludens, zonula adherens, gap junctions and desmosomes have been shown to connect the non-pigmented epithelium one to another. However, gap junctions, puncta adherentes and desmosomes attach pigmented epithelium cells one to another and to non-pigmented epithelium cells (Raviola & Raviola, 1978).

The zonula occludens or tight junctions, are found between the apical portions of the non-pigmented epithelium cells only (Raviola and Raviola, 1978). They form the main constituent of the blood-aqueous barrier and act as a barrier for paracellular diffusion (Cunha-Vaz, 1979). The functions of the tight junction are two-fold (Hirsch et al, 1985 and Bill, 1986). Firstly, they prevent the passage of molecules and ions through the space between cells. So materials must actually enter the cells (by diffusion or active transport) in order to pass through the tissue. This pathway provides control over what substances are allowed through. Secondly, they block the movement of integral membrane proteins between the apical and basolateral surfaces of the cell, thus the special functions of each surface,for example receptor-mediated endocytosis at the apical surface or exocytosis at the basolateral surface can be preserved.

Gap junctions are the only known cellular structures that allow a direct transfer of signalling molecules from cell-to-cell by forming hydrophilic channels that bridge the opposing plasma membranes of neighbouring cells. The crucial role of gap junction-mediated intercellular communication for coordination of development,

tissue function, and cell-homeostasis is now well documented; and mutations in gap junction channel protein-encoding genes can result in a number of diseases that include deafness, cataracts, severe dermatological disorders, and cancer. In addition, recent findings indicate that gap junction-mediated intercellular communication also plays a significant role in transient cell-cell contacts, and that gap junction hemi-channels (connexons) by themselves can function in intra/extra-cellular signalling (Segretain, et al., 2004).

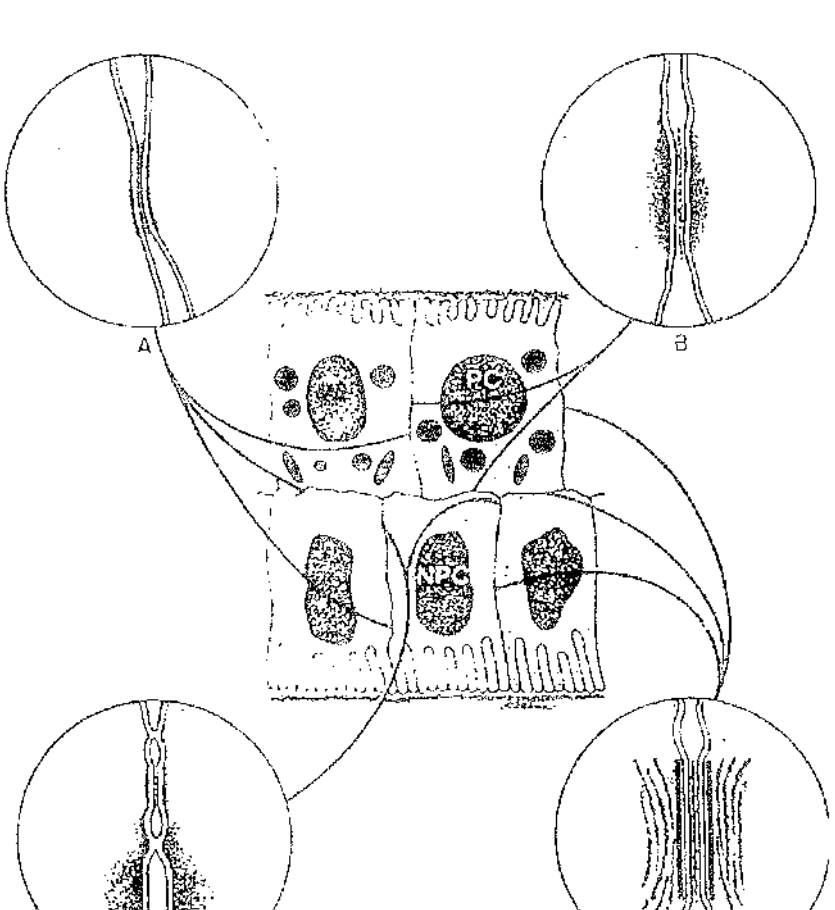

Figure 1.4: Diagram of the intercellular junctions in the ciliary epithelium (Raviola, 1977).

Desmosomes function as adhesive intercellular junctions and as linkers of intermediate filaments in epithelia (Figure 1.4). In the ciliary epithelium, desmosomes are thought to be especially important in forming mechanically stable cell junctions to withstand the forces exerted on the ciliary zonule by contraction and relaxation of the ciliary muscle during accommodation of the lens (Raviola, 1971). Puncta adherentia are large and abundant and located between pigmented and nonpigmented cells and the interface between epithelial layers (Raviola and Raviola, 1978).

1.2 Aqueous humour

The aqueous humour is the clear, watery fluid that fills the complex space in the front of the eye, which is bounded at the front by the cornea and at the rear by the front surface or face of the vitreous humour. Aqueous humour and its production are essential to normal function of the eye. Since the eye is an optical device and must obey the laws of optics, it has to maintain a certain size and shape, or the quality of the image produced and focused on the retina will suffer. The balance between production and drainage of the aqueous humour is one of the important ways in which this is accomplished (To et al., 2002).

The aqueous humour provides oxygen and glucose to the avascular cornea and lens. It also removes toxic metabolic products from the iris and cornea. Additionally, it is involved in maintaining intraocular pressure (To et al., 2002). The aqueous humour enters the posterior chamber, exits through the pupil, and from there enters the

anterior chamber. A very intricate outflow pathway from the anterior chamber ensues through a trabecular meshwork located at the interior junction of the iris and cornea/limbus. This meshwork is lined by trabecular cells, which maintain the hydration state of the meshwork as well as serve a phagocytic function for debris in the aqueous. After percolating through the meshwork, the aqueous in the human eye enters Schlemm's canal (Friedrich S. Schlemm, 1795-1858, a German anatomist) and from there drains into intrascleral and episcleral venous plexuses and finally into conjunctival, ciliary, and vorticose veins (Miller & Kaufman 1995).

The production of the aqueous humour is a constant process; and its removal is vitally important: the balance between production and drainage determines the intraocular pressure. Normal pressures maintain the normal shape and size of the eyeball, fulfilling the mechanical requirements to keep an image in focus. If the balance is disturbed, vision problems are the normal result (To et al., 2002 and Krupin et al., 1995).

1.2.1 Composition of aqueous humour

The composition of the aqueous humour is difficult to determine, as it largely depends on the nature of the freshly secreted fluid, the succeeding passive and active solute exchanges across local tissue, and the rate of drainage from the eye. Basically, this means that diffusional and metabolic alterations of the aqueous humour occur constantly. Of course the aqueous humour is largely water, but contains a number of components: glucose, which it feeds into the cornea; lactic acid, which the cornea

pumps out to the aqueous humour; oxygen; amino acids, including alanine, valine, lysine, leucine, and arginine in humans; a small amount of proteins; immunoglobulins, part of the immune system; and traces of coagulation systems, fibrinolytic systems, and cell growth inhibitors (Reily, 1983 and Spector et al, 1981). Some of the other inorganic substances found in the human aqueous include bicarbonate, chloride and a trace of phosphate (Cole, 1984). Other organic substances found in the human aqueous (all in relatively small concentrations) include ascorbate, citrate and hyaluronate (Tripathi 1989 and Raviola 1977). The composition of aqueous humour of both human and bovine eye is shown in Table 1.3.

Table 1.3: The concentration of electrolytes, low molecular weight solutes and protein in bovine and human Aqueous Humour (Spector et al, 1981).

Substance (mM)	Bovine aqueous humour	Human aqueous humour
Sodium ion	143.8-149.5	142
Potassium ion	4.5-7.1	4
Chloride ions	116.3-124.0	131-136
Bicarbonate ions	36	20
Ascorbate	2	1.0-1.1
Lactate	7.6-8.2	4.5
Glucose	2.17	2.8-3.9
Protein (mg/mL)	0.2-0.8	0.3-0.7

1.2.2 Aqueous humour formation

Aqueous humour is produced by the ciliary processes at approximately 2-3 μL/min in humans and the entire volume of aqueous humour is replaced every 90-100 minutes. Aqueous humour formation is a complex process and it can be subdivided

into four stages (Freddo, 2001). Firstly, the blood flows to the vascular bed of the ciliary processes. Secondly, an ultrafiltrate is passed through the fenestrated capillaries of the ciliary processes, which have high protein permeability, into the interstitial spaces between the vessels and the ciliary epithelium. Thirdly, a number of solutes are transported from the ultrafiltrate to the posterior chamber across the ciliary epithelium. Finally, the osmotic gradient established by the solutes facilitates the passive flow of water into the eye by osmosis (Viggiano, 1994).

The movement of solutes across the ciliary epithelium can be achieved by three inter-dependent mechanisms. The first one includes diffusion, which is defined as the passive movement of solutes across the ciliary epithelium in response to a concentration gradient. The second one consists of ultrafiltration, which is defined as the passive movement of water and water soluble substances across cell membranes as a result of the balance of hydrostatic and oncotic pressures between the ciliary stroma and the aqueous humour. The third mechanism consists of active transport, which is an energy consuming process involving the movement of solutes across the ciliary epithelium against their concentration gradients (To et al., 2002).

The formation of aqueous humour is widely believed to occur by ultrafiltration of blood plasma into the stroma and then by active transport of ions and diffusion of water across the pigmented and non-pigmented epithelia into the posterior chamber (Bill, 1975, and Brubaker, 1991). Bill reported that the difference in the hydrostatic pressure was less than the difference in the oncotic pressure between ciliary stroma

and aqueous humour. This differential pressure across the ciliary epithelium tends to move water from the posterior chamber into the ciliary processes (absorption) rather than facilitate ultrafiltration into the posterior chamber (Burstein et al, 1984).

1.3 Epithelial fluid transport

1.3.1 Introduction

During aqueous secretion water may flow either via the transcellular route, through cells, or the through the paracellular route, between cells. However, the formation of aqueous humour appears to be mainly dependent on the paracellular route, which has been discounted (Cole, 1977). The transfer of water and solutes from the stroma to the aqueous humour is carried out through three main stages. It includes firstly, the uptake of solutes and water at the stromal surface by pigmented epithelial cells.

Second, solutes and water in the pigmented epithelial cells are transferred to non-pigmented epithelial cells via the gap junctions through passive movement across cell membranes as a result of the hydrostatic and oncotic pressures between the ciliary stroma and the aqueous humour. The third step involves the transfer of solutes across the ciliary epithelium against their concentration gradients.

1.3.2 Intracellular ion transport

Although the HCO^-_3 details remain to be defined, it is clear that the underlying driving force for trans-endothelial ion-linked water transport ultimately resides in the

ouabain-sensitive sodium pump (Na^+/K^+-ATPase). Na^+/K^+-ATPase is a plasma membrane protein composed of two or three polypeptides (Wetzel et al., 2001). The critical role of the Na^+/K^+-ATPase in the transfer of the solute and water from the non-pigmented epithelial cells to aqueous humour has been demonstrated (Ellis et al, 2001). Na^+/K^+-ATPase which contributes to the transport of ions and the formation of aqueous humour in the ciliary processes catalyses the transfer of $2K^+$ from the extracellular space into the cell and the extrusion of $3Na^+$, while hydrolysing adenosine triphosphate to adenosine diphosphate and inorganic phosphate (Ellis et al, 2001). The resultant electrochemical gradient is harnessed by other cellular proteins, including ion transporters and cotransporters, and is thought to constitute the major energy source that drives the transepithelial transport of ions needed in the formation of aqueous humour.

Current evidence indicates that chloride ions may be the major, or lead, ion in the transport of fluid from the blood into the eye. Chloride channels in the non-pigmented epithelial cells have been suggested to be critical to the formation of aqueous humour, as well as in volume regulation of these cells (Jacob and Civan, 1996). The nature of the volume-activated chloride current in non pigmented epilithial cells has been investigated using pharmacological, molecular and immunocytochemical techniques. In rabbit non-pigmented epithelial cells, it was found that increases in cell volume increased intracellular calcium and activated a chloride channel, although there was no evidence that calcium or cyclic adenosine

monophosphate was involved in the regulation of the chloride channel (Botchin and Matthews, 1995).

Recent studies identified that at least 60% of the aqueous humour is formed by active secretion in bovine eyes (Shahidullah et al., 2003). Transport of anions, particularly the chloride ion, through the ciliary epithelium plays a crucial role in aqueous humour formation. In this investigation, an in vitro whole-eye preparation was used to study the effects of known inhibitors of ion transport on aqueous humour formation. The inhibitors used included Ouabain, a selective inhibitor of Na^{-}/K^{+}-ATPase; bumetanide, a specific inhibitor of NKCC (Na-K-2Cl) cotransport; furosemide, a nonspecific inhibitor of anion transport; (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) DIDS which is believed to inhibit the Cl^{-}-HCO^{-}_3 exchanger, Na^{+}-HCO^{-}_3 cotransporter and chloride channel; NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid), the chloride channel blocker in nonpigmented cells; and acetazolamide, a carbonic anhydrase inhibitor.

The finding of this study (Shahidullah et al., 2003) was that ouabain (1.0 mM), played a key role in producing a significant reduction in aqueous humour formation (46% and 42% when added to the stromal or aqueous side, respectively). When added to both sides it produced a reduction of 61%. Bumetanide (0.1 mM) and furosemide (0.1 mM), produced 35% and 45% reductions when applied to the stromal side. DIDS (0.001–0.1 mM), produced a dose-dependent reduction when added to the stromal side. The inhibition was 55% by the highest concentration used.

5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; 0.1 mM), produced a 25% reduction when applied to the aqueous side. Acetazolamide (0.1 mM), applied to the stromal side, produced 31% reduction.

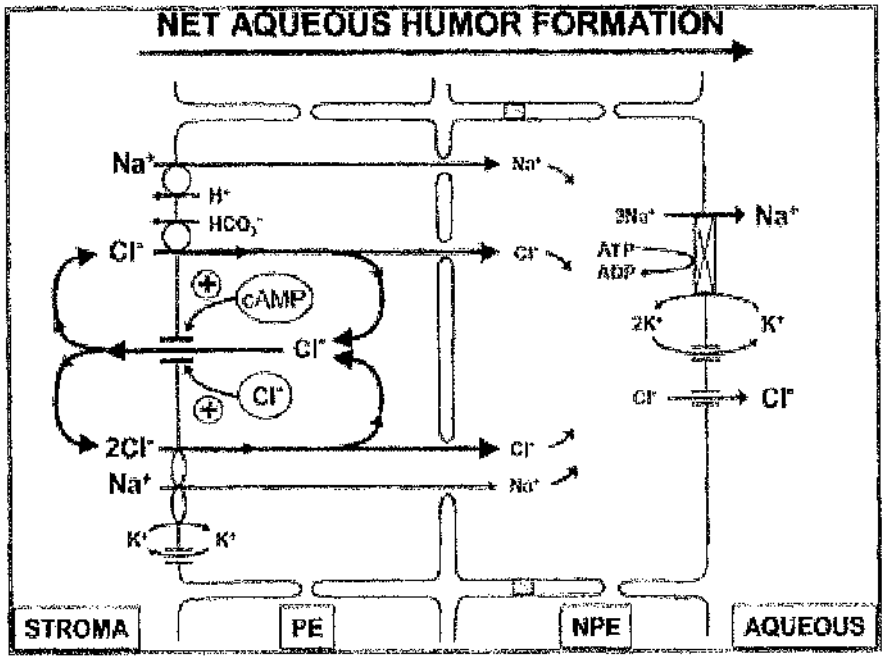

Figure 1.5: Formation of aqueous humour: abridged hypothesis, emphasising possible role of Cl^- recycling. Na^+-K^+-$2Cl^-$ cotransporter and coupled Cl^-/HCO_3^- and Na^+/H^+ parallel exchangers are principal pathways for the uptake of Na^+ and Cl^- into the pigmented epithelial (PE) cells. Tight junctions are present only between nonpigmented epithelial (NPE) cells and not between PE cells. (Civan et al., 2004)

These data support the conclusion that in the isolated bovine eye the aqueous is formed mostly by processes involving active secretion and chloride transport, indicating that anionic, specifically chloride, rather than cationic transport, is involved in the aqueous humour formation (Crook et al., 2000; Holland et al., 1970).

Figure 1.5 illustrates a simplified model of aqueous humour formation. Chloride ions can enter from the ciliary stroma into the pigmented epithelial cells through two main electroneutral pathways: the Na^+-K^+-$2Cl^-$ co-transporter (electroneutral symport) and parallel Na^+/H^+ and Cl^-/HCO_3^- exchangers. Chloride ions can then diffuse through the intercellular gap junctions to the non-pigmented epithelial cells and finally exit through chloride channels to the posterior chamber of the eye (Civan et al, 2004)

1.3.3 Regulation of aqueous humour drainage

Understanding the potential regulatory mechanisms influencing the aqueous humour outflow facility has important implications for unravelling the aetiology of glaucoma and developing better therapy. The trabecular meshwork has smooth muscle like properties and is actively involved in aqueous humour dynamics through contractile mechanisms (Khurana et al., 2003). The contractile and relaxation properties of trabecular meshwork and Schlemm's canal cells are believed to influence morphological and cell adhesion characteristics and thereby through these mechanisms could alter paracellular permeability and the geometry of the aqueous outflow pathway (Wiederholt et al., 2000).

The cGMP/NO system has long been recognised to play a vital role in regulating aqueous humour drainage (Nathanson et al., 1987). Direct application of membrane-permeable cGMP or activation of the intracellular cGMP/NO system by organic nitrates or non-nitrate vasodilators had a relaxing effect on ciliary muscle and trabecular meshwork, more pronounced in trabecular meshwork (Weiderholt et al.,

2000). This mechanism should lead to an increase of aqueous humour outflow and a reduction of intraocular pressure. The relaxing effect of cGMP/NO in trabecular meshwork cells was demonstrated to be mediated via a calcium-dependent maxi-K^+ channel (Stumpff et al., 1997). This channel appears to be most important for the regulation of smooth muscle tone and is a target protein mediating the effect of various relaxant substances.

1.4 Mobilisation of intracellular calcium

1.4.1 General introduction

The universality of calcium as an intracellular messenger depends on its enormous versatility. Cells have a calcium signalling toolkit with many components that can be mixed and matched to create a wide range of spatial and temporal signals (Berridge, 1997). This versatility is exploited to control a vast array of cellular functions (probably including aqueous humour secretion). In most cells, the generation of receptor-induced Ca^{2+} signals is complex involving two interdependent and closely coupled components: rapid, transient release of Ca^{2+} stored in the endoplasmic reticulum, followed by slowly developing extracellular Ca^{2+} entry (Berridge, 2003).

1.4.2 Calcium-dependent signal transduction

Calcium mobilizing receptors require phospholipase C hydrolysis of the membrane lipid phosphoinositide 4,5-bisphosphate into the second messengers, inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG) to affect the intracellular calcium levels (Chen et al., 1996). $InsP_3$ is a messenger that controls many cellular processes by generating internal calcium signals. It operates through receptors whose molecular and physiological properties closely resemble the calcium-mobilizing ryanodine receptors of muscle (Berridge, 1997). This family of intracellular calcium channels displays the regenerative process of calcium-induced calcium release responsible for the complex spatiotemporal patterns of calcium waves and oscillations. Many of the cellular actions of calcium are mediated by its binding to specific calcium-binding proteins, forming complexes which in turn activate specific kinases (Berridge, 1997).

In recent years, signal transduction mechanisms have been the subject of major investigation. Emerging evidence has made it increasingly clear that different cellular signalling systems interact and that signal transduction is not simply a linear process. The enhancement of Ca^{2+} release obtained from the concomitant or sequential activation of two types of receptor that are coupled to different G-proteins is of particular interest (Werry et al., 2003). For example, in rabbit non-pigmented ciliary epithelial cells, adenosine and adrenaline potentiate the elevation in $[Ca^{2+}]i$ induced by acetylcholine (ACh) through a mechanism involving communication between Gi- and Gq-proteins (Farahbakhsh et al., 1997). Bradykinin, carbachol and

histamine among many others, were demonstrated to activate the $InsP_3$/ diacylglycerol pathway within transformed human non-pigmented ciliary epithelial cells (Yang et al., 1998).

Spontaneous intracellular calcium oscillations were reported in both ciliary epithelial cell layers, although much more commonly in the non-pigmented epithelial cells (Berridge, 1993). Within the same cell layer, heterogeneous patterns of calcium oscillations were found with respect to frequency, duration and peak amplitude, even in adjacent cells, possibly indicating a lack of continuity via gap junctions. The results support the idea that the $InsP_3$ sensitive Ca^{2+} stores are a major contributor to Ca^{2+} oscillations. Calcium oscillations mediated by $InsP_3$ are probably relevant for modulation of the secretory process in many cell types (Berridge, 1993). Spontaneous and generated Ca^{2+} oscillations, so well delineated, suggest an important function in these electrically inexcitable secretory ciliary epithelia (Giovanelli et al., 1996).

1.5 Drainage of aqueous humour

The aqueous humour drains from the eye via two routes, the so called conventional and uveo-scleral (or unconventional) routes (Brubaker et al, 1982). In the human eye, most of this aqueous humour (70–90%) leaves via the trabecular outflow while the remainder exits through the uveoscleral outflow (Forrester et al., 2002).

1.5.1 Trabecular outflow (conventional) route

Most aqueous humour drains via specialised tissues situated in the angle of anterior chamber, located at the conjunction of iris, cornea, and sclera. Beginning at the anterior chamber and moving exteriorly, these tissues are the trabecular meshwork, a porous connective tissue; Schlemm's canal, a collecting duct lined by a vascular-like endothelium; and the collector channels/aqueous veins. The aqueous humour leaving the anterior chamber percolates through the trabecular meshwork into the canal of Schlemm which encircles the anterior chamber (Grierson, 1979; Greaney et al., 2002).

1.5.2 Uveoscleral (unconventional) route

The uveoscleral pathway is located behind the trabecular meshwork and is called the "unconventional" pathway. The uveoscleral outflow pathway was investigated using ^{125}I-labelled albumin injection (Bill, 1966). Label was observed in the iris, ciliary body, and in a thin tapering strip, extending from the tail of the ciliary body most of the way back to the optic nerve, that appeared to correspond to the choroid. The study indicated that uveoscleral outflow passes from the anterior chamber to the interstitial spaces among the ciliary muscle fiber bundles, to the suprachoroidal space where it transits toward the back of the eye, and exits through the sclera and, possibly, also through choroidal vessels. In the monkey eye in vivo, it may account for as much as 70% of total outflow (Bill, 1971; 1975). In human eyes, estimates

have ranged between 10% and 30% of total outflow depending on the age of the eye and method of measurement (Bill and Phillips, 1971).

1.6 Glaucoma

1.6.1 General introduction

Glaucoma is a generic term for an aetiologically heterogeneous disease causing progressive visual impairment that results from dysfunction and death of retinal ganglion cells. It is the leading neurodegenerative cause of blindness, and the second leading cause of blindness worldwide after cataract; (Quigley, 1996; Quigley; 2002; Marquis and Whitson. 2005). In Europe it is estimated that on average 13% of all blindness is caused by glaucoma and that 160,000 persons or 0.035% of the total population in the European Union are blind because of glaucoma (Poulsen at al. 2005). On the other hand there will be 60.5 million people with open angle glaucoma and angle closure glaucoma in 2010, increasing to 79.6 million by 2020, (Quigley, and Broman, 2006).

1.6.2 Intraocular pressure

Intraocular pressure (IOP) is the pressure caused by the fluid inside the eye that helps maintain the shape of the eye. This fluid, or aqueous humour, nourishes the cornea, iris, and lens, and it helps the eye maintain its globular shape. The typical human eye, produces about 4 mL of fluid a day, which is circulated and then drains out of the eye. The ocular walls have both elasticity and rigidity that helps to prevent

significant expansion of the eye if the intraocular pressure increases. The intraocular pressure is maintained by equilibrium between the forces pumping aqueous humour into the eye and those which tend to hinder its escape. If the drainage system becomes clogged or if too much fluid is produced, pressure inside the eye can build up.

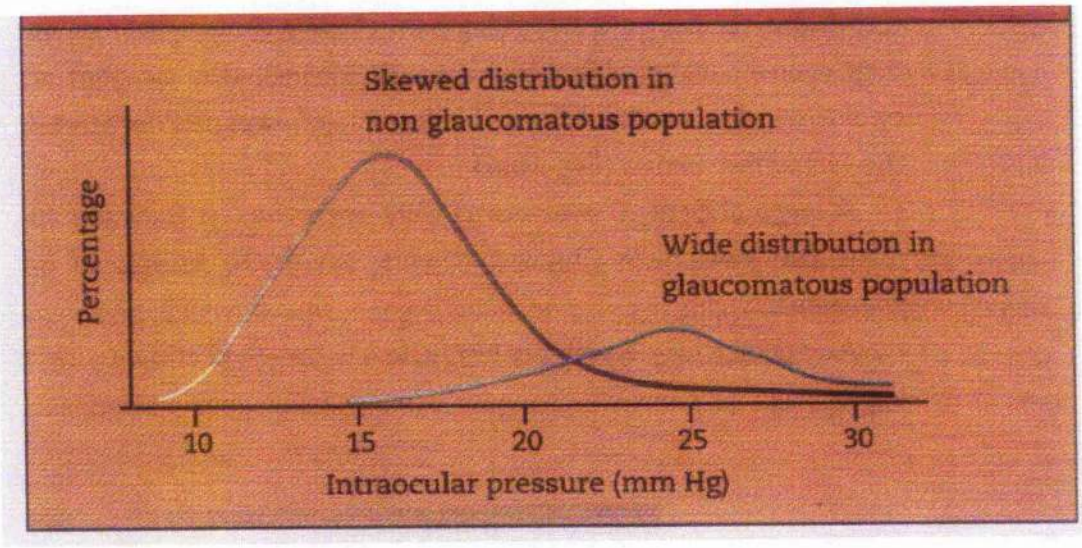

Figure 1.6: Distribution of intraocular pressure in a normal and glaucomatous population (James et al., 1997)

Normal eye pressure usually ranges between 10 and 21 mm of mercury, with an average of 16 mm Hg (Figure 1.6, James et al., 1997). Physical activity, stress, rapid fluid intake and caffeine can account for a small plus or minus change in an intraocular pressure reading. Pressure that is consistently above 21 mm Hg indicates ocular hypertension.

permeability of the skin and retina of type 1 diabetic rats and suggests that B_1-R antagonists could have a beneficial role in diabetic neuropathy and retinopathy (Lawson et al., 2005).

1.11 Aims and objectives of research

The project was aimed at investigating the actions and mechanism that BK and cGMP may have upon aqueous production within the eye. The effects on aqueous formation of BK and cGMP will be measured in the bovine isolated perfused eye. The mechanism of any effects of bradykinin will be studied using the drugs which are known to block several of the different mechanisms involved in the action of BK in other tissues. The procedure used in this study, was previously described by Shahidullah and Wilson (1993). If, as suspected, BK and cGMP reduce aqueous humour formation, an inhibitor of nitric oxide synthesis (L-NAME), an inhibitor of guanylate cyclase (ODQ) and the protein kinase G inhibitor (KT5823) will be used, to demonstrate whether the effect is mediated through nitric oxide or cyclic GMP. Other inhibitors will be used to test the involvement of a prostaglandin or EDHF in the mechanism. Published literature exists describing some aspects of the roles that $[Ca^{2+}]_i$ plays in aqueous humour formation. It was therefore, the intention to investigate the effect of both BK and cGMP on the movements of intracellular Ca^{2+} in cultured non-pigmented epithelial cells. The drugs detailed above to be used in the perfused eye will be also used to test the involvement of NO and cGMP in the release of mechanism Ca^{2+}. Other inhibitors will be used to test the involvement of a prostaglandin or EDHF. It is expected that the research will bring more insight into the function and mechanism that BK and cGMP may have upon aqueous production within the eye.

Chapter 2: Material and Methods

2 Materials and methods

2.1 The bovine perfused eye model

The method for dissection and setting up the constant flow method for the bovine perfused eye as well as the estimation of the aqueous humour formation rate were described by Wilson, et al (1993) and Shahidullah, et al (2003) (Figure 2.1). Bovine eyes were obtained from the local abattoir (Sandyford Abattoir, Renfrewshire, Scotland) within15 minutes of slaughter and transported to the laboratory. Temperatures during transportation depended on seasonal variation ranging from 2-20°C. The eyes were not transported on ice since the resulting solidity of orbital fatty tissue may hamper dissection and cannulation (Wilson et al., 1993). Immediately after transportation to the laboratory, excess adnexal tissue was trimmed from the eye. 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 was left attached to the globe. The ophthalmic artery was cleared of fat and cannulated with a polythene cannula distal to the point where it divides into the two long posterior ciliary arteries. After cannulation the eye was placed in a warming jacket at 37°C and covered with an insulated plastic cup to keep it warm and to prevent drying. The long posterior ciliary artery was perfused with Krebs' solution at 37°C within 60 minutes of enucleation from the animal.

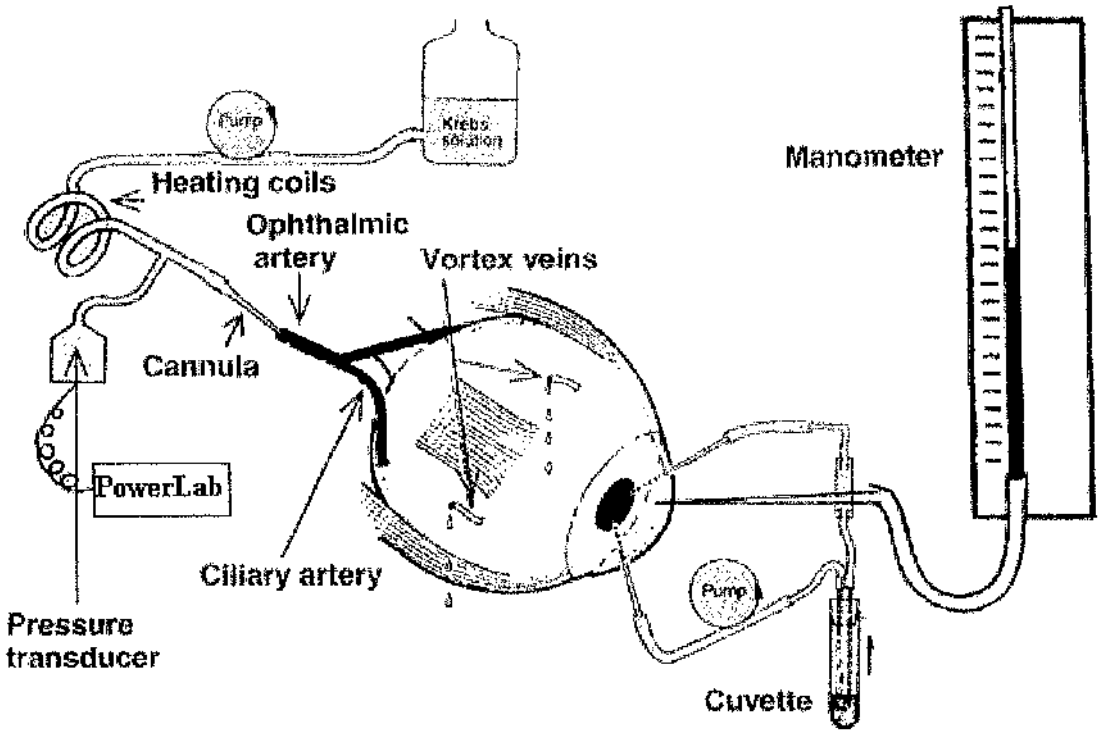

Figure 2.1: The bovine isolated eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under conditions of constant flow rate. Perfusion pressure and intraocular pressure were both monitored (after Wilson et al., 1993).

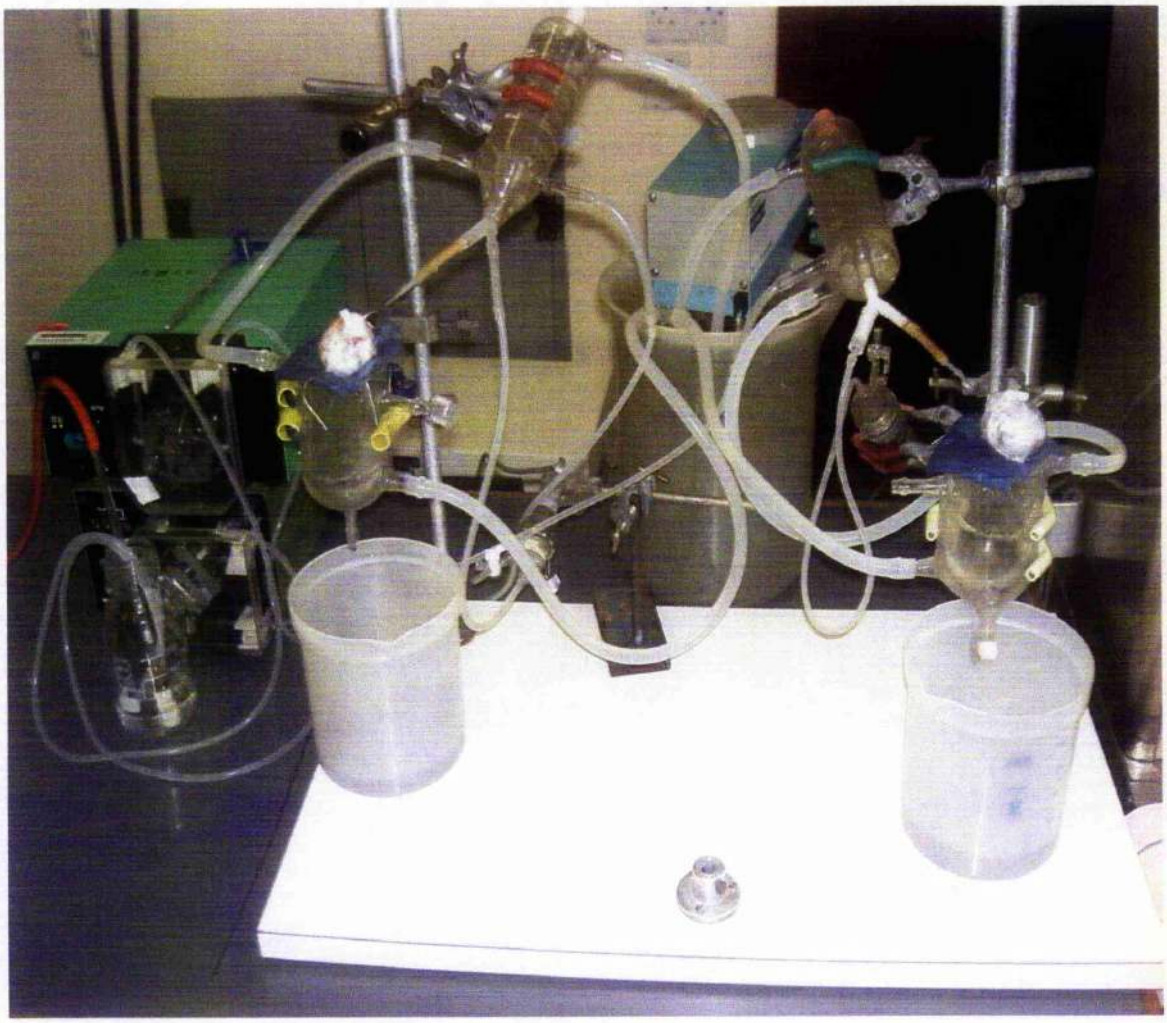

Figure 2.2: The bovine isolated eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under conditions of constant flow rate. Perfusion pressure and intraocular pressure were both monitored (after Wilson et al., 1993).

The Krebs' solution consisted of a mixture comprising (mM) sodium chloride (118), potassium chloride (4.7), magnesium sulphate (1.2), calcium chloride (2.5), sodium bicarbonate (25), potassium dihydrogen phosphate (1.2), glucose (11.5) and ascorbate, (0.05). The pH of this solution was adjusted to 7.4 by bubbling with oxygen containing 5% carbon dioxide both prior to and throught out the perfusion. Flow of Krebs' solution was induced through the vasculature using a peristaltic pump (Watson Marlow, Cornwall, England). At least two vortex veins out of four 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 (Fig.2). At the beginning of the experimental procedure the flow rate of Krebs solution was initially adjusted at 0.3 mL/min and was gradually increased to 2.25 mL/min over a 25 minute period.

2.1.1 Measurement of vascular pressure

The arterial perfusion pressure was monitored by a pressure transducer (Gould Statham, P32ID) close to the arterial cannula. Pressure was recorded by a Powerlab data acquisition system (AD instruments, Hastings, UK). Flow rate of Krebs solution was increased stepwise in such a way that perfusion pressure did not exceed 100 mmHg. Thereafter, the blood vessels gradually relaxed so that the perfusion pressure generally remained in the range 30-50 mmHg throughout the experiment. Eyes exhibiting vascular perfusion pressure above 100 mmHg were excluded from the study.

2.1.2 Measurement of intraocular pressure

After approximately 40 minutes the aqueous humour secretion restarted and the cornea was cannulated with a 23G needle, after the anterior chamber was carefully observed to make sure that the eye was firm and there was no folding of the iris. Intraocular pressure was measured manually with a water manometer at 5 min intervals. Silicon rubber tubing (i.d 0.5mm) connecting the needle and the manometer was filled with aqueous humour substitute: comprising (mM): NaCl, 110; KCl, 3; $CaCl_2$, 1.4; $MgCl_2$, 0.5; KH_2PO_4, 0.9; $NaHCO_3$, 30; glucose, 6; ascorbate, 3 and fluorescein, 0.0186. The pH of this solution was adjusted to 7.6 by bubbling oxygen containing 5% carbon dioxide. Eyes showing IOP above 160 mm H_2O were excluded from this study.

2.2 Estimation of aqueous humour formation rate

The rate of aqueous humour was determined using the fluorescein dilution technique, with the aqueous fluorescein concentration being continuously monitored by a Perkin Elmer LS-3B fluorescence spectrophotometer. Another two 23G needles were inserted into the anterior chamber enabling circulation of the aqueous humour via a Watson–Marlow peristaltic pump through a micro-cuvette in a spectrophotometer. The aqueous humour returned to the anterior chamber via the second 23G needle. In order to optimise the mixing process, the tips of the two circulating needles in the anterior chamber were kept crossed. The aqueous humour was transferred continuously from the anterior chamber through silicon rubber tubing to the fluorescence spectrometer cell

vehicle administration (-30 to +10 min control) was then compared statistically with those during the first period (+ 10 to +50 min after drug or vehicle administration) and the second period (+ 50 to + 100 minute after drug or vehicle administration) using one-way analysis of variance and paired Student's t-test.

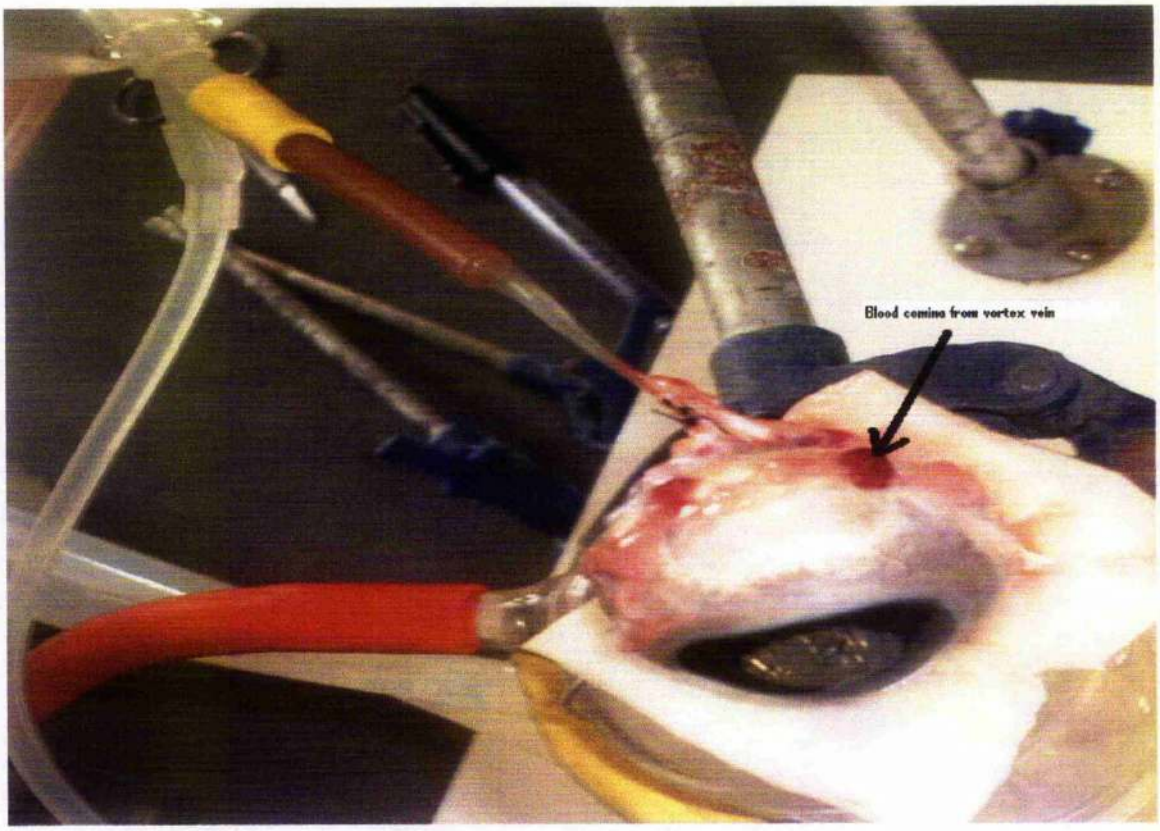

Figure 2.3: Perfused eye where appearance of small amounts of blood from the ends of the vortex veins indicate successful perfusion.

where the fluorescence of the solution was measured. The experimental set up is shown in Figure 2.2.

The capacity of the micro-cuvette was approximately 200 μl and of the entire system (mico-cuvette plus tubing system) was 1.04 ml aqueous humour substitute comprising (mM): NaCl, 110; KCl, 3; $CaCl_2$, 1.4; $MgCl_2$, 0.5; KH_2PO_4, 0.9; $NaHCO_3$, 30; glucose, 6; ascorbate, 3 and fluorescein, 0.0186. The pH of this solution was adjusted to 7.6 by bubbling oxygen containing 5% carbon dioxide. After the initial mixing period which lasted approximately 15 minutes and every 5 minutes thereafter the fluorescence of the solution was recorded at an excitation wavelength of 490 nm and emission of 515 nm using the fluoro-photometer. The data were collected using Microsoft Excel and the natural log of the fluorescence concentration was plotted against time using Minitab. The rate of aqueous humour formation (K_{out} min^{-1}) was determined using the slope of the regression line obtained.

After approximately 30 to 40 minutes, when visual inspection of the data indicated a constant rate of aqueous humour formation, the drug or vehicle under examination was added to the arterial perfusate and the fluorescence of the aqueous humour solution was recorded at 490 nm for a further 90 minutes. Throughout the experiment progressively weaker signals were obtained due to continuing dilution of fluorescein by freshly formed aqueous humour. The rate of fluorescence decrease in the 30 minutes before drug or

2.2.1 Criteria for accepting/rejecting eyes

Drugs were introduced to any perfused eye, which after an equilibration period of 30 to 60 minutes, maintained a stable IOP ranging between 60 to 180 mm H_2O, an arterial perfusion pressure below 100 mm Hg at any time of the experiment and had at least two vortex veins flowing and a steady decline in fluorescence indicating aqueous humour formation.

2.3 Administration of drug

In most cases, the drug examined was BK in the presence/absence of another drug, in order to determine the mechanism by which BK altered aqueous humour formation. Prior to administering the drug or vehicle, a stable IOP, the required perfusion rate (2.25 ml/min) and steady fluorescence decline had all been attained for a period of least 30 minutes. The drug solution or vehicle was administered by one of two routes:

1. By addition of drug to the perfusate reservoir at an exact concentration.
2. As a bolus dose injected intra-arterially in a volume of 3-10 µL using a micro syringe.

Intra-arterial injections were administered immediately proximal to the arterial cannula through re-sealable natural rubber tubing. Stock solutions were prepared by dissolving the drugs either in dimethylsulphoxide (DMSO) or distilled water according to their solubility.

2.3.1 Bradykinin

BK was dissolved in the perfusate reservoir at concentrations of 10^{-9} M, 3 x 10^{-9} M or 10^{-7}M. There was approximately 10 minutes lag time (determined empirically) before the compound entered the anterior segment. Fluorescence within the closed anterior chamber perfusion system was monitored continuously by the spectrofluorometer, though readings were recorded only once every 5 minutes. The data were recorded for 30 min prior to, and 90 minutes after addition of the drug.

2.3.2 8-Bromo-cGMP

In this study, the concentration of 8-Bromo-cGMP added to the perfusate was between10^{-6}M and 10^{-11}M. The introduction of the 8-bromo-cGMP solutions into the eye was carried out using the procedure described in Section 2.3.1. Once more, the data were recorded for 30 min prior to, and 90 minutes after addition of the drug 8-Br-cGMP.

2.3.3 Accessory drugs

The effect of each drug on the aqueous humour formation in the perfused eye was investigated in the presence and absence of BK. In each experiment, the rate of aqueous humour formation was calculated every ten minutes after the drug administration using the fluorescein dilution technique, with the aqueous fluorescein concentration being continuously monitored by the fluorimeter.

2.3.4 L-NAME

The effect of nitric oxide synthase inhibitor, L-NAME, on the action of BK was investigated in order to show whether or not nitric oxide is involved when BK decreases aqueous humour formation. After the equilibration period of about 30 minutes, L-NAME was added to the Krebs reservoir for continuous infusion. Once a stable rate of aqueous humour formation was established, responses to L-NAME (10^{-4}M) or a mixture of BK (10^{-9}M) with L-NAME (10^{-4}M) were assessed. As usual, the data were recorded for 30 min prior to, and 90 minutes after addition of the drug.

2.3.5 ODQ

The effect of ODQ, an inhibitor of soluble guanylate cyclase and a combination of ODQ with BK were investigated. Once a perfusion pressure suitable for conducting the experiment was established, responses to ODQ (3×10^{-7}M) or a mixture of BK (10^{-9}M) with ODQ (3×10^{-7}M) were assessed and the data were recorded for 30 min prior to, and 90 minutes after addition of the drug.

2.3.6 UK-114,542

The effect of solutions containing UK-114,542, a selective inhibitor of cGMP- specific phosphodiesterase type five, or a mixture containing UK-114,542 (10^{-6} M) and BK (10^{-9}M) were evaluated. Fluorescence within the closed anterior chamber perfusion system

was monitored continuously by the spectrofluorometer, and readings were recorded every 5 minutes. The data were recorded for 30 min prior to, and 90 minutes after addition of the drug.

2.3.7 KT-5823

KT-5823, a specific inhibitor of protein kinase G, was injected as a bolus dose of 3 nmol, every 15 minutes for a total of four injections. Following the initial dose, in a separate series of experiments, BK ($3x10^{-9}$M) was added to the Krebs solution at the same time as the first of the four doses of KT-5823.

2.3.8 Clotrimazole

Clotrimazole, an inhibitor of the action of EDHF, was added to the Krebs solution at a concentration of $3x10^{-5}$M. In a separate series of experiments, a combination of Clotrimazole ($3x10^{-5}$M) and BK (10^{-9}M) were added to the perfusate reservoir Once more, the data were recorded for 30 min prior to, and 90 minutes after addition of the drug.

2.3.9 Flurbiprofen

The effects of the cyclo-oxygenase inhibitor, flurbiprofen, were investigated. Furbiprofen was added to the perfusate at concentrations of 10^{-5}M alone or in combination with BK (10^{-9}M). The effects of the flurbiprofen alone or the mixture of

flurbiprofen and BK were evaluated using the experimental procedure described in section 2.3.8.

2.3.10 Ascorbate

For the majority of experiments ascorbate was added to the modified Krebs solution as recommended by Wilson et al (1993). In a number of experiments the ascorbate was omitted from the Krebs solution. Under these conditions the effects of BK (10^{-9}M) or cGMP (10^{-8}M) were tested using the experimental procedure described in section 2.3.8.

2.3.11 DMSO

The effect on aqueous humour formation and/or intraocular pressure of dimethyl sulphoxide (DMSO) used in dissolving KT-5823 was assessed by adding DMSO 3 µl to the Krebs solution perfusing the vasculature.

2.4 Intracellular free calcium measurements within ciliary epithelial cells

The membrane-permeant acetoxymethyl ester form of the Ca^{2+}-sensitive dye fura-2 was used to monitor changes in intracellular free calcium in cultured ciliary epithelial cells freshly prepared from bovine eyes. A rise in $[Ca^{2+}]_i$ causes a corresponding rise in the Fura-2 fluorescence ratio recorded from cells loaded with this dye, and this allows receptor mediated changes in $[Ca^{2+}]_i$ to be monitored using standard, microspectrofluorimetric techniques (Grynkiewicz et al., 1985).

2.4.1 Dissection of bovine eye

During dissection, great care was taken to avoid damage to corneal surfaces (epithelial and endothelial). All eyes were carefully examined, and those presenting defects, were discarded. Eyeballs were dissected using a posterior approach. In order to expose the vitreous body, two large linear cuts were made in the form of a cross, passing as far as the ora ciliaris retinae on each side and avoiding the optic nerve, thus allowing the reflection of the sclera. The vitreous body was then removed exposing the posterior lens capsule, which was then carefully removed together with the lens and anterior lens capsule in a single operation. The removal of these parts enabled the exposure of the ciliary processes. The tips of the ciliary processes lying on the iris were harvested with fine scissors.

2.5 Cell-culture of ciliary's epithelial cells

2.5.1 Addition of calcium-free buffer solution

The incubation of the ciliary processes was carried out in a Petri dish holding 15mL calcium-free buffer solution. Buffer solution included the combination of (mM), sodium chloride (142), potassium chloride (13.41), HEPES (4.82) and EDTA (0.25). The incubation was carried out by placing the Petri dish in an incubator with light agitation

for half an hour. The use of EDTA in the absence of calcium starts the process of disrupting the tissue.

2.5.2 Addition of collagenase solution

The incubated ciliary processes were then transferred to a solution of collagenase A. The preparation of 0.1% collagenase solution was carried out using a buffer containing (mM) NaCl, (66.73), KCl, (13.41), HEPES, (3.84); $CaCl_2$ (2.5), which was filter-sterilised using a single-use syringe-driven 0.22 µm Gelman filter (Gelman Sciences). The incubation of the ciliary processes using collagenase medium was conducted at 37° C and lasted half an hour.

2.5.3 Neutralization of collagenase solution

The neutralisation of the enzymatic effect was carried out by adding equal volumes of newborn calf serum (10%) and foetal calf serum (10%) to the collagenase solution. Dulbecco's modification of Eagle's Medium (DMEM) was then added to the partly digested tips collected. The processes were then disturbed by gently pipetting and expelling small volumes using a glass Pasteur pipette with a short tip that had been smoothed by heat. After this time, the ciliary processes were separated, leaving the DMEM solution containing suspended ciliary epithelial cells.

2.5.4 Separation by centrifugation and seeding onto coverslips

The DMEM solution containing suspended ciliary epithelial cells was then transferred into test tubes, centrifuged at a nominal speed of 300 g for approximately 3 minutes, after which time, pellets were collected from the test tubes and immersed in 5 mL of DMEM. The DMEM was prepared by using equal volumes of newborn calf serum (10%) and fetal calf serum (10%) containing gentamicin (200 µL/mL) prior to seeding onto five sterile coverslips (thickness of 0.1 mm and diameter of 22 mm) and incubated for a period of time ranging between 20 and 30 hours. The cells were incubated at 37°C in a humid incubator, containing 95% air and 5% CO_2.

At the end of the incubation period, the attached epithelial cells growing on the coverslips were transferred to 5 mL DMEM solution containing bovine serum albumin (1%) and fura-2 (1.5µM). The cells were then loaded with fura-2 for 40 min at 37° C with continuous mild agitation in a darkened environment containing 95% air and 5% CO_2.

After 40 mins loading, the cells were washed with modified Krebs' solution, mM composition was as follows: NaCl, (118); KCl, (4.8); $NaHCO_3$, (2.4); $MgSO_4$, (1.0); glucose, (11.0); HEPES, (10.0); $CaCl_2$, (1.8). After 20 minutes, the coverslips were mounted into a superfusion chamber attached to the stage of an inverted Nikon Diaphot

microscope and continually superfused with the same modified Krebs solution at a flow rate of 5 ml min^{-1}.

2.5.5 Fura-2-imaging

Loaded cells were imaged in 2D using an inverted Nikon Diaphot microscope equipped with a Nikon 40× oil immersion Fluor objective lens (NA = 1.3) and a cooled digital Cool Snap-HQ CCD camera (Roper Scientific/Photometrics, Tucson, AZ).

Epifluorescence excitation light was generated by an ultra high point intensity 75 W xenon arc Optosource lamp (Cairn Research, Faversham, Kent, UK) coupled to a computer controlled Optoscan monochromator (Cairn Research, Faversham, Kent, UK). The monochromator was set to 340/10 nm and 380/8 nm for the sequential excitation of Fura-2. Dichroics and emission filters were all purchased from Chroma Technology Corp., (Rockingham, VT). Fura-2 excitation light was reflected through the objective lens using a 400DCLP dichroic. Fura-2 fluorescence emission was passed through a 510 nm long pass emission filter and images were collected using a Cool Snap-HQ digital camera operated in 12-bit mode. Computer control of all electronic hardware and camera acquisition was achieved using Metafluor software (version 4.6.9; Molecular Devices Corp., Downing, PA).

Sequential images (2 × 2 binning) were collected every 2 s, exposure to excitation light was 80 ms/image, and all experiments were undertaken in the presence of extracellular Ca^{2+} in modified krebs solution as in section 2.2.4).

Ratio images were presented in MetaFluor intensity-modulated display mode (Tsien and Harootunian, 1990), which associates the color hue with the excitation ratio value and the intensity of each hue with the source image brightness. A region of no fluorescence adjacent to the cell was used to determine the average background level of fluorescence in the 340 and 380 nm channels. The background amount was then subtracted from each pixel in each channel. The background subtracted images were then expressed as a 340/380 nm ratio of each pixel. After determination of the upper and lower thresholds, the ratio value of each pixel was associated with one of the 24 hues from blue (low $[Ca^{2+}]_i$) to red (high $[Ca^{2+}]_i$). Agonist-evoked $[Ca^{2+}]_i$-signals were quantified by measuring the fluorescence ratio at the peak of each Ca^{2+}-response and subtracting from it the ratio measured immediately prior to stimulation, (*i.e.* difference between the base-line resting ratio level and that attained at the peak response). An example of the pseudo-colour allocated to the fluorescence ratio is shown (Figure 2.4).

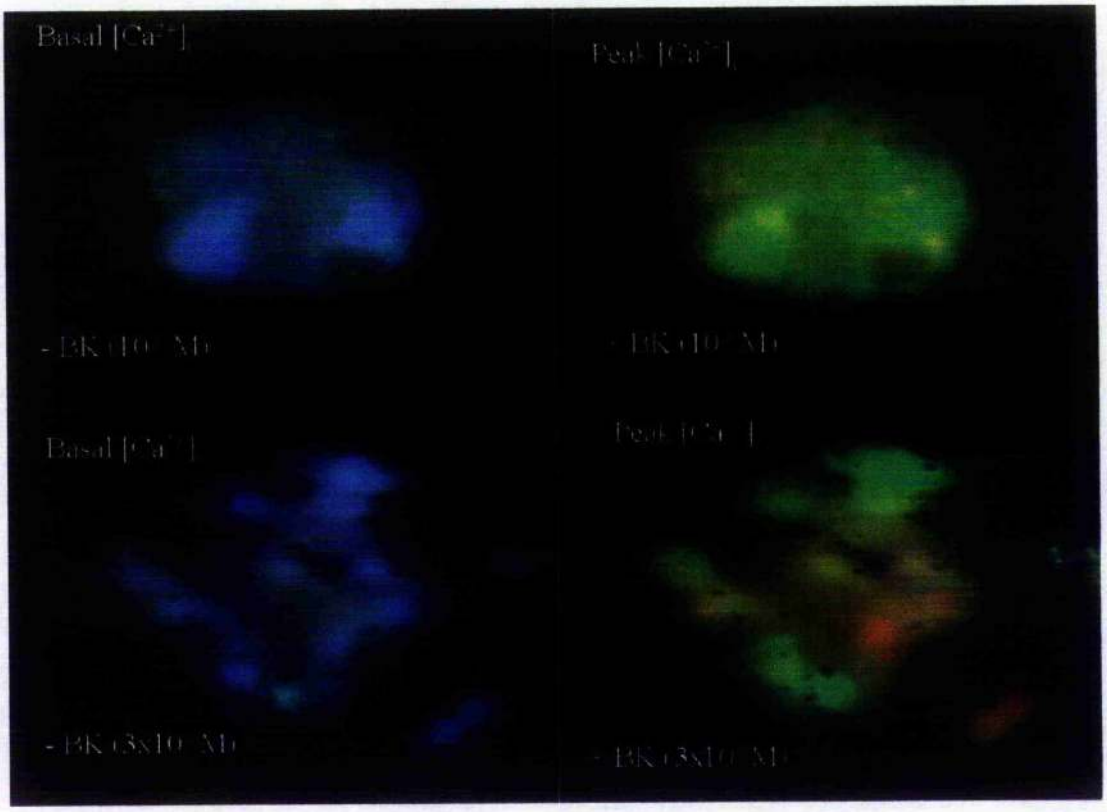

Figure 2.4: Examples of the Pseudo-colour allocated to the fluorescence.

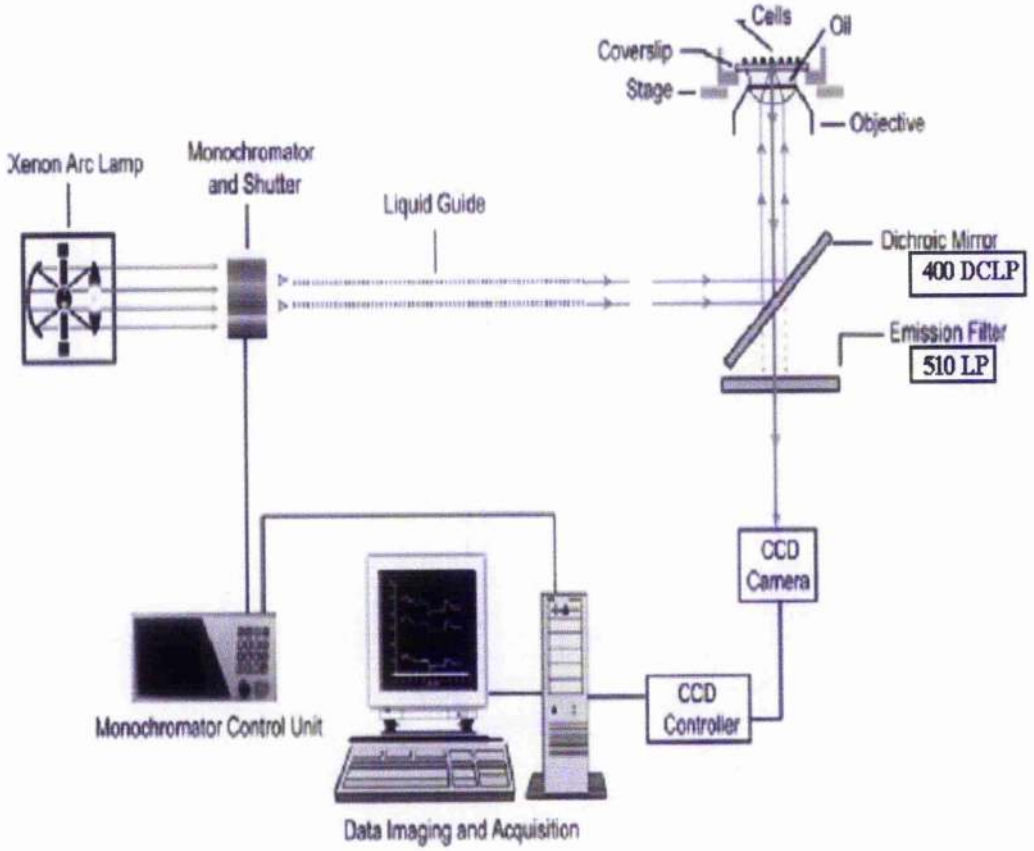

Figure 2.5: Experimental set up for the measurement of intracellular calcium.

2.5.6 $[Ca^{2+}]_i$ image analysis

Pooled average intensity-modulated display ratio intensity values measured from single cells were exported into PRISM 4.03, (GraphPAD Software, San Diego, CA) for statistical analysis and graphing. All data were expressed as the mean ± S.E. mean of at least 4-40 cells with the vertical lines representing S.E. of mean. Values of *n* refer to the number of cells in each experimental group, and the statistical significance of any difference between mean values was determined using a Student's *t* test.

An increase in $[Ca^{2-}]_i$ will cause a rise in the fluorescence (340/ 380 nm) ratio recorded from cells loaded with fura-2, thus allowing changes in $[Ca^{2+}]_i$ to be recorded using dual ratio excitation epifluorescence microscopy (Grynkiewicz et al. 1985).

Absolute calibration of fura-2 $[Ca^{2+}]_i$ -signals is very difficult to achieve as the Kd for fura-2 is extremely pH-dependent (Highsmith et al. 1986). Therefore without knowing what the pH is in the volume that contains fura-2, absolute $[Ca^{2+}]_i$ calibration measurements are next to impossible to achieve. F or example, if pH changes by only a few tenths, of one unit, the fura-2 dye would indicate a change in $[Ca^{2+}]_i$ when none had actually taken place. Due to this problem, changes in $[Ca^{2+}]_i$ were recorded from individual cultured non-pigmented epithelial (NPE) cells and expressed as a fura-2 fluorescence ratio intensity values. In order to minimise variation in the responses,

testing of BK and of BK in presence of each accessory drug (L-NAME, ODQ, etc.) was carried out on the same batch of cultured cells.

2.6 Addition of drug under investigation

An initial period of approximately 3 minutes was observed, during which the fluorescence ratio was monitored, as described above. Following this period the drug under investigation was added to the superfusing solution. Individual protocols varied slightly, as described, depending on whether pre-treatment or post-treatment addition of another compound was required. The reagents were all added to the modified Krebs' solution. By the use of a switch system it was possible to change easily between the solutions containing different drugs.

2.6.1 Bradykinin

After an initial 3-minute 'steady-state' period BK was added to the superfusing solution at concentrations of 10^{-9}M, $3x10^{-9}$M, 10^{-8}M or $3x10^{-8}$M and superfused for 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.2 L-NAME

BK at a concentration of 3 x 10^{-9}M or 10^{-9}M was tested in the presence of L-NAME (10^{-4}M). An initial 3 minute 'steady-state' period, was observed. L-NAME (10^{-4}M) was then added to the superfusing solution for a period of a further 3 minutes. After this time, both L-NAME and BK (3 x 10^{-9}M or 10^{-9}M) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.3 ODQ

BK at a concentration of 3 x 10^{-9}M or 10^{-9}M was tested in the presence of ODQ, (3 x 10^{-7}M). An initial 3-minute 'steady-state' period, was observed. ODQ, (3 X10^{-7}M) was then added to the superfusing solution for a period of a further 3 minutes. After this time, both ODQ and BK (3 x 10^{-9}M or 10^{-9}M) were superfused, for a further period of 3 minutes. A final 3 minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.4 Effects of 8-Br-cGMP

BK at a concentration of 3 x 10^{-8}M was tested in the presence of 8-Br-cGMP. An initial 3-minute 'steady-state' period was observed. 8-Br-cGMP at concentrations of 10^{-8}M, 10^{-9}M, 10^{-10}M, or 10^{-11} M was then added to the superfusing solution for a further period of 3 minutes. After this time, both 8-Br-cGMP and BK (3 x 10^{-8}M) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.5 KT-5823

BK at a concentration of 3x10^{-8}M or cGMP at a concentration of 1x10^{-8} M was tested in the presence of KT-5823, a specific inhibitor of protein kinase G. BK was used at a concentration of 3 x 10^{-8}M and cGMP 10^{-8} M. In each case an initial 3 minute 'steady-state' period was observed. KT-5823 at 2.5 x 10^{-7} M was then added to the superfusing solution for a further period of 3 minutes. After this time, KT-5823, BK (3 x 10^{-8} M) and cGMP (10^{-8}) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.6 UK-114,542

BK at a concentration of 3 x 10^{-9}M was tested in the presence of the phosphodiesterase inhibitor,UK-114,542. An initial 3-minute 'steady-state' period was observed. UK-114,542 (10^{-6} M) was then added to the superfusing solution for a further period of 3 minutes. After this time, both UK-114,542 and BK (3 x 10^{-8}M) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.7 Clotrimazole

BK at a concentration of 3 x 10^{-8}M was tested in the presence of Clotrimazole, an inhibitor of EDHF. An initial 3-minute 'steady-state' period was observed. Clotrimazole (3 x 10^{-5} M) was then added to the superfusing solution for a period of 3 minutes. After this time, both Clotrimazole and BK (3 x 10^{-8} M) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously.

2.6.8 *Flurbiprofen*

BK at a concentration of 3 x 10^{-8}M was tested in the presence of Flurbiprofen. An initial 3-minute 'steady-state' period was observed. Flurbiprofen (10^{-5} M) was then added to the superfusing solution for a period of a further 3 minutes. After this time, both Flurbiprofen (10^{-5} M) and BK (3 x 10^{-8} M) were superfused, for a further period of 3 minutes. A final 3-minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout the duration of the experiments the fluorescence ratio was recorded continuously

2.7 Testing the integrity of the blood –aqueous barrier

A series of preparation experiments was run in which eyes were perfused for 120 minutes under the usual conditions but with bovine serum albumin (BSA, 1%) added to the Krebs solution throughout. Perfusion pressure, aqueous humour formation and intraocular pressure were monitored as before. At the end of each experiment with albumin dissolved in the Krebs solution, a sample of aqueous humour up to a volume of 1.5 mL was withdrawn from the anterior chamber of the eye. Aqueous humour samples from each eye were transferred to Eppendorf tubes for storage at –20°C and assay. The amount of protein contained in the aqueous humour was measured by protein assay.

2.7.1 Protein assay

The amount of protein in the aqueous humour was determined using the Bicinchoninic Acid (BCA) assay, an improved version of the Lowry (1951) procedure with several advantages. The protein assay was done using a Dynatech plate reader employing a 96-well plate. The assay uses Cu^{2+} ions that form bonds with certain amino acids (e.g. tryptophan, tyrosine) and are reduced by Bicinchoninic Acid under alkaline conditions to Cu^{2+} ions, forming a purple chelate that has maximum absorbance at a wavelength of 540 nm. The intensity of this colour, determined spectrophotometrically is proportional to the concentration of protein present in the well at low concentrations. At high protein concentrations standard curves determined by this method cease to remain linear resulting in a plateau.

2.7.2 Plate set-up

Each plate consists of 96 wells, with each well containing a maximum volume of 300 µL. Each sample was assayed in duplicate. A blank (distilled water) and 5 protein standards were included in the plate. This standard consisted of a solution of BSA (2 $mg.ml^{-1}$) and was used to construct a standard curve from which the protein content of the unknown samples can be calculated. Bicinchoninic Acid solution was added to 4% copper sulphate pentahydrate solution in the ratio of 50 parts to 1 part; the first two column of a 96 well microplate were filled with various mixtures of bovine serum albumin solution and distilled water, as shown in Table 2.1.

Table 2.1: Diagram indicating set-up of 96 well plate for assay of protein in aqueous humour. DW= distilled water, BSA = protein standard (2 mg.ml-1),AHS=Aqueous Humour Sample.

	1	2	3	4	5	6	7	8
A	10µL DW	2µL DW+8µL BSA	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS
B								
C	8µL DW+2µL BSA	0µL DW+10µL BSA	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS
D								
E	6µL DW+4µL BSA		190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS
F								
G	4µL DW+6µL BSA		190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	190µL BCA+10µL AHS	
H								

2.7.3 Calculation of results

The plate reader calculated the average absorbance of the duplicate samples, and then subtracted the absorbance readings of the blank wells from the absorbance readings for the standards and samples in the rest of the plate. The concentration of protein in each sample was calculated by identifying the absorbance reading on the Y-axis of the standard curve and reading the corresponding value on the X-axis. Any sample dilution factors were taken into account to find the concentration of protein in the aqueous humour.

2.8 Drugs and solvents

Acetic acid (from Fisher Scientific), Ascorbate (from May and Baker), bovine serum albumin (from Sigma Chemical Company), BK triacetate (from Sigma Chemical

Company), calcium chloride (from BDH Laboratory Supplies), Collagenase A (from Roche Diagnostics), Dulbecco›s modification of Eagles medium (DMEM) (from Gibco), Dimethyl sulphoxide (DMSO) (from Sigma Chemical Company), *D*-glucose (from Fisher Scientific), EDTA (ethylenediamine-tetraacetic acid disodium salt) (from Sigma Chemical Company), EGTA Ethylene Glycol Tetraacetic Acid (from Sigma Chemical Company), Fetal calf serum (From Gibco), Fluorescein sodium (2%) (from Chauvin), Gentamicin (From Gibco), HEPES, (from Sigma Chemical Company), Fura2/AM (from Calbiochem), Hydrochloric acid (from BDH), Newborn Calf serum (from Gibco), Potassium chloride, Magnesium Chloride, Magnesium Sulphate, Potassium Dihydrogen orthophosphate and Potassium Hydroxide (all from BDH Laboratory Supplies), Sodium Carbonate, Sodium Chloride, Sodium Hydrogen Carbonate and Sodium Hydroxide (All from Fisher Scientific), L-NAME (Ng-nitro-L-arginine methyl ester), (from Sigma Chemical Company), ODQ (1-H-[1,2,4] oxadiazole[4,3-a] quinoxalin-1-one), (from Sigma Chemical Company), Flurbiprofen, (gift from pro.W. Martin lab), Clotrimazole (gift from pro. W. Martin lab), UK-114,542; cyclic guanosine monophosphate (from Pfizer), KT-5823 (PKG inhibitor) (from Calbiochem Corporation), 8-bromo cyclic GMP (from Sigma Chemical Company), Bicinchoninic acid solution (From Sigma Chemical Company). Stock solution of drugs were prepared by dissolving either in distilled water or in DMSO according to their solubility. Stock solutions of drugs were added to the perfusate to obtain intended final concentration.

Chapter 3: Results

3 Results

3.9 Effects of BK on AHF within the bovine isolated arterially perfused eye

3.9.1 Control eyes

As illustrated in Figure 3.1, the start of fluorescein mixing in the perfused anterior chamber coincided with a fall by about 50% of the fluorescence reading. The mixing can be defined as the beginning of aqueous humour flow into the cuvette containing the fluorescein solution, resulting in the dilution of the concentrated fluorescein solution. This drop is one of the criteria for a successful experiment, since it shows that the anterior chamber perfusion has commenced.

As illustrated in Figure 3.1, after about 15 minutes of fluorescein mixing a steady-state aqueous humour formation rate was observed through monitoring the fluorescein dilution. This was continued for at least half an hour preceding drug addition. The collection of the data related to aqueous humour formation rate continued after administration of each drug, with 10 minutes allowance being made for the drug to travel from the Krebs reservoir through the warming coil and reach the ciliary artery. The data collected was continued for a further 90 minutes. The time at which the drug under investigation was added to the reservoir of modified Krebs solution perfusing the long posterior ciliary artery was considered to be time zero for expressing any drug-induced changes in aqueous humour formation. The aqueous humour formation for the period -30 to +10 min was therefore compared

with that for each of the two post-drug stages, namely +10 to +50 min and +50 to +100 min. These arbitrary time intervals were chosen on the basis of previous work (Williams, 2005) which showed that the effect of BK was seldom apparent before 40 min following its administration.

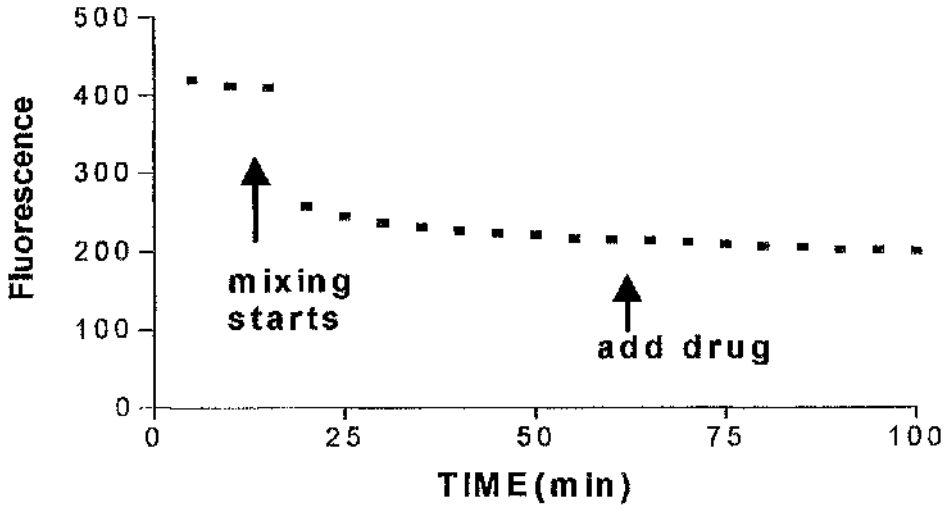

Figure 3.1: Experimental procedure for aqueous humour formation rate measurement.

3.9.2 Independent eye control

Further control experiments (Table 3.1) were carried out in which separate eyes were used as controls. During these experiments, aqueous humour formation rate was measured during the initial forty minutes stage, then in two further stages of 40 and 50 minutes each. Throughout the duration of these control experiments, the aqueous humour formation rate did not show any significant change. In these control eyes no drug was added at zero time. These experiments confirmed that in the absence of

any drug, aqueous humour formation remained constant for at least 2 hours, whether ascorbate was present or absent. (Refer to Table 3.1).

3.9.3 Same eye control

This procedure enabled each eye to act as its own control. The aqueous humour formation rate during the initial forty minutes stage preceding drug administration was compared that during the two later stages of 40 and 50 minutes each.

Table 3.1: Effect of ascorbic acid on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

	N	$K_{out}.min^{-1} \times 10^4$		Change (%)	$K_{out}.min^{-1} \times 10^4$	Change (%)
		Control -30 to +10 min	+10 to +50 min		+50 to +100 Min	
With ascorbic acid	29	30 ± 3	31 ± 2	3 †	28 ± 3	7†
Without ascorbic acid	13	31 ± 2	30 ± 3	3 †	30 ± 3	3†

†No significant change observed.

Changes shown in Table 3.1, indicate the percentage difference between the Aqueous humour formation observed during the initial control period (-30 to +10 min) and either the second (+10 to +50 min) or third (+50 to +100 min) periods. Each value is a mean of the rate of aqueous formation, expressed as $K_{out}.min^{-1} \times 10^4 \pm$ s.e. mean, of the number (N) of experiments shown.

3.9.4 Effect of 8-Bromo-cGMP on aqueous humour formation

The effect of 8-Bromo-cGMP on the aqueous humour formation rate was significant at all concentrations above 10^{-11} M. At high cGMP concentrations the aqueous

humour formation decreased considerably. The results suggested that the effect of cGMP on aqueous humour formation is concentration-dependent in the range 10^{-11} to 10^{-8} M. The data collected are shown in Table 3.2 and Figure 3.2.

Table 3.2: Effect of 8-Br cGMPon aqueous humour formation rate within the isolated arterially perfused bovine eye model.

8-Br cGMP (M)	N	$K_{out}.min^{-1} \times 10^{-4}$		Change (%)	$K_{out}.min^{-1} \times 10^{-4}$	Change (%)
		Control -30 to +10 min	+10 to +50 min		+50 to 100 Min	
10^{-6}	6	41 ± 5	26 ± 6	37***	24 ± 4	42***
10^{-7}	5	46 ± 6	29 ± 5	37*	27 ± 4	41**
10^{-8}	9	41 ± 3	26 ± 4	37***	25 ± 3	39***
10^{-9}	13	38 ± 4	29 ± 3	24*	28 ± 4	26*
10^{-10}	9	30 ± 4	25± 5	16†	21 ± 4	30**
10^{-11}	9	36 ± 4	32 ± 6	11†	30 ± 5	17†

†No significant change observed.

Aqueous humour formation rate was recorded during 30 min prior to drug infusion, then for 100 min after drug injection. Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period with the two following time intervals (+10 to +50 min and +50 to +100 min). Each value is a mean of the rate of aqueous formation, expressed as $K_{out}.min^{-1} \times 10^{4} \pm$ s.e. mean, of the number (N) of experiments shown. Significance of difference from initial "same eye" control period as indicated by paired Student *t*-test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

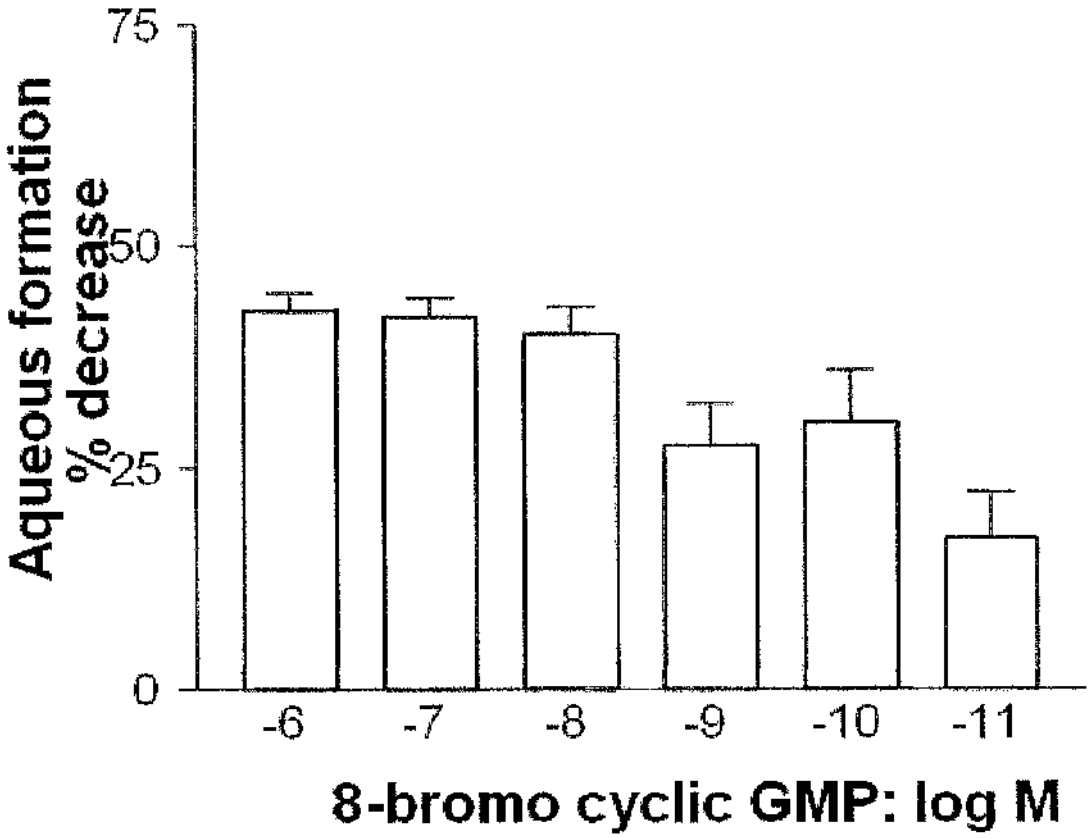

Figure 3.2. Data from table 2 are expressed graphically.

3.10 Effect of bradykinin on the aqueous humour formation

3.10.1 In the absence of any other drug

The effect of BK on the aqueous humour formation rate was considerable (Table 3). At high BK concentration (10^{-7} M) the aqueous humour formation decreased significantly throughout the second stage (40 minutes) to a rate of about 25±2, which suggests that BK had inhibited the aqueous humour formation. In the third stage (next 50 minutes), however, the aqueous humour formation rate decreased further. BKs at concentrations ranging from 3 x10^{-9} M to 10^{-9} M was also evaluated and the

results suggested that the effect was concentration-dependent and was somewhat delayed at 10^{-9} M, being only significant in the third time period.

Table 3.3. Effect of BK on aqueous humour formation rate within the bovine isolated arterially perfused bovine eye model.

BK concentration (M)	N	$K_{out}.min^{-1} \times 10^4$		Change (%)	$K_{out}.min^{-1} \times 10^4$	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to +100 Min	
10^{-7}	15	34 ± 2	25 ± 2	26***	20 ± 1	41***
$3x10^{-9}$	10	33 ± 2	22 ± 2	33***	12 ± 2	36***
10^{-9}	17	28 ± 2	25 ± 2	11†	22 ± 2	21**

†No significant change observed.

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 100 min after drug injection. Control ('same eye' control) represents values for eyes perfused with Krebs' solution before addition of any drug or reagent. Each eye thus acted as its own control, allowing comparison of aqueous humour formation rate over the initial period with the two following time intervals (+10 to +50 min and +50 to +100 min). Each value is a mean of the rate of aqueous formation (expressed as $K_{out}.min^{-1} \times 10^4$) ± s.e. mean of the number (N) of experiments shown. Significance of difference from initial "same eye" control period as indicated by paired Student t-test **p<0.01; ***p<0.001; † =not significant.

3.10.2 In the presence of nitric oxide synthase inhibitor

The experimental procedure used for BK testing was repeated in the presence of L-NAME, an inhibitor of nitric oxide synthase (Table 3.4.). L-NAME (10^{-4} M) infused on its own had no effect on the aqueous humour formation. However, when L-NAME combined with BK (10^{-9} M) was infused L-NAME blocked the inhibitory effect of BK.

Table 3.4: Effect of BK in presence of L-NAME, a nitric oxide synthase inhibitor, on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	K_{out}.min $^{-1}$ x 10^{4}		Change (%)	K_{out}.min $^{-1}$ x 10^{4}	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK 10^{-9} M	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
L-NAME 10^{-4}M	12	35 ± 2	34 ± 2	3†	33 ± 2	3†
L-NAME 10^{-4}M and BK 10^{-9} M	15	31 ± 2	26 ± 2	16†	29 ± 4	6†

Significance of difference from initial "same-eye" control period as indicated by paired Student t-test **$p<0.01$; † not significant.

3.10.3 In the presence of inhibitor of soluble guanylate cyclase

Experimental procedure similar to that used for L-NAME was utilised for ODQ. When the inhibitor of soluble guanylate cyclase ODQ (3 x 10^{-7} M) was administered on its own, the aqueous humour formation remained constant throughout the duration of the experiment suggesting that ODQ has no effect on the rate. When ODQ was administered simultaneously with BK (10^{-9}M), the rate remained constant suggesting that ODQ blocked the BK effect on the aqueous humour formation.

Table 3.5: Effect of BK in presence of ODQ, an inhibitor of soluble guanylate cyclase, on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	$K_{out}.min^{-1} \times 10^4$		Change (%)	$K_{out}.min^{-1} \times 10^4$	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3 †	28 ± 3	7†
BK 10^{-9} M	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
ODQ $3x10^{-7}$M	12	29 ± 2	29 ± 3	0	29 ± 4	0
ODQ $3x\ 10^{-7}$M and BK 10^{-9}M	13	37 ± 3	38 ± 3	3†	35 ± 3	5†

Significance of difference from initial "same eye" control period as indicated by Student t-test; **p<0.01, † not significant.

3.10.4 In the presence of the inhibitor of protein kinase G, KT5823

Injection of KT-5823 (10 nmol, four doses, one dose every 15 min starting at time zero) into the perfusate blocked the reduction of aqueous humour formation caused by BK ($3x\ 10^{-9}$ M) (Table. 3.6). Similar injection of KT 5823, into the Krebs perfusate in the absence of BK produced no effect (Table. 3.6).

Table 3.6: Effect of KT 5823 on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	$K_{out}.min^{-1} \times 10^4$		Change (%)	$K_{out}.min^{-1} \times 10^4$	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
DMSO (separate eye) (control)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK (3×10^{-9} M)	10	33 ± 2	22 ± 2	33*	21 ± 2	36**
KT-5823 in DMSO	8	38 ± 2	38 ±3	0	38 ±3	0
KT-5823 and BK (3×10^{-9}M)	13	39 ± 3	42 ± 6	8†	42 ± 6	8†

Significance of difference from initial "same eye" control period as indicated by paired Student t-test *p<0.05; **p<0.001, † not significant.

3.10.5 In the presence of UK-114,542 (phosphodiesterase type 5 inhibitor)

UK-114,542 on its own, at a concentration of (10^{-6}M), had a small but significant inhibitory effect on aqueous humour formation. When UK-114,542 (10^{-6}M) was infused simultaneously with BK (10^{-9}M), it appeared to increase the inhibitory effect of BK (Table.3.7).

Table 3.7: Effect of UK-114,542 on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	K_{out}.min^{-1} x 10^{4}		Change (%)	K_{out}.min^{-1} x 10^{4}	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK (10^{-9} M)	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
UK114,542 (10^{-6}M)	9	29 ± 2	27 ± 4	7†	24 ± 2	17*
UK114,542 (10^{-6}M) and BK (10^{-9}M)	8	35 ± 3	29 ± 4	17*	23 ± 2	34**

Significance of difference from initial"same eye" control period as indicated by paired Student t-test *$p<0.05$; **$p<0.001$, † not significant.

3.10.6 In the presence of clotrimazole

Clotrimazole is an EDHF inhibitor. Clotrimazole ($3x10^{-5}$M) on its own had no effect on aqueous humour formation. When clotrimazole ($3x10^{-5}$M) was infused simultaneously with BK (10^{-9}M), it did not block the effect of BK (Table. 3.8). If anything, clotrimazole produced an apparent increase in the effect of BK.

Table 3.8: Effect of clotrimazole on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	K_{out}.min $^{-1}$ x 10^{4}		Change (%)	K_{out}.min $^{-1}$ x 10^{4}	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK (10^{-9} M)	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
Clotrimazole (3x10^{-5}M)	18	30 ± 2	30 ± 3	0	29 ± 2	3†
Clo (3x^{-5}M) and BK(10^{-9}M)	18	30 ±1	25 ± 2	16†	19 ± 2	34 ***

Significance of difference from initial "same eye" control period as indicated by paired Student t-test **p<0.01; ***p<0.001; † not significant.

3.10.7 In the presence of flurbiprofen

FBP on its own, at a concentration of 10^{-5} M, had no effect on aqueous humour formation. When FBP (10^{-5}M) was infused simultaneously with BK (10^{-9}M), it did not block the effect of BK (Table 9). Indeed, there is if anything an apparently greater degree of aqueous humour formation inhibition by BK in the presence of FBP (Table. 3.9).

Table 3.9: Effect of Flurbiprofen on aqueous humour formation rate within the bovine isolated arterially perfused eye model.

Drug concentration	N	K_{out}.min $^{-1}$ x 10^{4}		Change (%)	K_{out}.min $^{-1}$ x 10^{4}	Change (%)
		Control -30 to +10 min	+10 to +50 Min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK (10^{-9} M)	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
FBP (10^{-5} M)	12	35 ± 2	35 ± 3	0	33 + 6	3†
FBP (10^{-5} M) and BK (10^{-9} M)	11	27 ± 2	23 ± 2	11**	20 + 2	26**

Significance of difference from initial "same eye" control period as indicated by paired Student t-test; **p<0.001, † not significant.

3.10.8 Effect of ascorbic acid on BK response

In this experimental procedure two sets of experiments were carried out. The first one included the addition of BK at time zero to the reservoir of standard modified Krebs that contained 0.05 mM ascorbic acid. In the second experiment, BK was added to the reservoir of modified Krebs prepared without ascorbic acid. The effect of BK on aqueous humour formation was similar in both experiments, suggesting that ascorbic acid had no effect on the response to BK. The results are shown in (Table 3.10.)

Table 3.10: Effect of BK on aqueous humour formation rate in presence and absence of ascorbic acid within the bovine isolated arterially perfused eye model.

Drug concentration	N	K_{out}.min^{-1} x 10^{4}		Change (%)	K_{out}.min^{-1} x 10^{4}	Change (%)
		Control -30 to +10 min	+10 to +50 min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
BK (10^{-9} M)	17	28 ± 2	25 ± 2	11†	22 ± 2	21**
Control without ascorbic acid	13	31 ± 2	30 ± 3	3 †	30 ± 3	3 †
BK (10^{-9} M) with no ascorbic acid	11	31 ± 2	27 ± 3	13†	23 ± 2	26**

Significance of difference from initial "same eye" control period as indicated by paired Student t-test; **p<0.001, † not significant.

3.10.9 Effect of ascorbic acid on cGMP response

In this experimental procedure two sets of experiments were carried out. The first one included the addition of 8-BrcGMP to the reservoir of modified Krebs that contained 0.05 mM ascorbic acid. In the second experiment, 8-BrcGMP was added

at time zero to the reservoir of standard modified Krebs prepared without ascorbic acid. The results are shown in (Table. 3.11).

Table 3.11: Effect of 8-Br-cGMP on aqueous humour formation rate in presence and absence of ascorbic acid within the bovine isolated arterially perfused eye model.

Drug concentration	N	$K_{out}.min^{-1} \times 10^{4}$		Change (%)	$K_{out}.min^{-1} \times 10^{4}$	Change (%)
		Control -30 to +10 min	+10 to +50 min		+50 to 100 Min	
Zero (separate eye)	29	30 ± 3	31 ± 4	3†	28 ± 3	7†
CGMP (10^{-8} M)	9	41 ± 2	26 ± 4	37***	25 ± 3	39***
cGMP 10^{-8}M with no ascorbic acid	11	38 ± 2	29 ± 3	24*	22 ± 3	42***

Significance of difference from initial "same eye" control period as indicated by paired Student t-test *$p<0.01$; ***$p<0.0001$, † not significant.

Williams (2005) suggested the hypothesis that barrier breakdown as indicated by high protein in aqueous humour may be correlated to a large increase in aqueous humour formation during the course of an hour perfusion experiment, to explain a rise in aqueous humour formation the perfusion of BK (10^{-7}M). The results are here expressed as ratio final/starting value of aqueous humour formation following such a perfusion experiment. The figure and table show that this correlation was poor (r^2 value=0.04) and so the hypothesis is not valid.

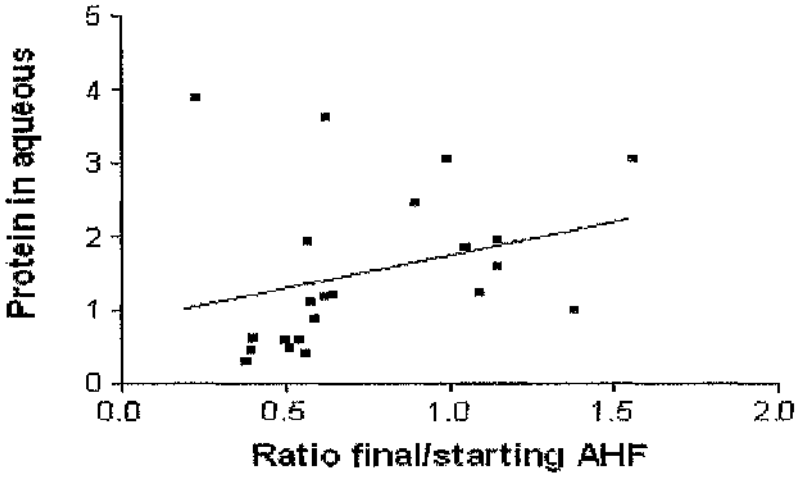

Figure 3.3: The relationship between the effect of BK (10^{-7} M) on aqueous humour formation and on the appearance of protein in the aqueous humour in the bovine perfused eye.

3.11 Effect of Bradykinin on intracellular calcium releases within non-pigmented ciliary epithelial cells

3.11.1 Arbitrary steady-state baseline fluorescence

As the calibration of absolute calcium concentration for a micro-spectrophotometric system for single cells was difficult to carry out, the intracellular calcium concentration was both recorded and expressed from individual cultured non-pigmented epithelial cells as the fluorescence ratio. The initial values for the fluorescence ratios (340/380 nm) were in the range 2.2 to 3.2 and the release of intracellular calcium by BK was revealed by a rise in the ratio that had an onset between 20 and 40 seconds after the start of drug superfusion (Figure. 3.4). The absolute resting values of $[Ca^{2+}]_i$ have been determined to be 120 nM in a population of bovine cultured non-pigmented epithelial cells (Shahidullah et al. 1997).

The $[Ca^{2+}]_i$ fluorescence baseline for a number of cells was obtained and the variation in the rate of onset of the BK effect was measured. The results were used to select an arbitrary time interval for data collection of 180 seconds period to BK superfusion. Of this 180-second delay, approx. 25s was accountable to the time taken between switching perfusate solutions and arrival of the drug in the chamber.

3.11.2 Ca^{2+} release by BK

Figures 3.4 and 3.5 show the release of calcium from bovine cultured ciliary epithelial cells by BK. The effect is clearly concentration-dependent. The time taken to reach the peak response also depends to some extent on concentration.

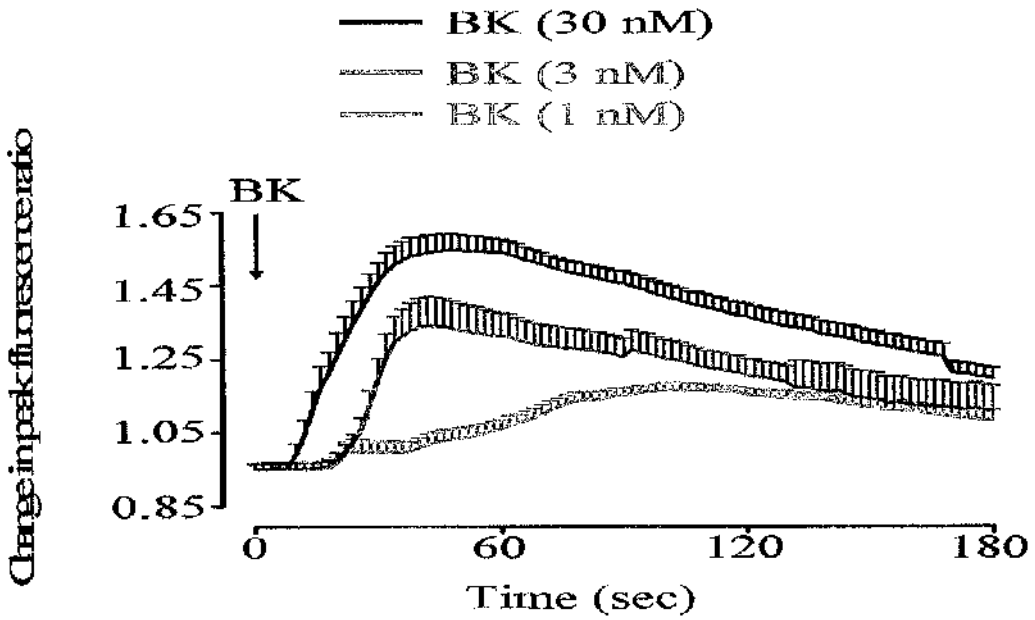

Figure 3.4: The time-course of effect of BK (10^{-8} M) on the release of calcium within a single isolated non-pigmented ciliary epithelial cell.

Using fura-2 as a calcium-binding fluorescent marker the change in intracellular calcium concentration was observed. The peak response was established as the highest fluorescence ratio and the base line value was taken to be at an arbitray time interval of 180 seconds prior to the peak. Each point on the curves is a mean of values from 4-33 cells, with on S.E.M. shown for each point.

Figures 3.5 to 3.11 show the effect of L-NAME, ODQ, UK,144-542, Flurbiprofen and clotrimazole on calcium release in bovine cultured ciliary epithelium cells. In each case is shown the response to BK alone followed by the responses to BK and the other accessory drug together, then the control response to the accessory drug alone. All values are mean responses from the number of cells shown ± s.e.m. The magnitude of the response to BK ($3x10^{-9}$ M) was increased significantly by L-NAME and ODQ. In contrast, the response to BK ($3x10^{-8}$ M) was decreased significantly by UK,144-542, L-NAME and ODQ. The BK response was unaffected by flurbiprofen or clotrimazole. All of these accessory drugs had negligible effects when used in the absence of BK.

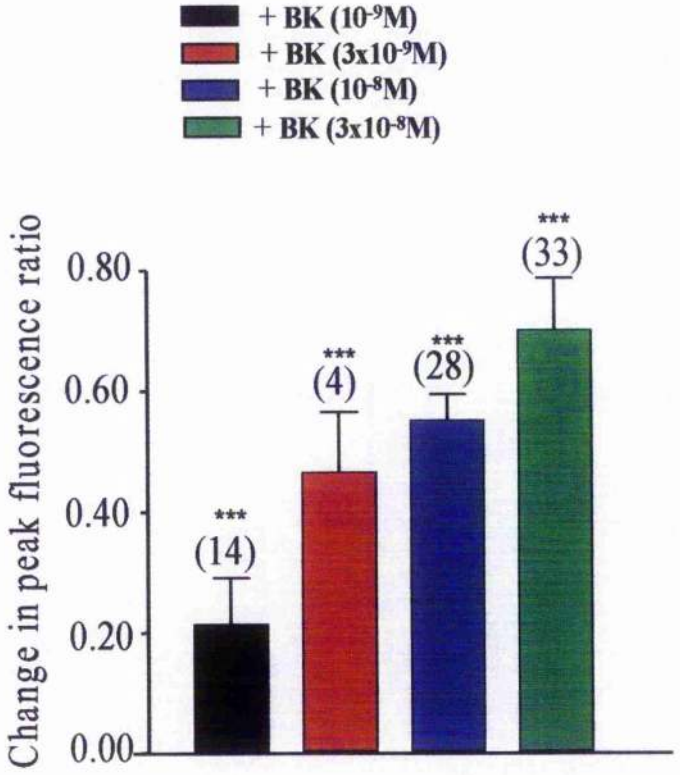

Figure 3.5: The effect of graded concentrations of BK (from 10^{-9} M to $3x10^{-8}$ M) on the calcium release in bovine NPE cells. Each column is the mean +/- SEM of the number of experiments shown in brackets. BK produced a concentration-dependent release of calcium.

Significance of difference from zero: ***P<0.001

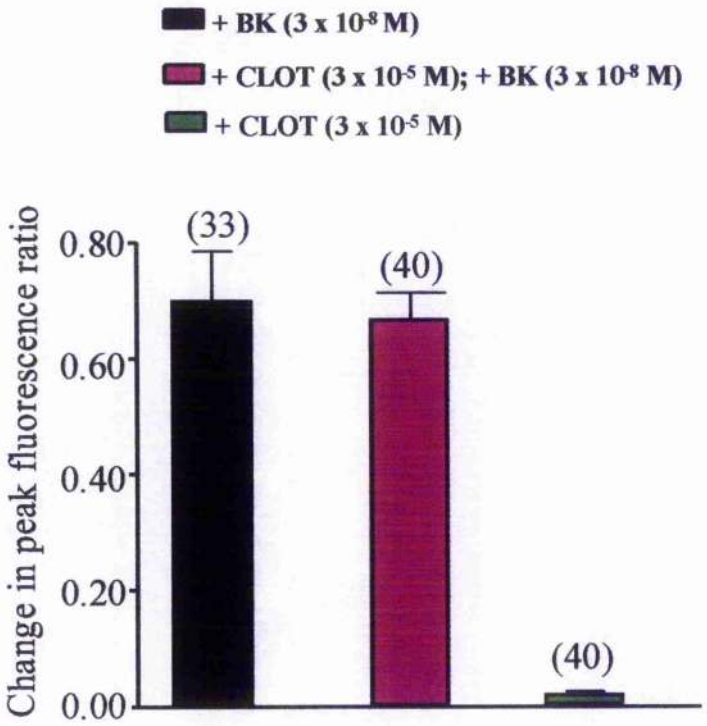

Figure 3.6: The effect of clotrimazole on the mobilisation of intracellular calcium in bovine NPE cells of BK.

Each point represents the mean (s.e mean) of the number of experiments shown in brackets.

Significance of difference from response to BK alone: not significant $P<0.809$ Student t-test.

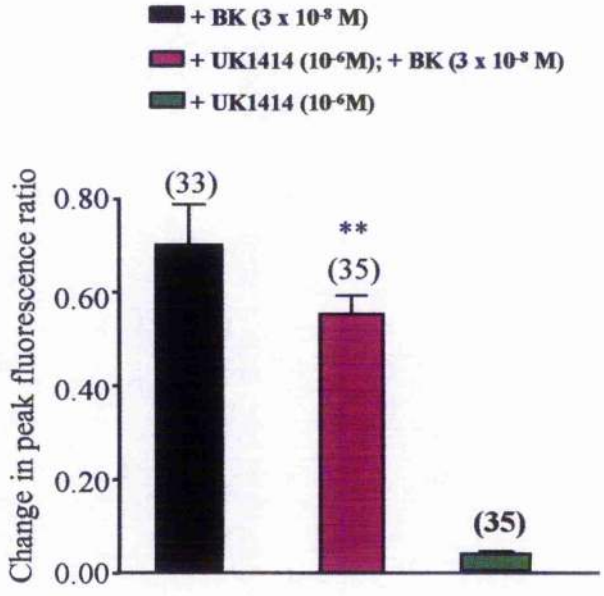

Figure 3.7: The effect of UK-114,542 on the mobilisation of intracellular calcium in bovine NPE cells of BK.

Each point represents the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: **P<0.01, Student t-test.

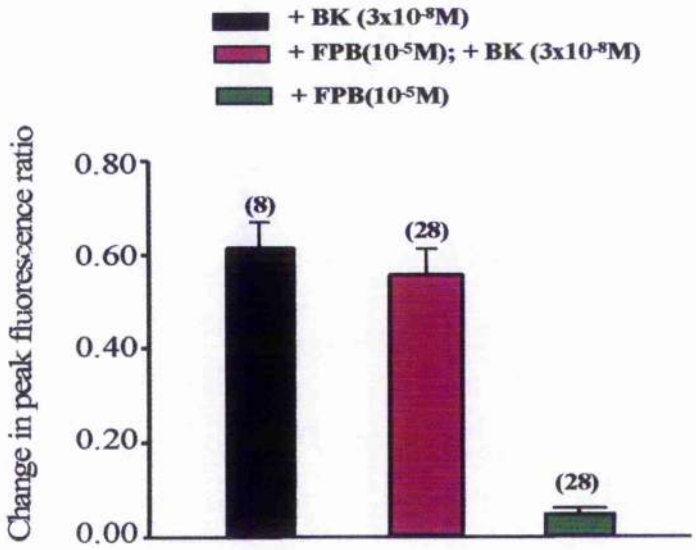

Figure 3.8: The effect of Flurbiprofen on the mobilisation of intracellular calcium in bovine NPE cells of BK.

Each point represents the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: not significant $P<0.809$,

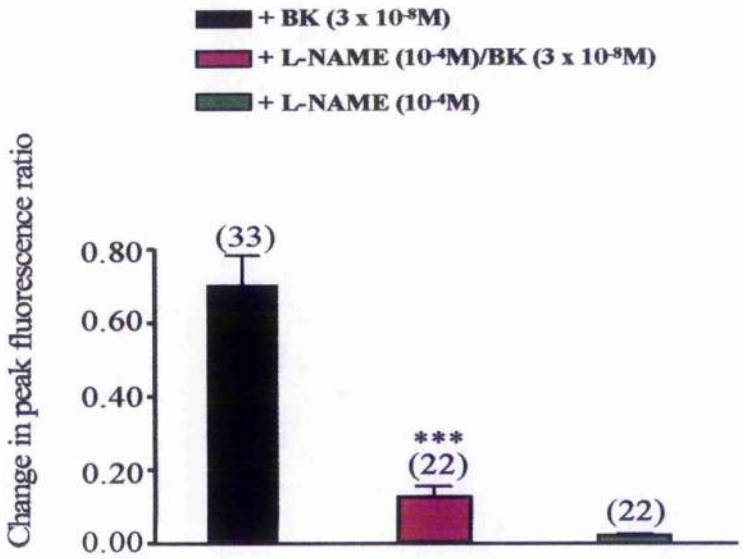

Figure 3.9: The effect on the mobilisation of intracellular calcium in bovine NPE cells of BK in the presence of L-NAME.

Each point represents the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: ***P<0.001, Student t-test.

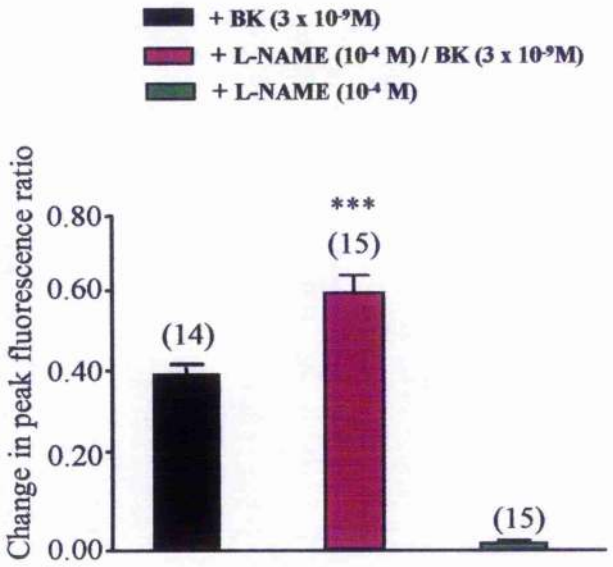

Figure 3.10: The effect on the mobilisation of intracellular calcium in bovine NPE cells of BK in the presence of L-NAME.

Each point represent the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: *** $P<0.001$, Student t-test.

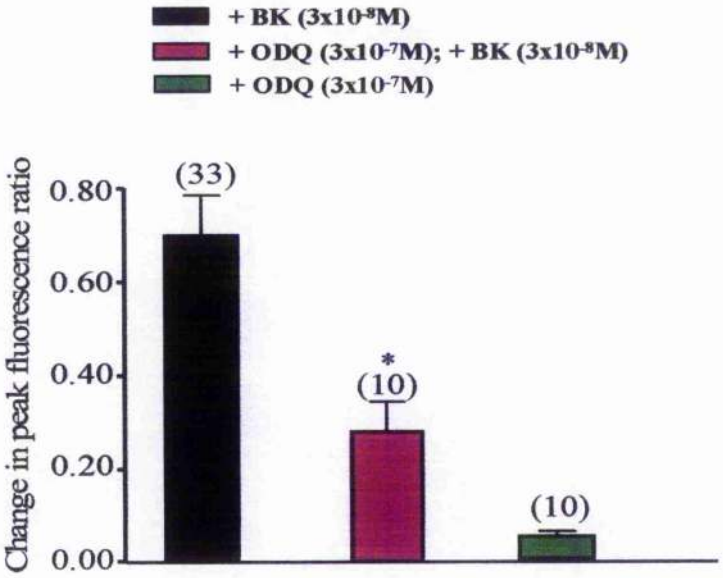

Figure 3.11: The effect on the mobilisation of intracellular calcium in bovine NPE cells of BK in the pr esence of ODQ.

Each point represent the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: *P<0.05, Student t-test.

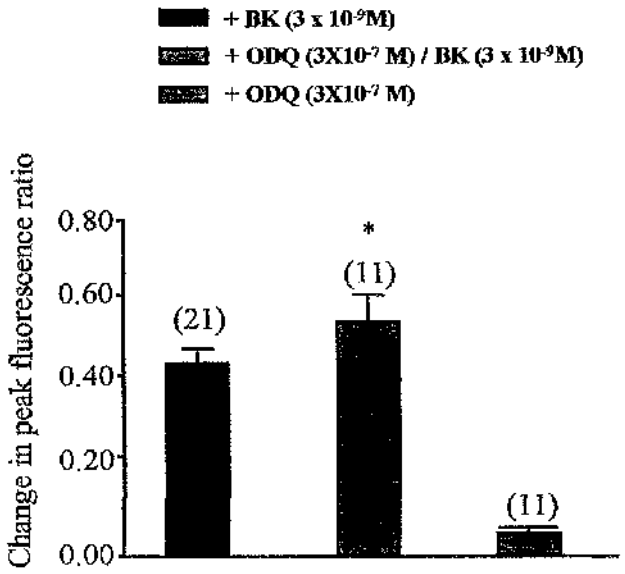

Figure 3.12: The effect of on the mobilisation of intracellular calcium in bovine NPE cells of BK in the presence of ODQ.

Each point represent the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of difference from response to BK alone: * $P<0.042$, Student t-test.

Figure 3.13 shows the effect of cGMP (10^{-11} to 10^{-6} M) on calcium release induced by BK (3 x10^{-8} M) within the epithelium cells. A clear concentration-dependent inhibition of Ca^{2+} is exerted by cGMP.

Figure 3.14 shows shows that when BK-induced release of Ca^{2+} is inhibited by cGMP (10^{-8} M), this inhibition is blocked by the presence of KT-5823 (2.5 x 10^{-7} M).

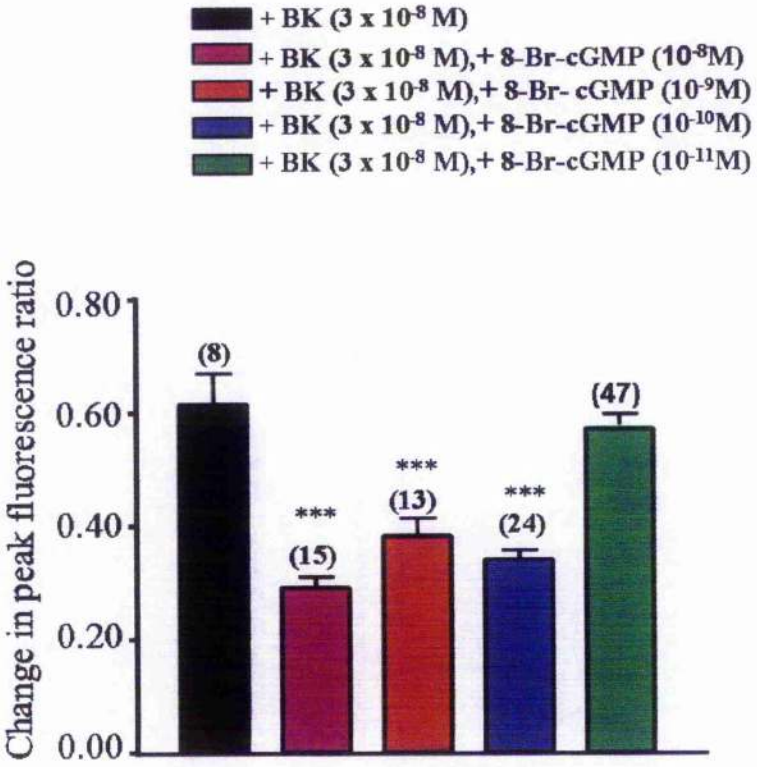

Figure 3.13: The effect on the mobilisation of intracellular calcium in bovine NPE cells of BK in the presence of 8-Br-cGMP

Each point represent the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of differences from response to BK alone as follow:

***P <0.001, and for 10^{-11}M. P <0.094 Student t-test

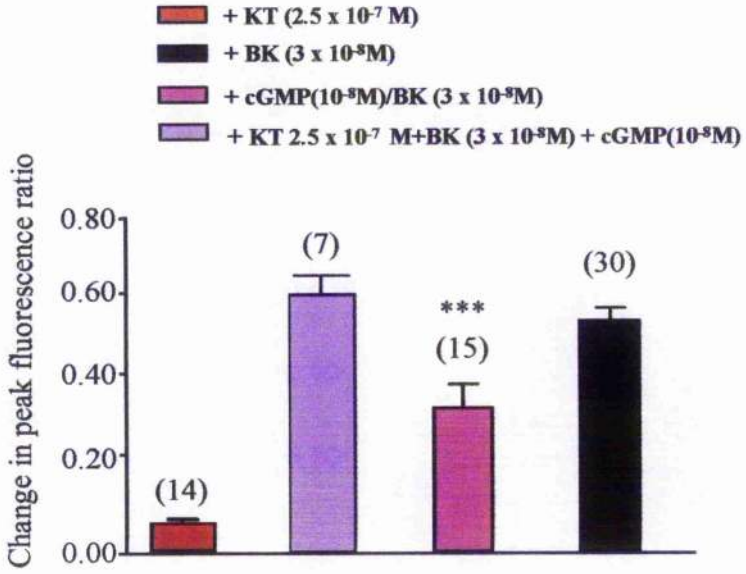

Figure 3.14: The effect on the mobilisation of intracellular calcium in bovine NPE cells of BK in the presence of cGMP and/or KT.

Each point represents the mean (± s.e. mean), of number of experiments shown in brackets.

Significance of differences between BK and BK + cGMP; ***P<0.001. No significant difference between BK (3 x 10^{-8} M) and BK (3 x 10^{-8} M) in the presence of cGMP (10^{-8} M) and KT-5823 (2.5 x 10^{-7} M): P<0.339, Student t-test.

Chapter 4: Discussion

4 Discussion

4.1 The Isolated perfused eye

The investigation of ocular function has been done commonly using arterially perfused eye as a model (Niemeyer, 1981). The study of pharmacology and physiology of aqueous humour has utilised isolated eyes, feline (Macri and Cevario, 1978; Chiou et al., 1980; Liu and Chiou, 1980; Macri et al; 1980; Van Alphen and Macri, 1981) or bovine (Kishida et al., 1985; Wilson et al., 1993, Gomez-Cabrero, 2005) and more recently porcine (Shahidullah et al., 2005, Townsend, 2006). Due to its availability, cheapness and good handling size for experimental use, the bovine eye provides an attractive tool for perfusion experiments.

The pharmacology of vascular dynamics can be studied quantitatively in perfused eyes because of the maintained integrity of the choroidal, retinal and ciliary circulation (McNeish et al., 2001). It has been shown that under adequate arterial perfusion an isolated eye can maintain structural integrity, physiological retinal performance, and sensitivity down to the range near human psychophysical thresholds (Shahidullah et al 2005); it is free from systemic effects in terms of absorption , metabolism and action of drugs; the isolated whole eye is free from the influence of any systemic nervous or hormonal effects; it allows simultaneous measurements of the arterial flow rate, arterial pressure, aqueous humour formation and intraocular pressure; it allows the use of high drug concentration that would

otherwise be impossible in the whole animal; it allows both baseline and control data to be obtained from the same eye; bovine eyes are readily available from local abattoirs. However, disadvantages include: reperfusion of the eye may be hindered by blood clots in the ocular vasculature if the eyes are not fresh; occasionally, fine air bubbles in the perfusion system block small arteries and thereby increase the perfusion pressure beyond the acceptable limit of 100 mmHg; despite precautions, some blood vessels are severed in the dissection process and perfusate may leak out from the eye (Shahidullah et al 2005, and Overby et al., 2002). Any delay in start of perfusion beyond one hour after slaughter significantly limited the possibility of achieving a stable intraocular pressure (Wilson et al., 1993).

Several classes of drugs have been shown to promote a decrease of intraocular pressure and aqueous humour formation in this way (Shahidullah and Wilson, 1995 and 1999). The bovine arterially perfused eye has been kept viable for almost five days when perfused with oxygenated serum-free Minimal Essential Medium under organ-culture conditions (De Coo et al., 1993). Assurance of the physiological viability of this preparation has recently been boosted by recording of multifocal electroretinograms from bovine arterially perfused eyes (Shahidullah et al., 2005). The bovine perfused eye has also been used to evaluate drug-induced changes in regional blood flow using labelled microspheres (Millar et al., 1992). The perfused eye preparation has also provided data on the relationship between aqueous humour formation and ciliary cyclic AMP (Shahidullah and Wilson, 1995) and on the

mechanism by which atriopeptin and other vasodilator drugs lower intraocular pressure (Shahidullah and Wilson, 1999).

In the present research study, bovine eyes were cannulated and perfused with Krebs solution with or without ascorbic acid or Krebs solution supplemented with BSA, according to the "constant flow method" of Wilson and co-workers (1993). After cannulation, a few eyes were discarded on the basis that: perfusion pressure was above 100 mm Hg at any stage; failure of the perfusate flow from at least two vortex veins; perfusion pressure displayed large fluctuations caused by air bubbles or blood clots blocking an artery; failure to establish a stable intra-ocular pressure. Application of these criteria resulted in the rejection of approximately 30% of eyes before drug or vehicle administration.

The relatively high rejection rate prior to drug administration was partly caused by damage during enucleation at the abattoir by unqualified operators. A further 8% of eyes were discarded after drug administration due to the emergence of air bubbles in the perfusate. A few eyes were found to be unhealthy through injury or infection of the cornea or cataract.

In the present work, an average value of 0.0030±0.0003 (n= 29) was found for the rate constant for aqueous humour formation ($K_{out}.min^{-1}$) in control eyes. In these eyes, ascorbic acid was included in the Krebs solution. The effect on aqueous

humour formation of omitting ascorbic acid from the Krebs solution was investigated in otherwise untreated control eyes. The mean K_{out} value was unchanged at 0.0031±0.0002 (n = 13).

Other published data indicate control values for K_{out} in bovine eyes ranging between 0.0046 and 0.0025 (Wilson et al., 1993; Shahidullah et al., 1999; Williams, 2005). In the present work, control values compare well with these. Factors affecting the rates observed in the present work include: the considerable time period over which the present work was conducted and the lack of control over the breed, sex and age of cattle from which the eyes were obtained. All these parameters may play a part in aqueous humour formation. An additional possible variable was seasonal variation. The hormonal variation that occurs with the seasons may also affect aqueous humour formation. Whether any of these factors actually modulate aqueous humour formation within the isolated bovine eye is currently not clear.

Mean $K_{out}.min^{-1}$ values in isolated arterially perfused cat eye and rabbit eye were found to be 0.0088 (Macri and Cevario, 1973) and 0.018 (Kodama et al., 1983) respectively. The lower $K_{out}.min^{-1}$ value reported for bovine eyes could be interpreted as a species difference or as a reflection of the much larger eye. Since the metabolic requirements of the aqueous are more a function of the internal surface area of the anterior chamber than of its volume, a larger eye may not require such a high flow of aqueous or it could be that the ciliary cells are in some way compromised by the perfusion process.

Since DMSO was used to dissolve one of the drugs used in this study, the effect of DMSO in the absence of drug on the aqueous humour formation was also investigated and found to be negligible (Table. 3.6). A similar lack of effect of DMSO was found elsewhere using pigs eye (Shahidullah, 2005).

4.2 Effect of BK on bovine perfused eye

Studies carried out previously in the same laboratories (Williams 2005 and McNeish 2003) investigated BK responses using a wide range of agonist concentrations. The lower limit of aqueous humour formation response was obtained for a BK concentration of 10^{-9} M and the highest response was obtained for concentration of 10^{-7} M. In conclusion the effect of BK is concentration-dependent.

Several studies have demonstrated with the use of animal or human arteries that BK stimulates endothelial cells to release a number of vaso-relaxing factors, such as NO, prostanoids and EDHF (McCulloch et al., 1997; Perez et al., 2001). However, the relative contributions of NO, PG, and EDHF to the vascular relaxation caused by BK vary with species and anatomic origin of the blood vessels used (Cyr et al., 2001).

This study is a continuation of ongoing research in our laboratories, aiming to investigate the mechanism by which BK acts in the eye, which started by investigating the effect of BK on the ciliary artery (McNeish et al., 2003). They found that unusually BK dilates the ciliary artery largely via release of EDHF and

that ascorbate, if present for more than two hours, blocks this effect (McNeish et al., 2003). The present study focused on the effect of BK on the aqueous humour formation.

The objective of this research segment was to firstly investigate the effect of BK administration on the aqueous humour formation and ultimately reveal the possible pathway(s) and mechanism(s) by which BK affects the aqueous humour formation. In order to do this the effect of BK on aqueous humour formation in the presence of inhibitors was investigated. These included an inhibitor of nitric oxide synthase (L-NAME), of cycloxygenase (flurbiprofen) and of EDHF (clotrimazole).

Currently, it is generally accepted that NO is involved in the modulation of ocular blood flow, vasodilation, neurotransmission and intraocular pressure regulation (Zembowicz et al., 1991; Hangai et al., 1999; Wiederholt et al., 1994). Indication of nitric oxide release by BK has been detected in porcine ciliary artery (Zhu et al., 1997) porcine ophthalmic artery (Yao et al., 1991) in bovine pulmonary supernumerary artery (Tracey et al., 2002) and in hepatocytes (Sesti et al., 2005).

Evidence in the literature suggested that BK-induced vasodilator responses in the porcine isolated perfused eye were blocked by L-NAME (Meyer et al.; 1993). The use of L-NAME in conjunction with BK would be expected to eliminate the effect of BK on aqueous humour formation. The findings of the present study involving the

effect of BK in conjunction with L-NAME indicated that L-NAME did block the effect of BK (Table. 3.4) therefore it can be concluded that the pathway of BK in causing reduction in aqueous humour formation in bovine is mediated through nitric oxide. Lack of any change in aqueous humour formation when L-NAME was used on its own suggests that there is no baseline spontaneous release of NO in the bovine ciliary body. These findings are in accordance with the results obtained in a recent study (Shahidullah et al., 2005), where L-NAME was found to have no effect on aqueous humour formation in porcine eyes. Recent study by Sesti and co-workers (2005) shows that NO release by BK in rat hepatocytes was blocked by L-NAME.

The effect of sodium azide, a nitrovasodilator which releases NO on IOP has been investigated and also its effect on ciliary vascular tone in the bovine isolated eye that was perfused via the ciliary artery (Millar et al., 1997; 2001). When compared with controls, sodium azide (10 nmole bolus dose) was found to lower IOP via a reduction in aqueous humour formation. The IOP-lowering effect of azide does not appear to depend on its ability to activate guanylyl cyclase in vascular smooth muscle since the dose required for the vascular effect was much higher than for the effect on aqueous humour formation, but rather is likely a consequence of direct activation of ciliary epithelial guanylyl cyclase. The pharmacological efficacy of a topical formulation of the nonselective nitric oxide synthase (NOS) inhibitor, L-NAME was investigated in an experimental model of glaucoma in rabbits (Giufrida et al., 2003). This study provided evidence that L-NAME significantly reduces the IOP in a model of ocular hypertension.

The observation was supported by another study (Kiel et al., 2001). In this research, L-NAME reduced aqueous humour formation in anaesthetised rabbit. It was argued that this reduction in the aqueous humour formation was due to ciliary vasoconstriction, that is, blood-flow dependent. Conversely, aqueous humour flow did not change significantly after intake of an oral single dose of isosorbide-5-mononitrate by ten healthy volunteers participating in a randomized, double-masked and placebo-controlled cross-over study (Kotikoski et al., 2003a). In contrast, (Chuman et al., 2000) showed that intravenous injection of L-arginine reduced IOP in human eye and this went mainly through NO formation.

The reasonable conclusion is that in bovine eye, NO is implicated directly in the regulation of aqueous humour formation in addition to its effects on ocular vasculature and aqueous humour outflow (Behar Cohen et al., 1996; Schmetterer 2001; Bonfiglio et al., 2006). Indirect support for the postulated assumption is now rising. For example, constitutive NOS (cNOS) activity has been found in the bovine (Geyer et al., 1997) and porcine (Meyer et al., 1999) ciliary processes. Results from a research study have demonstrated that L-NAME inhibited basal nitric oxide production in the human and porcine ciliary processes (Haufschild et al., 2000). Na-K-ATPase activity of the bovine ciliary processes was demonstrated to be inhibited by carbachol or NO (Ellis et al., 2001) and depolarisation of the epithelial transmembrane potential in porcine ciliary processes were obtained by stimulation of the NO/cGMP pathway (Fleischhauer et al., 2000). Moreover, NO has also been

shown to be implicated in fluid transport in other epithelia such as the kidney (Ortiz and Garvin, 2002) and the salivary gland (Lomniczi et al., 1998).

NADPH diaphorase activity is used widely as a marker for detection of the enzymes involved in nitric oxide production and has been identified in the posterior ciliary arteries of both pigs and monkeys (Toda et al., 1997, 1998). This evidence suggests that the production of nitric oxide takes place in the vasculature supplying the ciliary body. Nitric Oxide plays a major role in the hyperaemia, blood-aqueous barrier breakdown and cellular infiltration present in uveitis (Mandai et al., 1994; Meijer et al., 1995; Parks et al., 1994). In these studies, pre-treatment with nitric oxide synthase inhibitor have shown that delays the ocular inflammation associated with the injection of endotoxin into the footpads of lewis rats (Parks et al., 1994). Inhibition of nitric oxide synthesis also reduces the hyperaemia seen in the animal model of allergic conjunctivitis (Meijer et al., 1995). The inhibition of nitric oxide synthase with L-NAME in isolated eye preparations resulted in as much as 40% decreased vascular perfusion compared to that of the control (Koss et el., 1999). The inhibition of nitric oxide synthase results in further reduction in choroidal blood flow in the diabetic human eye as compared to healthy human eye (Schmetterer et al., 1997).

The association of cGMP in restraining aqueous humour formation was first postulated by Nathanson (1987). After intravitreal injections of atriopeptin in rabbit, a significant decrease in IOP and aqueous humour formation and a concomitant

increase in cGMP in iris-ciliary body developed (Korenfeld et al. 1989). Topical application of 8-bromo cyclic GMP, a membrane permeable cGMP analogue was also found to lower IOP in rabbits (Becker, 1990) and decrease aqueous humour flow when injected intravitreally in monkey (Kee et al.,1994). In bovine eye atriopeptin amplifies cGMP levels in both cultured ciliary processes and in the ciliary body of the whole eye (Millar et al., 1997) and atriopeptin decreases aqueous humour formation in these eyes. cGMP is implicated in reducing aqueous humour formation within the bovine eye (Millar et al., 1997; Shahidullah et al., 1999) and recently in pigs eye (Shahidullah et al., 2005).

The second set of experiments in the present study examined the involvement of cGMP in the effect of BK on aqueous humour formation. In these experiments the analogue of cGMP, 8-Br- cGMP, was tested at concentrations ranging from 10^{-11} M to 10^{-6} M. The selected concentrations produced significant suppression of aqueous humour formation (Table and Figure 3.2) in a manner that was clearly concentration-dependent. It appears that the effect of cGMP reaches a maximum at around 10^{-8} M and that the ED50 of cGMP would be no greater than 10^{-10} M. For cGMP concentrations 10^{-9} M up to 10^{-6} M the effect was very significant in both the first and second stages of the experiment, whereas the reduction was significant only in the third stage (+50 to 100 min) in the case of 10^{-10} M cGMP. One remarkable feature of this data is the extremely low concentration at which an effect is first seen. Miller et al (2001) calculated that the endogenous cGMP level in bovine ciliary

epithelium is approximately 10^{-8} M. This strengthens the hypothesis that we are observing a truly physiological mechanism for cGMP.

ODQ, a soluble guanylate cyclase inhibitor, is the drug of choice when it is required to differentiate cGMP-mediated effects of NO from cGMP-independent effects. In canine isolated ciliary artery, nipradilol produces relaxation which is resistant to L-NAME but is significantly inhibited by ODQ (Kitaoka et al., 2000). Norepinephrine also increases cGMP levels in this artery. These findings indicate that nipradilol-induced vasorelaxation in the canine posterior ciliary artery occurs via stimulation of the guanylyl cyclase-cGMP pathway, without the involvement of NO.

ODQ (3×10^{-7} M), was found to have no effect on aqueous humour formation, when used on its own (Table. 3.5). The findings were supported by recent research in which no effect of ODQ on aqueous humour formation on pig eye was observed (Shahidullah et al., 2005). The findings in the present study involving the effect of BK in conjunction with ODQ indicated that ODQ did block the effect of BK (Table. 3.5). Therefore it can be concluded that the mechanistic pathway of BK causing reduction in aqueous humour formation is through cGMP.

For further confirmation of the participation of cGMP in the BK pathway in aqueous humour formation, the effects of KT-5823 were examined. This compound has previously been used in bovine eye to block the action of atriopeptin and sodium

azide (Shahidullah et al., 1999). KT-5823 alone did not have any effect on aqueous humour formation (Table. 3.6) suggesting that there is no background influence of endogenous cGMP on the basal rate of aqueous humour formation. However, when KT5823 was used with BK, it completely suppressed the BK effect on aqueous humour formation. This blockade of BK-induced suppression of aqueous humour formation strongly suggests that an increase in cGMP production by BK is the most likely mechanism of its effects on aqueous humour formation.

Another set of experiments using UK-114,542 (a specific inhibitor of phosphodiesterase type five) was carried out in order to test again the influence of endogenous cGMP and also its participation in the BK pathway. UK-114,542 alone had a small but significant effect on aqueous humour formation (Table. 3.7). This suggests that there is some baseline production of cGMP in the bovine ciliary body and that PDE inhibition enhances cGMP levels to a level at which aqueous humour formation is affected. When used with BK, UK-114,542 appeared to have enhanced the effect of BK, consistent with the result for UK-114542 alone and also strengthening further the argument that cGMP is the mediator of its effects on aqueous humour formation.

A possible role of EDHF in BK-induced reduction in aqueous humour formation was assessed next. It is reported that BK dilates the bovine ciliary arterial bed largely

through release of EDHF rather than nitric oxide (McNeish et al., 2001), in contrast to the situation in rats (Koss, 1998). While it therefore would seem possible that BK could also release EDHF in the ciliary epithelium, identifying this mechanism using the toxins which selectively inhibit K^+ channels (McNeish et al., 2001) would be technically difficult to implement and very expensive. Clotrimazole was used as an EDHF inhibitor in the present work, having been shown to attenuate (though not abolish) the vasodilator response to BK (Kenny et al., 2002).

The results of this study indicated that the aqueous humour formation rate was unaffected by administration of clotrimazole on its own. The reduction of the aqueous humour formation rate which was detected upon administration of clotrimazole combined with BK was, if anything, even greater than when BK was administered on its own. It therefore appears that EDHF is probably not involved in the action of BK on aqueous humour formation.

The other option, in which BK-induced reduction in aqueous humour formation in bovine eye could be due to a product of cyclo-oxygenase was tested using flurbiprofen. The results of the present study indicated that the aqueous humour formation rate was unaffected by administration of flurbiprofen on its own. Administration of flurbiprofen combined with BK resulted in a suppression of

aqueous humour formation which was, if anything, even more than when BK was administered on its own, though the difference was not statistically significant.

4.3 Bradykinin and the blood-aqueous barrier

The blood-aqueous barrier is a major anatomical and physiological entity involved in the exclusion from the eye of various compounds e.g certain organic acids which freely circulate in the blood. There are also some compounds (e.g ascorbate) for which the blood –aqueous barrier constitutes a means of maintaining a much higher concentration in the eye than in the blood. Administration of BK (10^{-8} M) straight into the anterior chamber in the rabbit eye has previously been shown to cause collapse of the blood–aqueous barrier, noticeable as aqueous flare (Bynke et al., 1983).

Williams (2005) reported that in the bovine perfused eye, BK (10^{-7} M) caused an increase in aqueous humour formation in contrast to the decrease caused by smaller doses. In the present study, we investigated whether this resulted from damage to the blood-aqueous barrier, by adding a high molecular weight marker, albumin to the perfusate. Due to its molecular weight, albumin would be unable to cross the blood–aqueous barrier under normal physiological conditions. If the barrier was damaged, then escape of albumin into the anterior chamber would be measurable at the end of

the experiment. A relationship between continually rising IOP and leakage of albumin into the chamber has previously been demonstrated (Wilson et al., 1993).

The results of the present study suggested that when BK (10^{-7} M) was included in the perfusate there was no significant association between protein accumulation in aqueous humour and an apparent rise in aqueous humour formation. Instead, the effect of this 10^{-7} M of BK was to suppress aqueous humour formation by 41%, a slightly greater inhibition than was exerted by any of the lower concentrations of BK tested. This result did not confirm the report by Williams (2005) but was nevertheless internally consistent in that a high concentration of BK produced a large suppression of aqueous humour formation accompanied by little evidence of barrier breakdown. When the data were analysed (Figure 3.14) there was little evidence, even for a few individual eyes, that a correlation existed between high aqueous formation and protein accumulation. Although BK caused barrier breakdown in the rabbit (Bynke et al., 1983), it is well known that this species is prone to such damage by various chemical agents.

4.4 Bradykinin in presence/absence of ascorbate

McNeish et al., (2002 and 2003) have presented much evidence that inclusion of ascorbate in the perfusate blocks vasodilation induced by BK in the bovine ciliary artery. This observation prompted Williams (2005) to examine the possibility that

ascorbate might also block the BKs effect on aqueous humour formation; he reported that ascorbate in the perfusate did indeed abolish this action of BK.

Attempts in the present study to duplicate Williams (2005) findings have not been successful. Both BK and cGMP were capable of suppressing aqueous humour formation whether in the presence or absence of ascorbate (Table. 3.10 and Fig.3.11). Our failure to demonstrate a similar effect of ascorbate to that reported by McNeish (2002, 2003) is consistent with the fact that these workers were using blood vessels where the mechanism of BK is largely dependent on EDHF, whereas the present study has made clear that BK in the ciliary processes is acting through NO/cGMP and not EDHF, nor prostaglandin. In conclusion, it can be said that the administration of the mechanism of action of BK involves the reduction in aqueous humour formation through NO and cGMP in bovine perfused eye.

4.5 Effect of BK on intracellular calcium release from non-pigmented ciliary epithelial cells

4.5.1 Introduction

In all cells, a very slow drift of the baseline fluorescence ratio was observed during the experimental procedure suggesting a constant slow influx of calcium. The baseline drift was perhaps due to either damage caused by the cell disruption

technique used (collagenase) or to some other consequence of culturing the ciliary epithelium. In order to overcome the variation in the rate of onset of the BK effect, an arbitrary time interval of 180 seconds prior to the peak of the response was selected for estimation of the baseline ratio. One major contributor to this response variation is the variation among individual cells.

Although the mechanisms of many drug effects on Ca^{2+} release have been studied in ciliary epithelial cells, little information exists about the way in which BK affects intracellular Ca^{2+} in ciliary epithelial cells. One aim of the present study was to investigate how the release of Ca^{2+} by BK is influenced by the various inhibitors and other drugs which were used in the first part of this work to analyse the effect of BK on aqueous humour formation.

4.5.2 Effect of bradykinin on calcium release in non-pigmented ciliary epithelial cells.

To test the effect of BK on Ca^{2+} release within non-pigmented epithelial cell, a series of BK concentrations ranging from 10^{-9}M to $3x10^{-8}$M were investigated. The responses produced by BK were slow, taking approximately 50 to 100s to peak, depending on concentration (Figure. 3.3). The corresponding time for release by ATP is reportedly less than 30 s (Shahidullah and Wilson, 1997). The subsequent decline was also slow ($t_{1/2}$ approx. 60s).

The results are shown in Figures 3.3 and 3.4. A significant rise in calcium release was observed with increasing BK concentration. The highest and fastest calcium release level was obtained following BK ($3x10^{-8}$M). The lowest and slowest calcium release level was obtained for the lowest BK concentration used in this set of experiments (10^{-9}M). This suggests that a dose-dependent increase in intracellular calcium release is obtained for BK with an EC_{50} of about 4.1×10^{-9} M. The speed of onset is presumably due to the rate at which BK accumulates in the tissue bath. Williams (2005) showed that BK produced a similar response in ciliary epithelial cells in both the presence and absence of extracellular calcium. This would strongly suggest that the BK-induced response is utilizing intracellular calcium stores rather than an extracellular influx. The results are in accordance with previous studies carried out in human transformed non-pigmented ciliary epithelial cells exposed to BK (Lee et al., 1989). BK was also found to induce an elevation of $[Ca^{2+}]_i$ in sensory neurons that was mostly independent of extracellular calcium influx (Thayer et al., 1988). In another ocular epithelium, BK B2 receptor agonists, BK and Lys-BK were also found to cause a concentration-dependent rise of $[Ca^{2+}]_i$ level in canine cultured corneal epithelial cells (Huang et al. 2001). In contrast, the same workers used thapsigargin to deplete intracellular stores of Ca^{2+} prior to instillation of BK, with no effect on the BK-induced increase in $[Ca^{2+}]_i$. This would suggest that the effect of BK on Ca^{2+} mobilization in canine cultured corneal epithelial cells is dependent on extracellular Ca^{2+}, rather than intracellular stores. BK–induced increase in $[Ca^{2+}]_i$ is therefore subject to both cell-type and species variation.

4.5.3 Effect of L-NAME on calcium release by BK in non-pigmented ciliary epithelial cells

To test the mechanism of BK on calcium release from non-pigmented epithelial cells, we investigated the effect of L-NAME at a concentration of 10^{-4} M in the absence and presence of BK (3×10^{-9} M). The results are shown in Figure 3.10. The amplitudes of calcium release in non-pigmented epithelial cell induced by the administration of L-NAME (10^{-4} M) on its own were very small compared to calcium release obtained with BK on its own. However, as shown in Figure 3.10 a significant rise in calcium release was observed when L-NAME was used in combination with BK (3×10^{-9} M). The rise in calcium release comprised approximately 60% ($P < 0.001$). The findings of this set of experiments indicated that L-NAME blocks NOS which means that production of NO is prevented. This in turn would block synthesis of cGMP, which we know to be inhibitory in relation to Ca^{2+} release in ciliary epithelial cells (Shahidullah and Wilson, 1999) when ATP is the agonist. In the present work, we also show directly the inhibitory effect of cGMP on BK-induced Ca^{2+} release (Figure 3.13). Hence, L-NAME was able to boost the calcium release by BK. This effect was highly significant. This is strong evidence of a contribution by NO in this BK pathway. This allows us to develop the theory that extracellular BK acts via B_2 receptors which usually act through the G-protein (Gq/11). Activation of this protein leads to activation of phospholipase C, which catalyses the formation of $InsP_3$ and DAG from phosphatidylinositol 4, 5-bisphosphonate. $InsP_3$ then causes release of Ca^{2+} from the endoplasmic reticulum stores (Berridge, 1987).

This calcium released into the cytosol then stimulates NOS, which will cause NO production; finally NO will stimulate cGMP production (Figure 4.1).

In contrast to the above, when a large concentration of BK (i.e. $3x10^{-8}$ M) in conjunction with L-NAME was used, it suppressed the BK-induced calcium release from the non-pigmented epithelial cells. There was 82% inhibition of calcium release by BK and the effect was significant ($p< 0.001$) (Figure 3.9). This result was anomalous and more work needs to be done in order to understand the mechanism. In a previous study, iminoEt-Orn and l-NNarg as NOS inhibitors were used to investigate the contribution of NO in BK-induced Ca^{2+} influx [Abdalla et al., 1996]. The results suggested that iminoEt-Orn and l-NNarg suppressed the BK-induced Ca^{2+} influx. Williams (2005) concluded that BK-induced increase in $[Ca^{2+}]_i$ in non-pigmented epithelial cells does not depend on Ca^{2+} influx. However, the range of [BK] tested in the absence of Ca^{2+} did not extend to $3x10^{-8}$ M, so it seems possible that at this high [BK], influx of a large $[Ca^{2+}]$ may occur. This might explain the anomaly described above.

4.5.4 Effect of ODQ on calcium release in non-pigmented ciliary epithelial cells

ODQ, a soluble guanylate cyclase inhibitor is probably the drug of choice when it is required to differentiate cGMP-mediated effects of NO from cGMP-independent effects ODQ ($3x10^{-7}$M) was found to have no effect on calcium release from non-

pigmented epithelial cells when used on its own. The experimental results in the present study, involving the effect of BK ($3x10^{-9}$ M) in conjunction with ODQ showed that calcium release was slightly increased compared to the use of BK on its own and the difference was significant (Figure. 3.12). These results suggest that ODQ blocks soluble guanylyl cyclase which will enhance calcium release above levels that were generated by BK used on its own, following the same logic as was used above to explain enhancement of the BK ($3x10^{-9}$ M) response by L-NAME. Therefore it can be concluded that the mechanistic pathway of BK triggering calcium release from non-pigmented epithelial cells leads to cGMP formation, which then limits the amount of Ca^{2+} released (Figure 4.1).

On the other hand, a higher concentration of BK ($3x10^{-8}$ M) in conjunction with ODQ suppressed significantly the BK-induced calcium release from non-pigmented epithelial cells (Figure. 3.10). This result seems anomalous in relation to the enhancement of the response to BK ($3x10^{-9}$ M) just described, but it is nevertheless very similar to the anomaly observed in the experiments with L-NAME. Both concentrations of BK in conjunction with ODQ had very different actions on the calcium release by BK but both demonstrated the involvement of cGMP in BK-mediated calcium response. It has been previously reported that NO causes an inhibition of Ca^{+2} release from IP3-sensitive stores such as endoplasmic reticulum through formation of cGMP (Clementi et al., 1995; Karaki et al., 1988). This is in line with the present study using L-NAME to block the production of NO by BK ($3 x 10^{-9}$M). Likewise, it is in agreement with using ODQ to block cGMP formation by

BK (3×10^{-9}M). In both cases, this concentration of BK is presumably nearer to levels which might be considered physiological than the the higher BK concentration (3×10^{-8}M) which gave the anomalous results. Also in both cases the level of Ca^{2+} was increased, suggesting that NO inhibition of Ca^{2+} release is through cGMP.

Such results imply that when BK triggers of calcium, the subsequent synthesis of cGMP then exerts a negative feedback by inhibiting further release of calcium (Figure 4.1). However, in contrast to the above, when either L-NAME or ODQ was used in the presence of a higher concentration of BK (3×10^{-8}M) the level of Ca^{2+} was decreased. This anomaly may be due to the involvement of other cGMP-independent pathways when cGMP is perturbed by the effect of ODQ. Recent studies indicated that NO can regulate Ca^{2+} release through direct stimulation of ryanodine receptors instead of cGMP-dependent pathway (Stoyanovsky et al., 1997; Galione et al., 1993). In contrast, Ruisheng et al. (2002) reported no significant differences in Ca^{2-} release observed in the presence or absence of ODQ when used to study NO pathways in cultured rat mesangial cells.

Several other experiments in the present work supported the negative feedback involvement of cGMP in limiting calcium release triggered by BK in ciliary epithelium.

In order to demonstrate the inhibitory effect of cGMP in BK-induced calcium release, the analogue of cGMP (8-Br- cGMP) was tested at concentrations ranging from 10^{-11} M to 10^{-8} M. These concentrations had no effect on calcium release on

their own but when used in conjunction with BK ($3x10^{-8}$ M) there was concentration-dependent inhibition of the Ca^{2+} transient induced by exogenous BK (Figure 11). Similar effects occur in bovine ciliary epithelial cells with atriopeptin, sodium azide or 8-Bromo cGMP, which all inhibit Ca^{2-} release caused by ATP (Shahidullah and Wilson., 1999).

In a previous research study carried out in other tissue, platelet activation and mobilisation of stored Ca^{2+} were both inhibited by nitrovasodilator, and this occurred through cGMP and protein Kinase G induced mobilization of stored Ca^{2+} (Geiger et al.,1992). A variety of mechanisms have been suggested for this inhibitory action of cGMP on Ca^{2-} signalling. Amongst the mechanisms advanced are inhibition of influx of extracellular Ca^{2-} (Pozzan et al., 1994); activation of re-uptake by stores (Raeymakers et al., 1988); activation of pumps in the plasma membrane (Vrolix et al., 1988) and inhibition of IP_3-induced Ca^{2-} release (Komalavilas & Lincolin, 1994). Further research is required to establish which mechanisms are involved in bovine ciliary epithelium, but since Williams (2005) has shown that calcium release by BK in ciliary epithelium is independent of extracellular Ca^{2+}, an effect on influx seems unlikely.

Several previous studies (Yang et al. 1994, Assender et al., 1997, Eguchi et al , 1997, Wassdal et al ., 1999) indicated that BK invariably increases cytosolic Ca^{2+} at least by release from intracellular stores via activation of a G protein-coupled receptor and phospoinositide phospholipase C causing production of IP_3 which then binds to its

receptor on the endoplasmic reticulum, thus mobilizing stored Ca^{2+}. The BK effect upon $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells appears to be mediated by B_2 receptors (Williams. 2005). Also BK is known to act at B2 receptors in the kidney (Maeda et al., 1996) and in mesenteric vascular beds (Peredo et al., 1997) to stimulate formation of eicosanoids and NO. It has been shown in canine cultured corneal epithelial cells that BK stimulates B_2 receptors resulting in a calcium influx (Huang et al., 2001).

4.5.5 Effect of KT-5823 and UK-114542 on calcium release in non-pigmented ciliary epithelial cells

For further confirmation of the participation of cGMP in the BK pathway in calcium release from endoplasmic reticulum, the effects of KT-5823 (a specific inhibitor of (PKG) were examined. This compound has previously been used in bovine eye to block the action of atriopeptin or sodium azide (Shahidullah et al., 1999). KT-5823 alone did not have any effect on calcium release (Figure. 3.13), but when KT-5823 was used in conjunction with BK ($3x10^{-8}$ M), and 8-Br-cGMP ($1x10^{-8}$ M), KT-5823 completely suppressed the inhibition by 8-Br-cGMP of BK-induced calcium release. Recent study (Jiang , et al ., 2000) showed that 8-Br-cGMP- induced activation of cardiac L-type Ca^{2+}-channels was shown to be inhibited by KT5823, suggesting that the influence of cGMP on influx of calcium is different from its effect on release of stored calcium.

Another set of experiments using UK-114,542 (a specific inhibitor of phosphodiesterase type five) was carried out in order to test for the presence of cGMP and its participation in the BK pathway. UK-114,542 alone had no effect on calcium release (Figure 3.6). When used with BK, UK-114,542 appeared to inhibit the effect of BK, strengthening further the argument that cGMP is synthesised in these cells in response to BK.

As reported in previous research studies, cyclic GMP inhibits ATP-induced Ca^{2+} release (Shahidullah et al., 1999). One may perhaps anticipate that inhibition of cGMP synthesis could potentiate BK-induced calcium release. Accordingly we have demonstrated that inhibition by KT-5823 of the cGMP effect stimulates BK-induced release of intracellular calcium in the non-pigmented ciliary epithelial cell. UK-114,542 inhibited BK induced release of Ca^{2+}. Therefore the endogenous cGMP level is apparently not enough to inhibit either aqueous humour formation or calcium release (as revealed by the KT-5823 experiments) but since UK-114,542 decreased aqueous humour formation, endogenous cGMP may be able to modulate aqueous humour formation if cGMP levels are raised by mediators like NO, BK etc.

It is known that NO released by BK from vascular endothelial cells diffuses to the vascular smooth muscle cells which then produce cGMP (Rang et al., 2003). Calcium release from ciliary epithelial cells is subject to negative feedback due to that fact that BK causes an enhancement of cGMP synthesis, therefore this suggests that ODQ, inhibiting cGMP formation, would increase calcium release. Moreover, since we cannot be certain that our culture of ciliary epithelium contains only non-

pigmented cells, there is a possibility that BK, through another 2nd messenger, may be acting wholly or partly upon the pigmented epithelial cells.

By analogy with blood vessels, BK might activate NOS in pigmented epithelial cells. NO released from these cells could then stimulate cGMP synthesis in non-pigmented cells. This possibility could be excluded if we could be sure that our cultures contained only non-pigmented cells.

In the ciliary epithelium, the effectiveness of the way that BK shows this result may be due to a physiological role of BK as a modulator. The hypothesis has been proven that intracellular calcium in the ciliary epithelium plays an imperative role in aqueous humour formation, as there is a close analogy between the very low concentration of BK needed to inhibit aqueous humour formation and influence calcium transition. Mitchell et al have shown that the regular production of aqueous humour can be affected by procedures that affect regular calcium oscillations in ciliary epithelial cells.

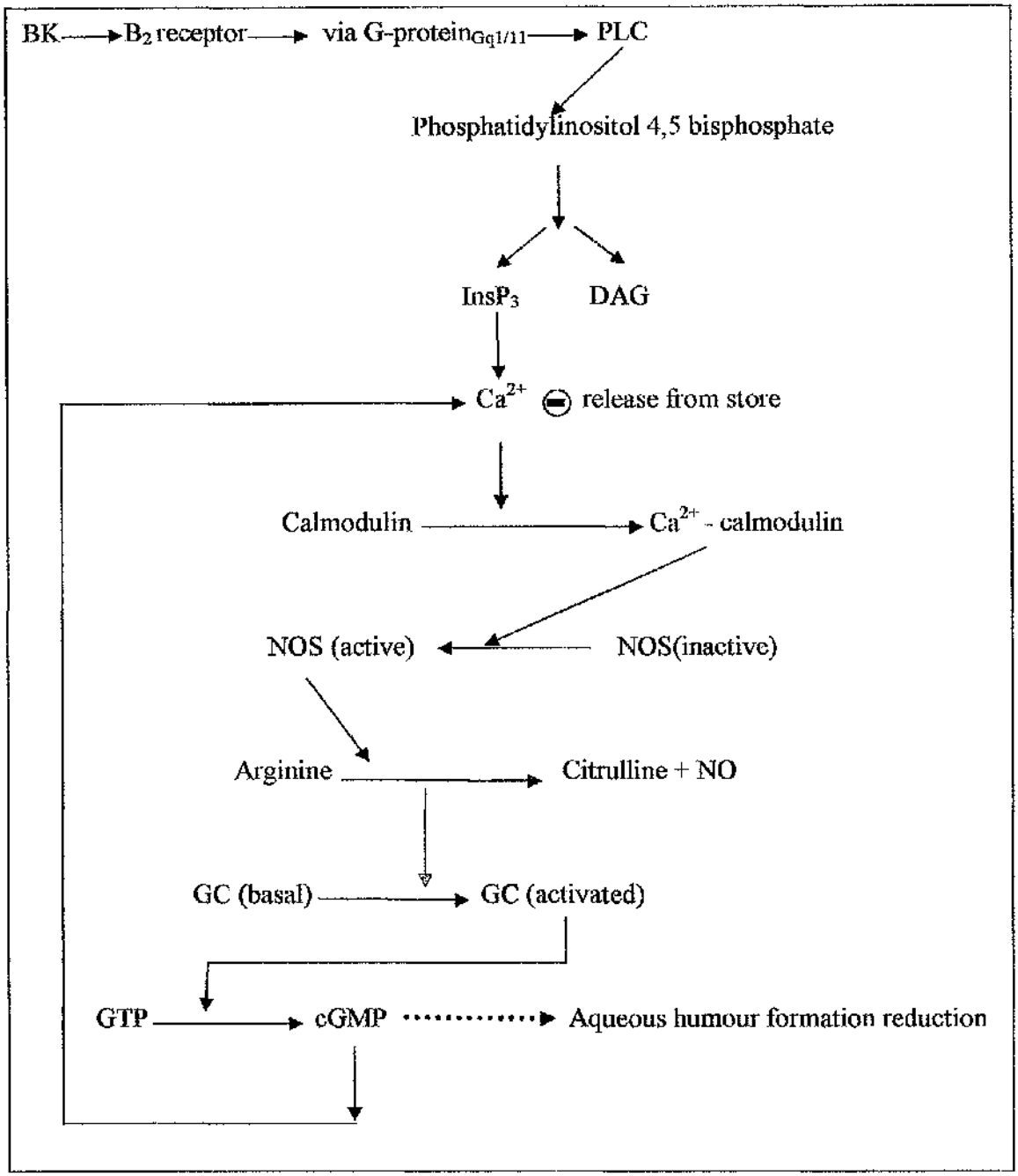

Figure 4.1: Possible pathway by which BK acting via the inositol trisphosphate ($InsP_3$) pathway results in NO release which eventually leads to the production of cGMP by guanylate cyclase (GC) and final reduction in aqueous humour formation (after Berridge, 1987).

The role of EDHF in BK-induced calcium release in non-pigmented epithelial cells was assessed. The results of this study indicated that the calcium release was unaffected by clotrimazole on its own. There was no change in calcium release following administration of clotrimazole combined with BK as compared with calcium release when BK was administered on its own (Figure 4.1). This suggests that EDHF is not involved in the action of BK on calcium release and contrast with the situation in some other tissues. In porcine ciliary artery, relaxation by BK involves a K^+-channel resulting in a relaxation induced by nitric oxide (Zhu et al., 1997). The relaxation caused by BK is almost completely eradicated by elimination of the endothelium. The activation of the K^+-channel is believed to lead to a transient rise in cytosolic free calcium, which in turn boosts the synthesis of nitric oxide (Schilling, 1989; Colden-Stanfield et al., 1987).

Another possibility, that BK-induced calcium release in non-pigmented epithelial cells was influenced by prostaglandins was tested by using flurbiprofen, a cyclo-oxygenase inhibitor. The results of the present study indicated that the calcium release was unaffected by flurbiprofen on its own. There was no change in calcium release following administration of flurbiprofen combined with BK as compared with calcium release when BK was administered on its own (Figure 4.6). This suggests that prostaglandins are not involved in the action of BK on calcium release in ciliary epithelial cells.

The present work has demonstrated that BK affects aqueous humour formation at very low concentrations, much lower than those reported by Bynke (et al., 1983) in relation to its damaging effect on the blood-aqueous barrier. This supports the possibility that BK has a physiological role in the control of aqueous humour formation.

Table 4.1: Data on the effect of various agents on calcium release and aqueous humour formation in bovine perfused eye is summarised from Shahidullah and Wilson, 1999 and the present work.

	Effect of calcium release	Effect of aqueous humour formation
ATP	+	↓
BK	+	↓
Azide, Atriopeptin	–	↓
cGMP	–	↓

Table 4.1 puts together the data from the present work with that from previous work in our laboratory. To seek a clear unifying theory from this data is not straightforward. Agents which suppress aqueous humour formation may trigger calcium release or may inhibit calcium release. Mitchell et al., (1998) have proposed that aqueous formation may depend on regular oscillations of calcium in the ciliary epithelia. Perhaps, therefore any of these agents can interfere with such oscillations and may thus suppress aqueous humour formation. We still cannot tell for sure whether calcium or cGMP is more directly responsible for effects on aqueous humour formation, but the above evidence suggests that BK increases cGMP via its

ability to release calcium, so strengthening the status of calcium as a primary mediator of intracellular events.

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