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RESEARCH INTO AQUEOUS HUMOUR PRODUCTION AND ITS CONTROL WITHIN THE BOVINE EYE

A thesis submitted to the

University of Glasgow

in candidature for the degree of

Doctor of Philosophy

in the

Faculty of Science

by

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Abstract

Aqueous humour formation within the isolated, arterially perfused bovine eye

The isolated, perfused bovine eye model has a long proven track record as an established experimental model allowing the study of the physiology and pharmacology of aqueous humour. **Wilson *et al.*, 1993** have shown this model in numerous experiments to provide reproducible results, consistent with *in vivo* models. **Shahidullah *et al.* (1995, 1999)** have since taken the use of the model further showing a number of categories of drugs that reduce aqueous production and hence intraocular pressure.

Bradykinin (10^{-9} to 10^{-8}M) suppresses aqueous humour formation (42-49% reduction). At higher concentrations ($3x10^{-8}$M and 10^{-7}M) aqueous production appeared to be increased (8-15% increase). It is possible that suppression of aqueous humour formation persists at higher bradykinin concentrations but at higher concentrations leakage of fluid from the vascular compartment into the chamber of the eye occurs -breakdown of the blood-aqueous barrier.

Bradykinin was found to act through the B_2 receptor. WIN 64338 ($3x10^{-8}$M), a B_2 receptor antagonist, abolished the effect of bradykinin on aqueous humour formation suppression (unpaired t-test: $p<0.01$).

L-NAME (10^{-4}M), a nitric oxide synthase inhibitor, significantly reduced the effect of bradykinin (10^{-9}M) on aqueous humour formation (bradykinin (10^{-9}M) alone 42% reduction, in presence of L-NAME 14% reduction: unpaired t-test $p<0.01$). L-NAME alone (10^{-4}M) had no significant effect on aqueous humour formation (paired t-test >0.1) suggesting that there is no tonic influence of endogenous nitric oxide in the bovine ciliary epithelium. Bradykinin appears to suppress aqueous humour formation via nitric oxide and cGMP since a soluble guanylate cyclase inhibitor (ODQ) also blocked the bradykinin-induced inhibition of aqueous humour formation (bradykinin (10^{-9}M) alone 42% reduction, in presence of ODQ ($3x10^{-7}$M) 4% reduction: unpaired t-test $p<0.004$).

The absence of ascorbate from the perfusate significantly reduced the inhibitory effect of bradykinin on aqueous humour production (bradykinin (10^{-9}M) in presence of ascorbate 42% reduction, bradykinin (10^{-9}M) in absence of ascorbate 5% reduction: unpaired t-test $p<0.004$). It is possible that the vasodilator effect of bradykinin in the absence of ascorbate (**McNeish *et al.*, 2003**) may counter the effect on aqueous humour formation.

Substance P (10^{-8}M) appears to inhibit aqueous humour formation (substance P (10^{-8}M) 38% reduction, paired t-test $p<0.001$). It has previously been suggested that bradykinin either releases substance P or acts via substance P receptors and therefore it may be that we are actually activating the same pathway with substance P as we are with bradykinin.

Intracellular calcium release from non-pigmented ciliary epithelial cells

A previously well described fura-2 fluorescence technique **(Shahidullah *et al.*, 1997)** enabled the effects on intracellular calcium in non-transformed cultured ciliary epithelial cells to be studied.

ATP (10^{-4}M) added to the solution superfusing the non-pigmented ciliary epithelial cells triggered an almost immediate (10-20s) increase in $[Ca^{2+}]_i$. Rapid release of Ca^{2+} from intracellular stores caused a peak within 40s of initial onset, which at first declined quickly ($t_{1/2}$ <30s), then more slowly. The response to bradykinin was much slower taking approximately 2 min to peak. The subsequent decline was also slower ($t_{1/2}$ approx. 60s). These findings may be due to involvement of different receptors and possibly different second messenger pathways with different end-effects.

Bradykinin (10^{-10}M to $3x10^{-8}$M) was found to have a concentration-dependent effect on $[Ca^{2+}]_i$ (Correlation coefficient log[bradykinin] (M): Pearson r =0.9638, p value = 0.008). Bradykinin (10^{-8}M), at a concentration ten thousand times less, produced an increase in $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells comparable in peak height to the effect of ATP (10^{-4}M) (2.72$\pm$ 0.34 compared to 3.11$\pm$ 0.24, mean $\pm$ s.e.; unpaired t-test, p>0.1). It appears that bradykinin utilizes intracellular calcium stores rather than an influx of extracellular calcium as a similar response occurred in both the presence and absence of extracellular calcium.

At a lower concentration of bradykinin (10^{-10}M), the prior treatment with bradykinin delayed the onset of the response elicited by ATP. The change in fluorescence ratio between baseline and peak was also reduced in the presence of bradykinin when compared to then effect of ATP alone (59.5 ± 6.0s compared to 38.6 ± 6.3s, mean ± s.e.; unpaired t-test, p=0.03). We are currently unsure as to the mechanism and relevance of this inhibition.

The bradykinin effect upon $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells appears to be mediated by B_2 receptors. The response was inhibited by WIN 64338, a B_2 receptor antagonist (bradykinin (10^{-8}M) vs. bradykinin (10^{-8}M) + WIN 64338 (10^{-6}M) 2.72± 0.34 compared to 1.31± 0.15, mean ± s.e.; unpaired t-test, p>0.001). Direct stimulation with a B_1 agonist, Lys-[des-Arg^9]BK, appeared to have no effect on calcium release in non-pigmented ciliary epithelial cells.

ODQ and L-NAME had no effect upon $[Ca^{2+}]_i$. Neither drug appeared to have any effect on the intracellular Ca^{2+} response to bradykinin (10^{-8}M) in these cells (bradykinin (10^{-8}M) vs. bradykinin (10^{-8}M) + ODQ (10^{-6}M) vs. bradykinin (10^{-8}M) + L-NAME (10^{-4}M) (2.72± 0.34; 2.65± 0.49; 2.34± 0.43: mean ± s.e. unpaired t-test >0.1). This would suggest, not surprisingly, that neither cyclic GMP nor nitric oxide play a part in the release of Ca^{2+} induced by bradykinin. If the hypothesis that interfering with calcium movements is the mechanism by which bradykinin suppresses aqueous humour formation is to be upheld, then bradykinin must generate nitric oxide somewhere in the vicinity of the ciliary epithelium, possibly in non-pigmented ciliary epithelial cells, and this nitric oxide could

then generate cGMP in pigmented ciliary epithelium.

Ascorbate is present in high concentration within aqueous humour. The presence of ascorbate appeared to have no effect on the bradykinin-induced increase in $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells (ascorbate absent 2.72± 0.33; ascorbate present 2.92± 0.44: mean ± s.e. unpaired t-test >0.1). It may be that the ciliary epithelial cells require prolonged pre-treatment with ascorbate before any effect on the bradykinin-induced calcium response is observed.

Substance P ($>10^{-9}$M) causes mobilisation of intracellular calcium within non-pigmented ciliary epithelial cells (substance P (10^{-8}M) 3.22± 0.29: mean ± s.e.). Substance P (10^{-7}M) and ATP (10^{-4}M) have a comparable delay in onset of calcium release and the size of the response (42.4 ± 3.7s compared to 38.6 ± 6.3s respectively, mean ± s.e.; unpaired t-test, p=0.03). It is possible that ATP and substance P utilize a common pathway and rather bradykinin uses an alternative pathway in its mobilization of intracellular calcium.

The close parallel between the very low concentrations of bradykinin required to suppress aqueous humour formation and to affect intracellular calcium movements, supports the hypothesis that intracellular calcium plays an important role in aqueous humour formation. It may also suggest a physiological role for bradykinin as a modulator in the ciliary epithelium.

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When Catherine took me on, three months into the project, I don't think she realised the commitment that would be needed. Since then we've married and have a beautiful daughter, Iris. Cold, lonely nights due to my laboratory visits obviously made the heart grow fonder.

I owe my parents for both financial and psychological support over the past five years; they were there even when I bought another Land Rover.

I am indebted to the Sandyford abattoir for their endless supply of fresh bovine eyes.

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Declaration

I hereby declare that this thesis has been composed by myself 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, between November 1998 and November 2004.

No part of this thesis includes work that has previously been presented for any degree either at this or any other university.

INTRODUCTION

General introduction

The eye is a highly developed structure that, in conjunction with the various regions of the brain and their inter-positioned pathways, allows visualisation, interpretation and understanding of the complex world around us. The eye is highly adapted, allowing it to perform its role of light perception.

Aqueous humour is a remarkable solution produced within the eye, the functions of which include delivering oxygen and nutrients coupled with the removal of waste products from the avascular ocular tissues with which it is in contact. Aqueous humour is a colourless medium, that is constantly produced and drained from the eye, maintaining intraocular pressure, keeping the globe of the eye formed, and so providing a pathway of constant length through which light may pass to the retina.

Anatomy of the bovine eye

The bovine and human eyes share a number of anatomical similarities. The most important of these are shown in Table 1. With regard to the present work the arrangement of the blood vessels is probably of most importance.

	Bovine eye	Human eye
Globe dimensions	Antero-posterior 28-30mm Diameter 30mm	Antero-posterior 21-26mm Diameter 23-25mm
Globe volume	14.135cm^3	7.853cm^3
Main arterial blood supply	External ophthalmic artery	Internal carotid artery
Distance from iris root to ora serrata/ ora ciliaris retinae	6.5mm	6mm
Ciliary muscle	Poorly developed	Well developed
Lens dimensions	Antero-posterior ~13.3mm Diameter ~19.5mm	Antero-posterior ~4mm Diameter ~10mm
Lens volume	2.2cm^3	0.2cm^3
Accommodation	Limited	Extensive
Termination point of retina/ start of ciliary body	Ora ciliaris retinae	Ora serrata
Scleral spur	Absent- well developed pectinate ligament	Present
Anterior chamber volume	1.7cm^3	0.24cm^3
Ciliary processes	90-110	70

Table 1

Anatomical differences between the human and the bovine eye

Adapted from Cole (1970), Doughty (1997), Forrester *et al.* (1996), Prince *et al.* (1960), Shahidullah (1994), Tripathi & Tripathi (1984), Williams & Warwick (1980), Robertson (1999).

Ocular blood supply in the human eye

The human eye is supplied by two separate vascular systems, these being the retinal vessels and the uveal blood vessels.

As the internal carotid artery emerges from the roof of the cavernous sinus it gives off the ophthalmic artery. The ophthalmic artery divides into the central retinal artery, 2 or 3 posterior ciliary arteries and several anterior ciliary arteries.

The posterior ciliary arteries branch further to form approximately 15 short posterior ciliary arteries and 2 long posterior ciliary arteries. Around the optic nerve the short posterior ciliary arteries pass through the sclera to supply the choriocapillaris. It is this vascular bed that supplies the outer retinal layers, consisting of the outer two thirds of the inner nuclear layer, outer plexiform layer, outer nuclear layer, photoreceptors and retinal pigmented epithelium.

The long posterior ciliary arteries pass anteriorly, entering the globe at the equator, where they continue anteriorly to anastomose with the anterior ciliary arteries, forming the major arterial circle of the iris. This anastomosis supplies the ciliary processes and the iris as well as providing a small contribution to the choriocapillaris.

The central retinal artery enters the optic nerve, before passing anteriorly to the optic disc where it branches into four retinal branches. These retinal artery branches supply the inner retinal layers comprising the nerve fibre layer, ganglion cell layer, inner plexiform

layer and inner third of inner nuclear layer.

Venous blood drains from the retina via the central retinal vein, passing along the optic nerve to the cavernous sinus. The choroidal circulation drains via the vortex veins, of which there are four, one in each quadrant.

Ocular blood supply in the bovine eye

In contrast to the human eye, where the majority of the blood supply is derived from the ophthalmic artery, within the bovine eye most of blood supply comes from the ciliary artery, a branch of the external ophthalmic artery (Figure 1).

Within the bovine species, the internal ophthalmic artery arises from the internal rete to enter the orbit through the optic foramen. The ciliary artery arises from the external rete, before dividing into the large medial and smaller lateral ciliary arteries. Both of these arteries bifurcate prior to the globe, producing a number of short posterior ciliary arteries. The two branches of the medial ciliary artery form the two long posterior ciliary arteries, one inserting at the medial side, and the other the lateral side of the globe. The long posterior ciliary arteries initially enter the sclera superficially, but penetrate fully before the equator is reached.

The small internal ophthalmic artery joins the medial long posterior ciliary artery just a few millimetres before entering the globe. The short posterior ciliary arteries form the

choriocapillaris, supplying the outer layers of the retina. The long posterior ciliary arteries pass through the choroid, anastomosing within the ciliary body with the anterior ciliary arteries to form within the peripheral iris, the major arterial circle **(Diescem, 1975).** The major arterial circle mainly supplies the iris and ciliary body and provides a small contribution to the choroidal circulation.

Blood returns from the choroid, iris and ciliary processes to drain via the vortex veins. There are four vortex veins, one in each quadrant. The vortex veins after leaving the globe, join the supraorbital vein, the inferior orbital vein and the external rete, before anastomosing to join the orbital vein **(Prince *et al.*, 1960)**.

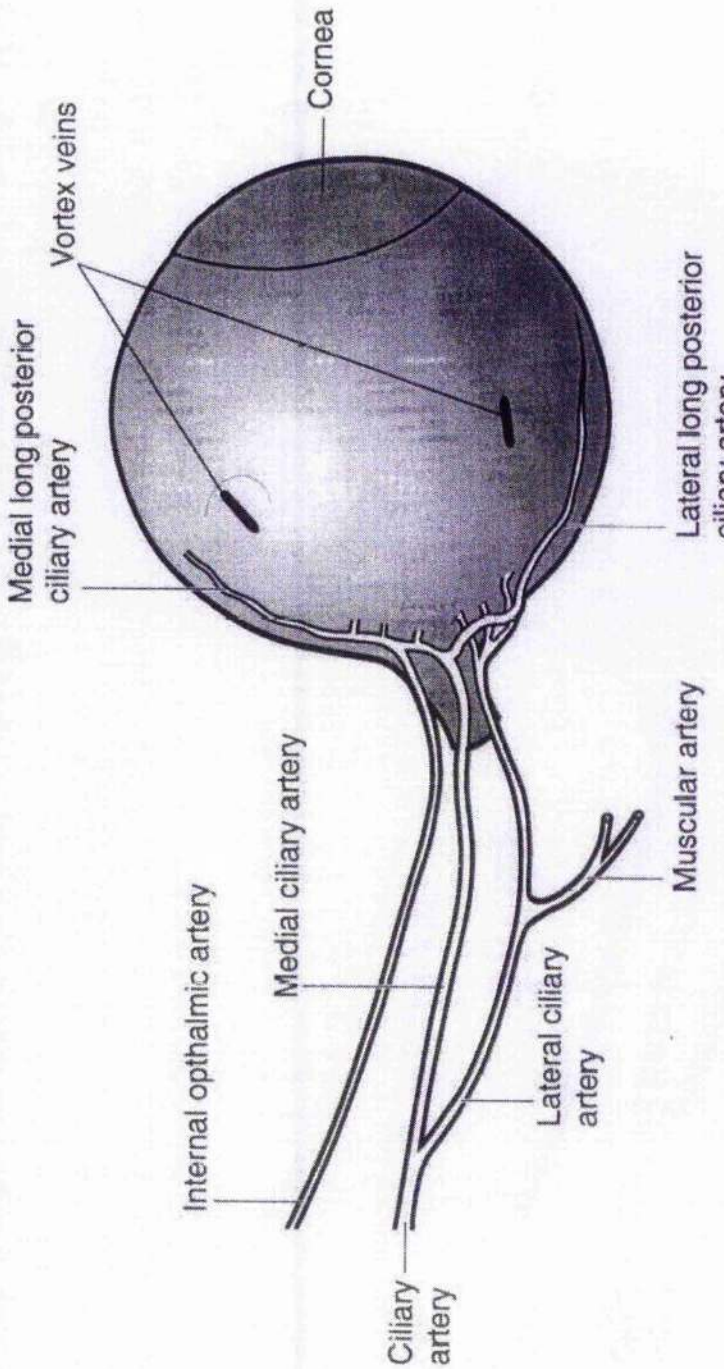

Figure 1 Schematic diagram of the vascular blood supply to the bovine eye. Adapted from Prince *et al.* (1960).

The ciliary body in the human eye

The ciliary body is a ring-shaped structure, situated between the iris anteriorly and the ora serrata posteriorly. It is divided into the pars plicata anteriorly, and the pars plana posteriorly. About seventy villus-like structures (ciliary processes) of approximately 2mm in length (Figure 2), project inward from the pars plicata. These consist of capillaries surrounded by stroma and a double epithelial layer, the ciliary epithelium (Figure 3).

The ciliary epithelium is made up of non-pigmented and pigmented ciliary epithelial cells. The pigmented cells are in contact with the ciliary body stroma and the non-pigmented cells make up part of the lining of the posterior chamber. The pigmented epithelial cells are cuboidal, contain numerous melanosomes, but have few intracellular organelles in comparison to the non-pigmented cells. The non-pigmented epithelial cells, on the other hand, are columnar and contain numerous mitochondria, as well as rough and smooth endoplasmic reticulum.

It has previously been shown that in the rabbit the non-pigmented ciliary epithelial cell contains more intracellular organelles, specifically mitochondria, and has a higher metabolic rate than pigmented ciliary epithelial cells **(Cameron *et al.*, 1963)**, suggesting that the non-pigmented ciliary epithelial cell play a greater role in aqueous production than pigmented ciliary epithelial cells.

Various types of intercellular junctions join the non-pigmented and pigmented epithelial cells, including desmosomes, gap junctions, puncta adherentes and tight junctions **(Raviola, 1977; Raviola *et al.*, 1978).** Tight junctions are always present at the apices of the non-pigmented cells. Gap junctions occur between the lateral surfaces of pigmented epithelial cells and between the non-pigmented cells and allow the electrical potential and ionic composition of the two layers to be similar, acting like a syncytium **(Green *et al.*, 1985, Bowler *et al.*, 1996, Wiederholt *et al.*, 1991).**

The tight junctions between non-pigmented cells exclude large or ionised molecules from passing between the capillaries and the aqueous humour **(Green, 1984).** In conjunction with the non-fenestrated iris capillaries, the tight junctions between non-pigmented epithelial cells make up the blood-aqueous barrier, which prevents many large molecules in the plasma from entering the aqueous, such that in the human, normal plasma protein levels are approximately 6g/100ml, compared with less than 20mg/100ml in the aqueous **(Krause *et al.*, 1969; Bill, 1964).**

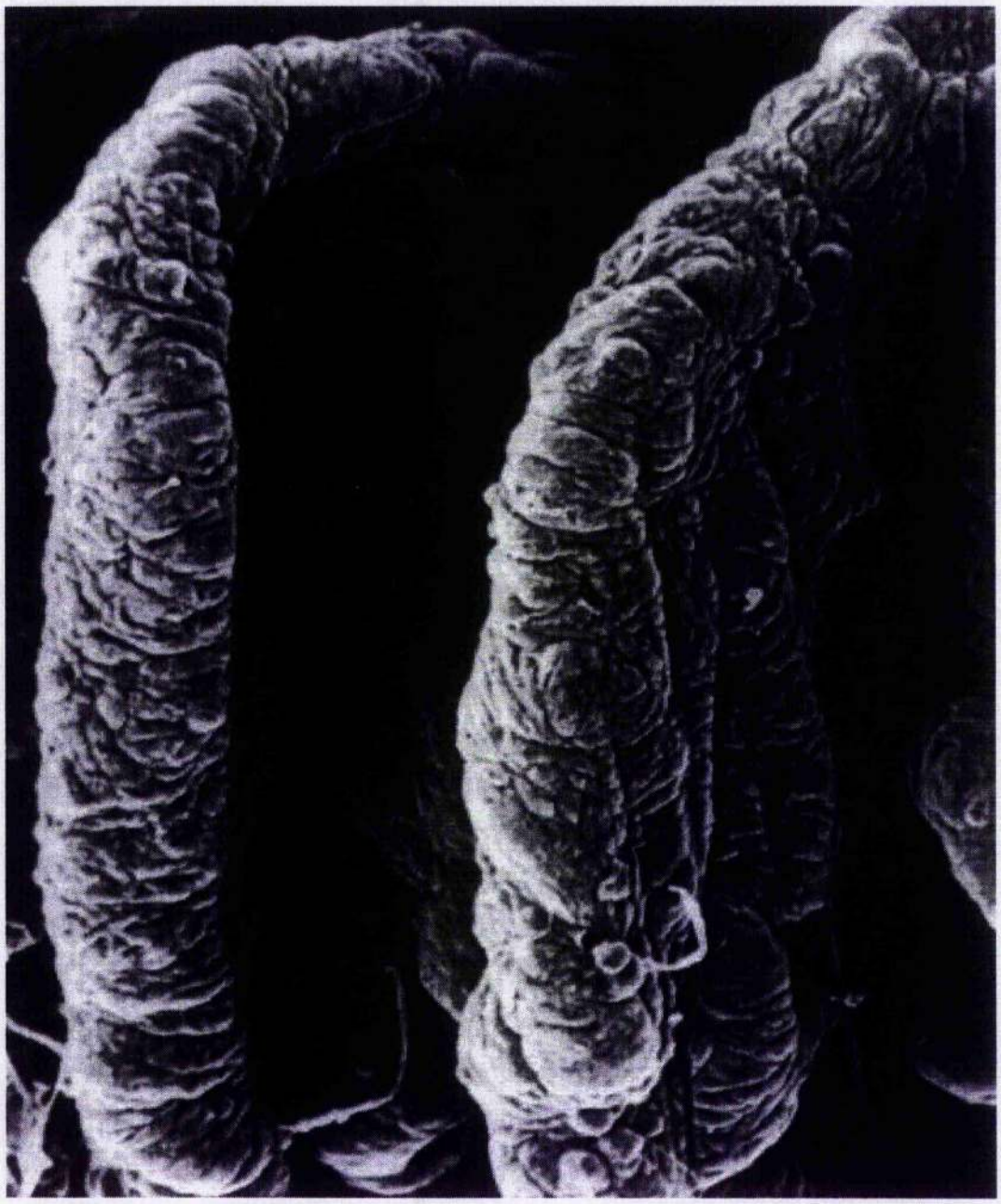

Figure 2

Scanning electron micrograph of the anterior tips of human ciliary processes (Magnification x1200)

(Caprioli, 1992)

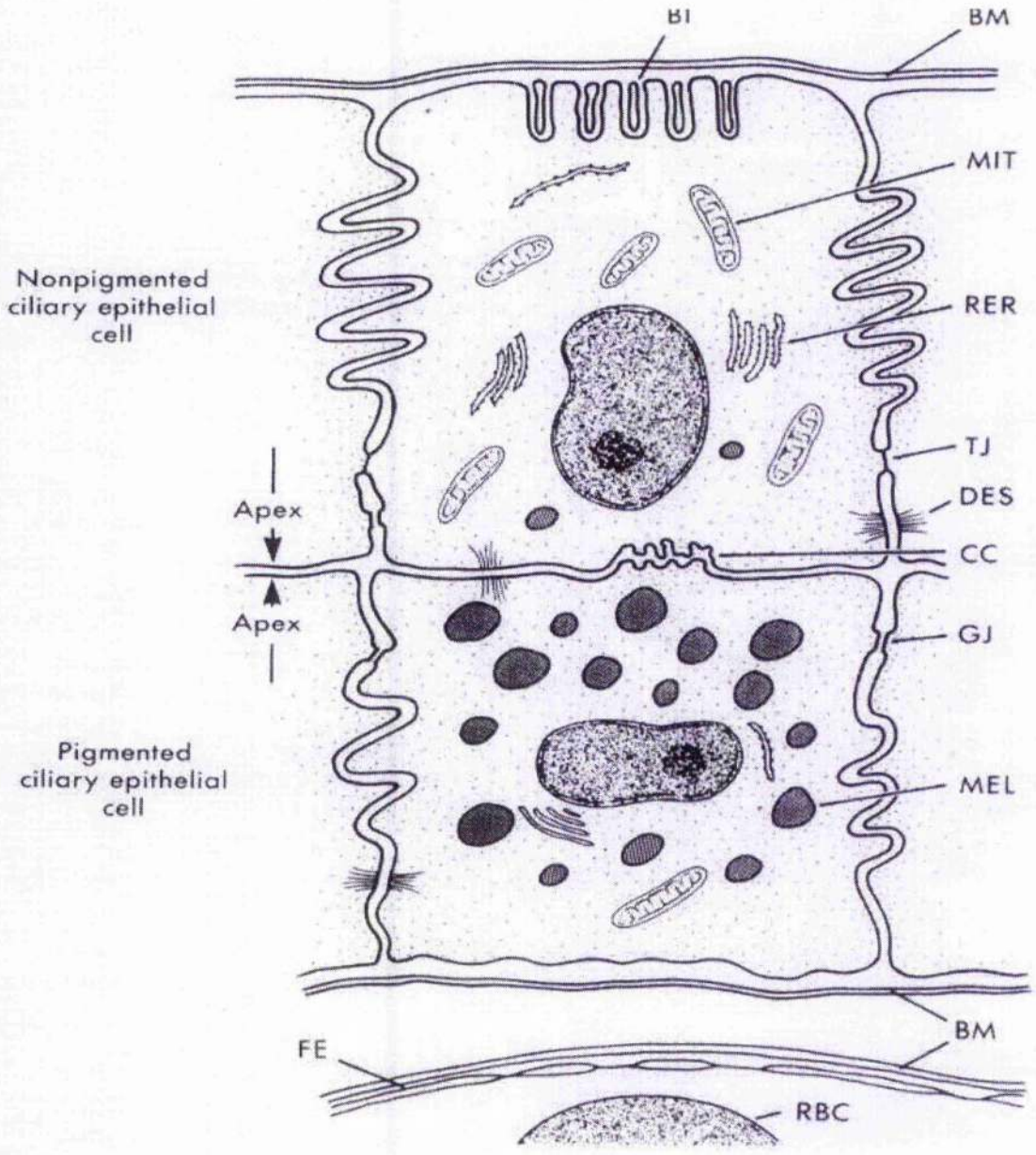

Figure 3

Schematic diagram of non-pigmented 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 body in the bovine eye

Unsurprisingly, much less is known about the ciliary body in the bovine eye (Table 1). As with the human ciliary body it extends from the ora ciliaris retinae (bovine equivalent of ora serrata) to the iris root **(Prince *et al.*, 1960).** The circular muscle fibres and radial muscle fibres are both very rudimentary, and there is no evidence of any great degree of functional activity **(Prince *et al.*, 1960).** The ciliary processes number between 90-110 and are large ranging between 3-5 mm in length **(Prince *et al.*, 1960).**

There is no scleral spur in the bovine eye, with rather the iris being supported by the pectinate ligament against the forces of the ciliary and pupillary muscles **(Walls, 1963).** The pectinate ligament is a very well developed structure consisting of connective tissue fibres running from the limbal region to the iris root **(Walls, 1963).**

The role of aqueous humour

It is currently believed that circulation of aqueous humour within the anterior chamber of the eye has four main roles **(Krupin *et al.*, 1995; Millar *et al.*, 1995):**

(1) The delivery of substrates to, and removal of waste products from the avascular anterior chamber tissue.

(2) To provide high levels of ascorbate to the anterior chamber, at concentrations approximately 25 times those found in the plasma, though its actual role remains uncertain.

(3) Involvement in local immune responses.

(4) Maintaining the shape of the globe to allow it to perform as an optical apparatus.

Aqueous production

Role of diffusion, ultrafiltration and active transport

There are three physiological processes that contribute to aqueous humour formation, these being diffusion, ultrafiltration and active transport, the greatest contributor being that of active transport. The active solute pump sets a concentration gradient that forces osmotic flow of fluid into the posterior chamber **(Barany 1963; Bill 1975; Kinsey 1971; Cole 1977).**

Diffusion occurs down a concentration gradient and requires the substance to move freely across cell membranes. It is therefore the major routes for lipid-soluble substances that pass easily across the cell membranes.

Ultrafiltration had been suggested to be involved in up to 70% of aqueous humour production **(Green, 1973)**. However, the values of the hydrostatic and oncotic forces in the ciliary processes do not favour ultrafiltration as an important mechanism of aqueous humour production **(Bill, 1973)**.

Previous calculations **(Green *et al.*, 1972)** have estimated that capillary hydrostatic pressure exceeding 50mmHg would be required to drive ultrafiltration sufficient to produce normal aqueous flow. This greatly exceeds that calculated for the rabbit, for example, estimated between 27-28mmHg **(Bill, 1975)** and 25-33mmHg **(Cole, 1977)**. Inhibition of Na^+/K^+ ATPase with ouabain results in a 70% reduction in aqueous humour formation **(Cole, 1977)**, if ultrafiltration were contributing such a large amount to aqueous humour formation then inhibition to this extent would not occur. The ultrafiltration component of aqueous humour formation is pressure-sensitive, decreasing with increasing intraocular pressure.

This phenomenon is quantifiable and is termed facility of inflow. As it is pressure-dependent, a pressure-induced decrease in inflow will appear as an increase in outflow when techniques such as tonography and constant-pressure perfusion are used to measure outflow facility. The ultrafiltration rate is approximately 0.02 $\mu l \times min^{-1} \times mm\ Hg^{-1}$ in the

monkey **(Bill, 1971; Kaufman, *et al.*, 1977)** and 0.06 µl x min^{-1}x mm Hg^{-1} in the human eye **(Gaasterland *et al.*, 1975)**, although the latter may be an overestimate.

Active secretion of certain solutes by the ciliary epithelium is the most important process in the formation of aqueous humour. The rate of active solute transport by the ciliary epithelium determines the rate of aqueous production approximately 2.0 µl to 2.5µ1 x min^{-1} **(Cole, 1977).** The active transport system is energy-dependent, acting via membrane-bound enzyme complexes. Active secretion is essentially pressure insensitive at near-physiological intraocular pressure. Current opinion is that chloride plays a significant role **(Jacob *et al.*, 1996)** (see page 17).

Movement of water via transcellular route

Water may flow either via the transcellular route, through cells, or the paracellular route, between cells. Formation of aqueous humour appears to be mainly dependent on the transcellular route rather than the paracellular route, which has largely been discounted **(Bill, 1973; Cole, 1960, 1977).**

Unidirectional transfer of water and solutes

The mechanism involved in the unidirectional secretion of solutes and water into the posterior chamber of the eye by the ciliary epithelium has been extensively investigated **(Civan *et al.*, 2004).** Active transfer of solute, predominantly NaCl, from the stroma to the posterior chamber creates an osmotic gradient along which water moves passively.

The unidirectional transfer of water and solutes from the stroma to the aqueous humour involves three specific steps.

(1) Solute and water uptake at the stromal surface by pigmented ciliary epithelial cells. Initially NaCl moves from the stroma into the pigmented ciliary epithelial cells by either the Na^+/H^+ and Cl^-/HCO_3^- antiports **(McLaughlin *et al.*, 1998; Counillon *et al.*, 2000; To *et al.*, 2001)** or a Na^+-K^+-$2Cl^-$ symport **(Edelman *et al.*, 1994; Crook *et al.*, 2000; To *et al.*, 2001; Dunn *et al.*, 2001)**;

(2) Transfer from pigmented to non-pigmented ciliary epithelial cells via gap junctions. Gap junctions then allow the solute to pass from the pigmented ciliary epithelial cells into the non-pigmented ciliary epithelial cells;

(3) Transfer of solute and water from non-pigmented ciliary epithelial cells to aqueous humour. From the non-pigmented ciliary epithelial cell membrane Na^+,K^+-ATPase channels release Na^+ into the posterior chamber and Cl^- channels **(Yantorno *et al.*, 1992; Coco-Prados *et al.*, 1995)** have a similar role for Cl^-.

Transfer of solute and water from non-pigmented ciliary epithelial cells to aqueous humour

This final step involves movement of solute and water from the non-pigmented ciliary epithelial cells into the aqueous humour. This involves a number of mechanisms (Figure 4):

(1) Na^+,K^+-ATPase. Aqueous humour formation is dependent on active transport by the Na^+,K^+ exchange pump **(Cole, 1977).** This being an active transport mechanism requires an energy source, in the form of ATP.

It has previously been reported that cAMP inhibits Na^+,K^+-ATPase in rabbit ciliary epithelium, presumably due to phosphorylation of the pump **(Delamere *et al.*, 1990; Delamere *et al.*, 1992).** The inhibitory effects of cAMP can, at least in part, be reversed

by the second messenger cGMP **(Carré *et al.*, 1995)**, possibly by direct stimulation of cAMP phosphodiesterase to lower the cAMP level **(Mittag *et al.*, 1987)**.

It has recently been shown that nitric oxide may modulate Na^+,K^+-ATPase function in certain cell types **(Bachmann *et al.*, 1994)**.

(2) K^+ channels. K^+ channels have two roles, firstly accumulating K^+ against an electrochemical gradient, and secondly to set the membrane potential of the ciliary epithelial syncytium at a large negative value. This provides the energy for driving Cl^- from non-pigmented ciliary epithelial cells into the aqueous humour **(Jacob *et al.*, 1996)**.

(3) Cl^- channels. It is well proven that the ciliary transepithelial potential is negative, in respect of the posterior chamber to the serosal side **(Burstein *et al.*, 1984)**. The chloride ion is the major anion found in aqueous humour and this negative potential was found to be linked to the transport of chloride ions **(Chu *et al.*, 1987)**. The Cl^- channel is the main route by which chloride ions pass from the non-pigmented ciliary epithelial cells to the aqueous humour **(Krupin *et al.*, 1995; Jacob *et al.*, 1996; Coca-Prados *et al.*, 1995)**. Current opinion is that the activity of Cl^- channels in the non-pigmented ciliary epithelium is the rate limiting step in aqueous humour production **(Coca-Prados *et al.*, 1995)**.

Chloride channels form a large heterogenous group of membrane proteins. Cl^- channels tending to be divided into groups dependent upon their gating mechanisms; the ClC

family of voltage-dependent Cl^- channels, the cAMP-activated transmembrane conductance regulator (CFTR), Ca^{2+} activated Cl^- channels (CaCC) and volume-regulated anion channels (VRAC) **(Jentsch *et al.*, 2002).**

Chloride channels, in contrast to cation channels, are not involved in the initiation or spread of excitation, but rather in the regulation of excitability. They are also involved in cell volume regulation, pH regulation and synaptic activity. The Cl^- channels are essential for transepithelial transport and the control of water flow **(Nilius *et al.*, 2003).** As the equilibrium potential of Cl^- is close to the cell's resting potential, movement of Cl^- requires relatively little energy. Na^+-K^+ ATPase activity results in an electrochemical gradient being provided by K^+ and Na^+ ions. This allows transporter mechanisms to move Cl^- against its electrochemical gradient. The crucial role of Cl^- channels for the transport of salt and water through various epithelia is well recognized **(Nilius *et al.*, 2003).**

In vitro work suggests that adenosine activates non-pigmented ciliary epithelial cell volume-regulated anion channels (Cl^- channels) by occupying A_3 adenosine receptors **(Mitchell *et al.*, 1999; Carré *et al.*, 2000).** It is thought that the non-pigmented ciliary epithelial cells themselves are the origin of the adenosine, as they release ATP that is then metabolised to adenosine **(Mitchell *et al.*, 1999).**

(4) H^+-ATPase. The exact mechanism and role of these channels it not yet clear **(Saito *et al.*, 1995).**

(5) Water Pores. Aquaporin-1 has been identified in non-pigmented ciliary epithelial cell membranes, though the clinical importance of these 'water pores' is currently uncertain **(Stamer *et al.*, 1994; Nielsen *et al.*, 1995).**

Although in general opinion is that aqueous humour formation results from net inflow due to unidirectional secretion across the ciliary epithelium, a number of investigators **(Sears, 1984; Civan *et al.*, 1996; McLaughlin *et al.*, 1998)** have identified both non-pigmented ciliary epithelial cell and pigmented ciliary epithelial transporters that may allow water and solute transfer from the posterior chamber to the stroma. Release of Cl^- via pigmented ciliary epithelial cell Cl^- channels, under the action of cAMP generated by ATP, is thought to be the final step in this reabsorptive pathway **(Fleischhauer *et al.*, 2001).**

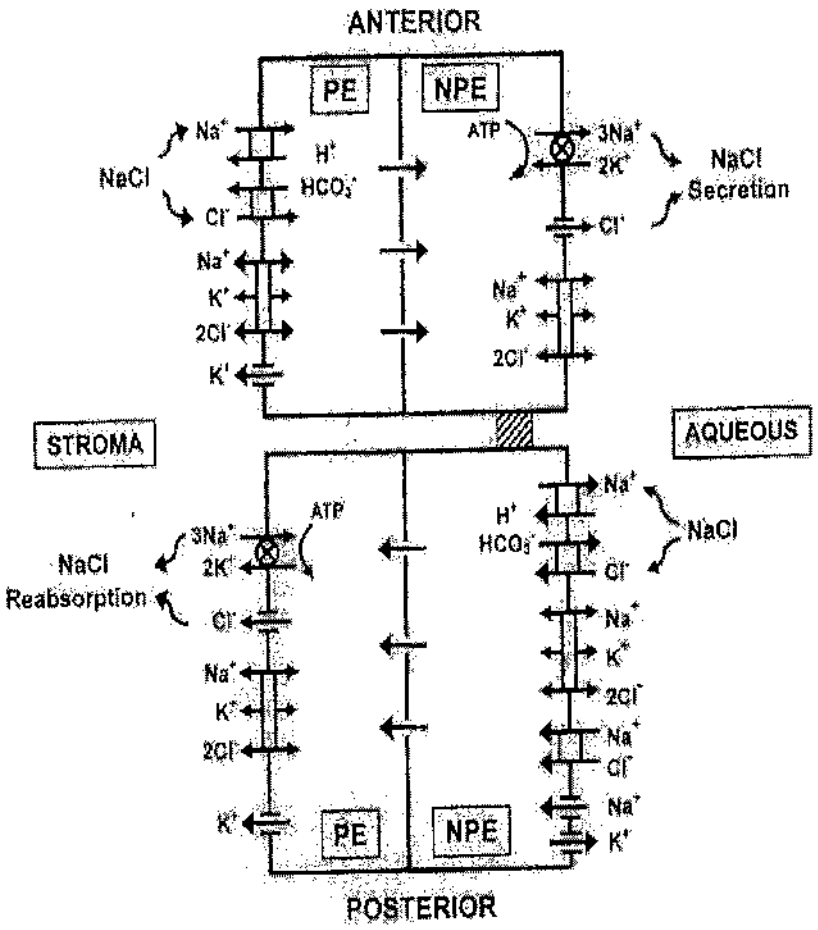

Figure 4

Model of aqueous humour formation.

Mechanisms thought to underlie unidirectional secretion are presented in the upper pair of pigmented ciliary epithelial (PE) and non-pigmented ciliary epithelial (NPE) cells, and those possibly involved in unidirectional reabsorption are illustrated in the lower pair of PE –NPE cells. **(Civan *et al.*, 2004)**

Receptors isolated on non-pigmented ciliary epithelial cells

Both parasympathetic and sympathetic nerves have previously been observed within the ciliary processes, though there is no evidence for direct innervation of the epithelial cells **(Polansky *et al.*, 1985).** It is suggested, though not yet confirmed, that both the sympathetic and parasympathetic systems may be involved in the control of secretion of aqueous humour **(Yamada, 1988),** and so their role remains uncertain.

In the rabbit both noradrenaline and adrenaline have been found to increase $[Ca^{2+}]_i$ but had little effect on human non-pigmented ciliary epithelial cells **(Ohuchi *et al.*, 1992).** The authors' suggestions were that in human non-pigmented ciliary epithelial cells there may be an alternative messenger for the α-adrenergic receptor. Alternative theories suggested that as fetal eyes were used the receptors may not have yet developed and finally that the normal cell physiology may be altered in cell culture.

It is well known in both humans and rabbits that a diurnal variation occurs in IOP **(Newell *et al.*, 1965).** Research would suggest that this diurnal variation is, least in part, under β-adrenergic control **(Yoshitomi *et al.*, 1991; Gregory *et al.*, 1985).**

Use of the confocal microscope in conjunction with calcium-sensitive dyes has allowed different patterns of calcium response to be identified within the rabbit **(Suzuki *et al.*, 1997).** It has been noted that non-pigmented ciliary epithelial cells contain predominantly muscarinic receptors, while pigmented ciliary epithelial cells appear to have a

predominance of α-adrenergic receptors. It is also suggested that both cADP and $InsP_3$ systems are involved in intracellular calcium regulation **(Suzuki *et al.*, 1997).**

It has previously been shown that rabbit non-pigmented ciliary epithelial cells have a significant number of active enzyme systems situated on their basolateral membranes. These include Na^+/K^+-ATPase, adenylate cyclase and carbonic anhydrase **(Kaye *et al.*, 1965; Mishima *et al.*, 1982; Shiose *et al.*, 1966; Tsukahara *et al.*, 1978).**

Regulation of aqueous humour formation experimentally

Cyclic AMP (cAMP) has for a long time been shown to play an important role in regulation of aqueous humour formation **(Neufeld *et al.*, 1972; Nathanson, 1980).** More recently alternative second messengers including cyclic GMP (cGMP) **(Becker, 1990; Shahidullah *et al.*, 1999)** and Ca^{2+} **(Lee *et al.*, 1989; Yoshimura *et al.*, 1995; Berridge, 1993; Shahidullah *et al.*, 1999)** have developed favourable interest.

Cyclic GMP has been shown to lower IOP in rabbits when given topically **(Becker, 1990).** Within the bovine eye **Millar *et al.* (1997)** reported that atriopeptin increased cGMP in both ciliary processes and cultured ciliary epithelial cells. The group also found a decrease in aqueous humour formation rate and IOP with atriopeptin. The case for the involvement of cGMP in aqueous humour formation has strengthened, with reports of sodium azide, a compound that increases cGMP production, though by an alternative mechanism to atriopeptin, also reduces aqueous humour formation **(Shahidullah *et al.*, 1999).** They also suppressed the atriopeptin and sodium azide-induced reduction in

aqueous humour production with the aid of KT-5823 (a specific inhibitor of protein kinase G). It would be reasonable to suggest cGMP as a common pathway of these drugs, the effect of which is to reduce aqueous humour formation.

Mobilization of intracellular calcium

The development of Ca^{2+} -sensitive dyes, including Fura-2, and fluorescent techniques have allowed monitoring of free $[Ca^{2+}]_i$ continuously in living cells **(Grynkiewicz *et al.*, 1985)**.

Within the resting cell most calcium is stored in the endoplasmic reticulum, sarcoplasmic reticulum and mitochondria. Free calcium is kept at a low level (approx. 10^{-7}mol/L) compared to extracellular Ca^{2+} concentration $[Ca^{2+}]_o$ found in the tissue fluid in which the cells are bathed (approx. 2.4 mmol/L). The gradient is produced by: (1) active transport mechanisms that either pump Ca^{2+} out through the plasma membrane or pump it into the endoplasmic reticulum, and (2) the relative impermeability of the plasma and endoplasmic reticulum membranes to Ca^{2+}.

Exogenous ATP has been shown to mobilise intracellular calcium **(Shahidullah *et al.*, 1997)**, acting via $P2Y_2$ surface receptor and utilizing phopholipase C, eventually resulting in a release of intracellular calcium from the endoplasmic reticulum. Atriopeptin, sodium azide and 8-bromo cGMP were shown to inhibit the ATP-induced release of intracellular calcium.

In other settings various ways by which cGMP may inhibit Ca^{2+} signalling have been postulated. These include inhibition of $InsP_3$-induced Ca^{2+} release **(Komalavilas *et al.*, 1994)**; activation of store reuptake **(Raeymakers *et al.*, 1988)** and inhibition of extracellular Ca^{2+} influx **(Pozzan *et al.*, 1994).**

If the mobilisation of intracellular Ca^{2+} does play an important final step in aqueous humour formation then it would be important to look at the signal transduction systems and second messenger systems in the ciliary epithelium.

The inositol trisphosphate ($InsP_3$)/ diacylglycerol (DAG) pathway has previously been shown to be involved in the release of intracellular Ca^{2+} from non-pigmented ciliary epithelial cells **(Crook *et al.*, 1991).** The $InsP_3$/DAG pathway involves receptor-mediated activation of phospholipase C, which catalyses the formation of $InsP_3$ and DAG from phosphatidylinositol 4, 5-bisphosphonate. $InsP_3$ causes release of Ca^{2+} from the endoplasmic reticulum stores, and DAG activates protein kinase C **(Berridge, 1987)** (Figure 5).

$InsP_3$ is formed as a second messenger in two pathways, one being via the family of G protein-linked receptors and the other via tyrosine kinase-linked receptors **(Berridge, 1993).**

The $InsP_3$/DAG pathway within the transformed human non-pigmented ciliary epithelial cells has been reported to be activated by histamine, bradykinin, vasopressin, carbachol and bombesin **(Crook *et al.*, 1989,1991,1992; Yang *et al.*, 1998). Yang *et al.* (1998)** also

reported, in canine cultured tracheal epithelial cells, an absence of $InsP_3$ formation and subsequent Ca^{2+} release normally produced by B_2 receptor activation, if the cells were pre-treated with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC). This would firstly suggest an association between B_2 receptor activation, $InsP_3$ formation and Ca^{2+} release, and secondly some sort of mechanism by which either PKC production, or a product of its making feeds back to inhibit the B_2 receptor response.

Calcium transients have been observed as oscillating Ca^{2+} spikes in ciliary epithelial cells **(Hirata *et al.*, 1998)**. It has been suggested that these transients may be involved in aqueous humour secretion **(Shahidullah *et al.*, 1999)**. An ionic basis for the oscillations has been suggested with involvement of Ca^{2+} -activated K^+ channels **(Jacob *et al.*, 1996)**. An alternative theory has been storage of ATP within the ciliary epithelial cells, the release of which would lead to Ca^{2+} release within the cytosol **(Mitchell *et al.*, 1998)**. **Yoshimura *et al.* (1995)** studied organ-cultured rabbit ciliary processes with both noradrenaline and carbachol causing release of calcium within non-pigmented ciliary epithelial cells, this contrasted with **Lee *et al.* (1989)** findings in human ciliary epithelial cells where both noradrenaline and carbachol had no effect.

A dose-dependent increase in intracellular calcium has previous been found in human non-pigmented ciliary epithelial cells in cultured cell lines exposed to carbachol, vasopressin, bradykinin, histamine and angiotensin II **(Lee *et al.*, 1989)**. Other investigators have shown an increase in $[Ca^{2+}]_i$ to various agonists, including adenosine, acetylcholine and epinephrine **(Farahbakhsh *et al.*, 1994; Lee *et al.*, 1989; Ohuchi *et***

***al.*, 1992; Crook *et al.*, 1992).** This would suggest a role for Ca^{2+} as an important second messenger in non-pigmented ciliary epithelial cells.

Research has suggested that calcium ions play a central role in aqueous production and that some drugs well known to decrease aqueous production also alter calcium movements within cells **(Lee *et al.* 1989; Crook *et al.*, 1992; Yoshimura *et al.*, 1995; Farahbakhsh *et al.*, 1997).** Over recent years various studies have been undertaken to look at the signal transduction systems and second messenger systems in the ciliary epithelium **(Shahidullah *et al.*, 1995; Hirata *et al.*, 1998).**

If intracellular calcium transients are involved in aqueous humour secretion **(Jacob *et al.*, 1996; Shahidullah *et al.*, 1999)** then it is possible that the ionic/electrophysiological basis for these transients may include involvement of calcium-activated potassium channels **(Jacob *et al.*, 1996).**

Though the cell signalling mechanism that leads to an increase in $[Ca^{2+}]_i$ has been extensively investigated by numerous researchers it currently still appears to be far from understood.

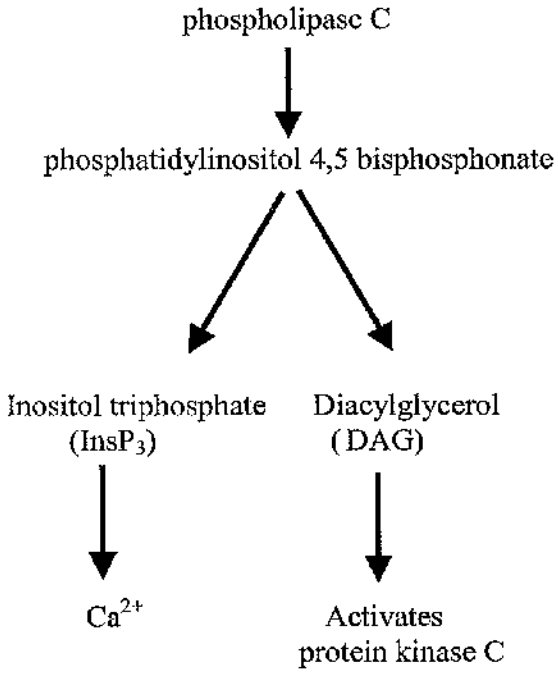

Figure 5

Inositol trisphosphate / diacylglycerol pathway

$InsP_3$, Inositol triphosphate; DAG, diacylglycerol

(Adapted from Berridge, 1987)

The role of non-pigmented and pigmented ciliary epithelial cells in the formation of aqueous humour

It appears that the interaction between the non-pigmented ciliary epithelial cells and pigmented ciliary epithelial cells is much more important than previously thought, though agreement is still that the non-pigmented ciliary epithelial cells play the major role in the active transport of solutes and water into the anterior chamber.

Hirata *et al.* (1998) used the confocal microscope to investigate interactions between pigmented ciliary epithelial cells and non-pigmented ciliary epithelial cells in isolated intact ciliary epithelial bilayers. They specifically looked at Ca^{2+} signalling. It was found that both the non-pigmented and pigmented ciliary epithelial cells were independently capable of generating Ca^{2+} signals, the non-pigmented ciliary epithelial cells via muscarinic receptors and the pigmented ciliary epithelial cells via α-adrenergic receptors. Stimulation however with epinephrine resulted in increased $[Ca^{2+}]_i$ in both layers, reflecting sequential Ca^{2+} signalling passing from the pigmented to the non-pigmented ciliary epithelial cells. The study suggested that $[Ca^{2+}]_i$ waves spread, via gap junctions, from the pigmented to the non-pigmented ciliary epithelial cells **(Hirata *et al.* 1998).**

The composition of aqueous humour

Aqueous humour has a low protein concentration compared to plasma, approximately 0.5% that of plasma **(Bito, 1977).** The composition of the protein component also differs between aqueous and plasma, with a much higher ratio of low molecular weight proteins, for example albumin, to higher molecular weight proteins, for example beta-lipoproteins, in aqueous compared to plasma.

The blood-aqueous barrier behaves as an isoporous membrane with a pore radius of 10^3nm **(Dernochamps *et al.*, 1975, 1977).** Previously the blood aqueous barrier was described as an epithelial barrier formed by the non-pigmented ciliary epithelium and an endothelial barrier in the form of the iris blood vessels **(Raviola, 1977).** The agreed theory was that tight junctions between the apico-lateral surfaces of the non-pigmented ciliary epithelial cells, and between endothelial cells of the iris vasculature, prevent the movement of plasma proteins into the aqueous humour **(Freddo *et al.*, 1982; Hirsch *et al.*, 1995).**

Recently a number of findings have questioned this theory **(Freddo, 2001).** Plasma-derived protein found in the aqueous humour originates from the fenestrated capillaries of the ciliary body stroma **(Freddo *et al.*, 1990).** Thus the main route of entry for protein into aqueous humour is directly into the anterior chamber, bypassing the posterior chamber. McLaren and colleagues **(McLaren *et al.*, 1993)** described a new model, identifying the iris stroma as a third compartment involved in the blood aqueous barrier.

Plasma proteins leak from the fenestrated capillaries of the ciliary body, though the tight junctions of the non-pigmented ciliary epithelial cells prevent them from entering the posterior chamber. The protein then diffuses to the iris stroma. As the anterior surface of the iris has no epithelium, the protein reaching the iris stroma passes into the anterior chamber. The combination of the barrier produced by the tight junctions of the iris epithelium and the forward, unidirectional flow of aqueous humour through the pupil, prevent flow back toward the posterior chamber. Thus the route by which aqueous humour is delivered the anterior chamber is different to the pathway delivering the plasma-derived proteins found in aqueous humour **(Freddo, 2001).**

Stur *et al.* (1983,1986), using timolol, had identified that as aqueous humour production was suppressed, the protein concentration in the anterior chamber increased to a level that it caused light scatter (flare) in the anterior chamber. As a result of this they postulated the above hypothesis.

Freddo (2001) suggests that the blood aqueous barrier, rather than intending to separate blood from aqueous, is rather separating the plasma constituents from the anterior and posterior chambers.

Table 2 shows the concentrations of electrolytes in the aqueous humour and in plasma. The aqueous concentration of glucose is approximately 80% of that of plasma. Lactic acid levels in the anterior chamber are much higher than those in plasma. Ascorbate is secreted actively by a specific, saturable transport mechanism into aqueous humour

producing high levels. Phospholipids are found in aqueous in concentrations ranging from 1/30 to ½ of the plasma concentrations **(Bito, 1977; Krause *et al.*, 1969).**

	Human Plasma	Human AH	Bovine AH
Electrolytes			
Na^+ (mM)	130-145	142	143.8-149.5
K^+ (mM)	3.5-5.0	4	4.5-7.1
Cl^- (mM)	92-125	131-136	116.3-124
HCO_3^- (mM)	24-30	20	36
Organic solutes			
Ascorbate (mM)	0.04-0.06	1.0-1.1	2
Lactate (mM)	0.5-1.9	4.5	7.6-8.2
Glucose (mM)	5.6-6.4	2.8-3.9	2.17
Protein ($mg.ml^{-1}$)	60-70	0.3-0.7	0.2-0.8

Table 2

The concentration of electrolytes, low molecular weight solutes and protein in bovine and human aqueous humour, and human blood plasma

Adapted from Bito *et al.* (1970), Caprioli (1992), Cole (1970), Davson (1990), Midelfart (1991), Novak *et al.*(1988), Pavao *et al.* (1989), Shahidullah (1994).

Ascorbate and the eye

Ascorbic acid is secreted by the ciliary epithelium, specifically via the SVCT2 Na^+-dependent L-ascorbic acid transporters **(Tsukaguchi *et al.*, 1999).**

The role of ascorbate within the eye is now widely accepted as involvement in the protection against free-radical formation, with potential damage to intraocular structures. For many years it had been suggested that its main role was to act as an electron donor to ascorbate peroxidase (APX), hydrogen peroxide-scavenging peroxidase. However it was not until relatively recently that ascorbate peroxidase activity was identified in bovine retinal pigment epithelium and choroid, thus strengthening the role of ascorbate in protection against free radicals **(Wada *et al.*, 1998).**

In the high concentrations of ascorbate present within the bovine eye, aqueous ascorbate has been shown to increase absorption and suppress fluorescence of radiation below 310nm wavelength. It has also been shown to reduce fluorescence emission in the UV-A range (320-400 nm) **(Ringvold, 1996).**

Measurement of ascorbate in the anterior bovine eye has shown highest concentrations to be found in the central corneal area overlying the pupillary area. This would be consistent with the idea of ascorbate acting as a UV filter, protecting the internal eye structures from radiation damage. Ascorbate concentration was also found to be present in a concentration comparable to that found within the anterior chamber within the corneal

stroma. This would suggest that the aqueous humour plays an important role in the distribution of ascorbate **(Ringvold *et al.*, 2000).**

Lower levels of ascorbate within the eye have been found to be associated with the condition exfoliation syndrome found in the human, which is thought to be related to impaired extracellular matrix metabolism **(Koliakos *et al.*, 2002)**. It is postulated by these authors that this condition may be a result of free radical formation in the presence of reduced ascorbate levels.

Other roles have been suggested for ascorbic acid. In the lung for example L-ascorbic acid has been shown in normal physiological concentrations to reduce cGMP accumulation by scavenging nitric oxide as well as direct inhibition of soluble guanylyl cyclase **(Shrammel *et al.*, 2000).** In contrast, ascorbic acid has been shown to actually increase nitric oxide synthesis in endothelial cells **(Heller *et al.*, 1999).** Opinion is that this action may play a part in the beneficial vascular effects seen with ascorbic acid. Evidence has yet to be found as to whether a similar role is present within the eye.

Trabecular meshwork and uveoscleral pathway

A stable intraocular pressure is the result of a balance between the production of aqueous humour and its outflow via the uveoscleral and trabecular meshworks. For a stable intraocular pressure, production should equal outflow.

There are two main pathways by which aqueous humour leaves the eye. The main outflow pathway is through the trabecular meshwork and is pressure-dependent. The trabecular meshwork is situated in the peripheral aspect of the anterior chamber. It is composed of a series of parallel layers of connective tissue, with each lamina containing pores.

The uveal meshwork is the innermost layer of the trabecular meshwork, and the corneoscleral meshwork is deep to this. Situated between the corneoscleral meshwork and the Schlemm's canal is the juxtacanalicular tissue, which provides the highest resistance to aqueous outflow. Having passed into the canal of Schlemm, aqueous humour flows via connector channels to episcleral veins.

The uveoscleral outflow pathway is the second pathway, with aqueous draining at the angle of the anterior chamber, immediately posterior to the cornea. The aqueous humour passes through the stroma of the peripheral iris and ciliary body before entering the suprachoroidal space and then out through the sclera **(Krohn, 2004).** The aqueous passes by bulk flow into blood vessels or through the scleral wall. This process is independent

of intraocular pressure **(Bill, 1966; Bill *et al.*, 1971).**

Models have now been developed that allow assessment of the human trabecular meshwork in isolation **(Erickson-Lamy *et al.*, 1991).**

Numerous chemicals have shown to have a direct effect on the ciliary muscle and trabecular meshwork of the bovine eye *in vitro*, reducing outflow resistance for example carbachol, pilocarpine and endothelin **(Wiederholt *et al.*, 1995)**, and thus increasing outflow facility **(Kaufman, 1984).**

Glaucoma

Glaucoma is a major cause of blindness worldwide **(Thylefors *et al.*, 1995).** Estimates suggested that by the year 2000, 67 million people world wide would be suffering from glaucoma **(Flanagan, 1998).** Its incidence in the United States is estimated at 3% of the over 65 population **(Johnson *et al.*, 1986).** It is a broad term involving intraocular pressure (IOP), optic nerve head damage, visual field loss and the drainage angle **(Sugrue, 1989).** If untreated, glaucoma may progress to blindness.

The pharmacological basis of ocular hypotensive agents

Physicians have treated glaucoma with topical agents for over one hundred years **(Zimmerman, 1981)** though our understanding of these agents still remains at best, basic.

Recent large studies, in humans **(Kass *et al.*, 2002),** have confirmed that in a population lowering intraocular pressure slows progression of glaucomatous visual field loss, thus preserving vision.

Intraocular pressure may be reduced by either reducing production within the eye or increasing the rate of outflow. Parasympathomimetic agents, adrenergic agonists, and prostaglandin analogues increase outflow rate, resulting in a reduction in IOP. Adrenergic antagonists and carbonic anhydrase inhibitors reduce aqueous humour production and so lower IOP. The current pharmacological preparations available are described below with, as far as possible, their mode of action.

Adrenergic agonists

α_1-adrenergic stimulation causes vasoconstriction within the ciliary body, reduction in aqueous humour formation and increased trabecular outflow. α_2-adrenergic inhibits noradrenaline release, resulting in reduced aqueous humour production and increased uveoscleral outflow. Both β_1 and β_2 stimulation within the ciliary epithelium increases

aqueous humour production. β_2 receptor activation increases trabecular meshwork outflow facility **(Zimmerman, 1981).**

Topically applied clonidine, predominantly an α_2-adrenergic agonist, reduces aqueous humour formation. Unfortunately it is readily absorbed systemically and causes systemic hypotension **(Harrison *et al.*, 1977; Leopold *et al.*, 1986).** This therefore makes clonidine not a feasible option as an ocular hypotensive agent.

Brimonidine and apraclonidine are derivatives of clonidine and with poor penetration of the blood-brain barrier have fewer of the side effects of clonidine with most of the IOP-lowering effect **(Abrams *et al.*, 1987; Serle *et al.*, 1991; Hurvitz *et al.*, 1991).** These agents may act locally via vasoconstriction resulting in decreased blood flow in the ciliary processes and thus reduced aqueous production **(Abrams *et al.*, 1987; Burke *et al.*, 1986).** Current opinion is that they also augment the release of prostaglandins at a local level, which may in turn increase uveoscleral outflow **(Crawford *et al.*, 1987; Poyer *et al.*, 1995).**

Beta-receptor antagonists

β-blockers are either nonselective, inhibiting β_1-receptors and β_2-receptors, or β_1-selective, specifically inhibiting β_1-adrenoceptors. β-blockers have potentially severe systemic side effects due cardiac β_1-adrenoceptor blockade, causing bradycardia, arrhythmia, congestive cardiac failure or syncope. β_2-adrenoceptor blockade can cause bronchospasm and exacerbation of both asthma and chronic obstructive airway disease.

Beta-blockers include agents such as timolol, betaxolol and levobunolol. B_2 receptors have been identified in rabbit and ovine ciliary processes **(Nathanson, 1980; Trope *et al.*, 1982)** and in non-pigmented ciliary epithelial cells in the bovine ciliary processes **(Elena *et al.*, 1984).** Interestingly, B_2 adrenoceptors have also been identified in human trabecular meshwork **(Jampel *et al.*, 1987).**

Timolol, a non-selective beta-adrenergic antagonist, reduces aqueous humour formation and thus lowers IOP **(Coakes *et al.*, 1978).** It has been suggested that timolol down-regulates adenylate cyclase by its inhibition of β_2-adrenoceptors on the ciliary processes. It is only effective during the waking hours, with little/no effect on aqueous humour production during sleep **(Coakes *et al.*, 1978; Topper *et al.*, 1985).** Timolol has no effect on outflow facility **(Zimmerman *et al.*, 1977; Sonntag *et al.*, 1978).**

Betaxolol, a β_1-selective adrenoceptor antagonist, reduces aqueous humour formation **(Reiss *et al.*, 1983),** though less effective at lowering IOP compared to than timolol. As

the ciliary body contains few β_1-adrenoceptors, it is thought to be acting via its much weaker β_2-blocking properties (Reiss et al., 1983). β_1-selective adrenoceptor antagonists minimise the potential β_2-mediated pulmonary effects and cardiovascular adverse effects including bradycardia and congestive cardiac failure **(Harris *et al.*, 1986; Roholt, 1987; Nelson *et al.*, 1987; Ball, 1987).**

Though extensively used as ocular hypotensive agents their mode of action still remains questionable. Available data suggests a local action of beta-adrenergic antagonists but whether their ocular hypotensive effect is due to blockade of beta-adrenergic receptors still remains uncertain **(Leopold *et al.*, 1988; Leopold *et al.*, 1986; Watanabe *et al.*, 1983).**

Wilson *et al.* (1988) and Shahidullah *et al.* (1997) found that both timolol and terbutaline did produce significant reduction in IOP. This would suggest that at least one of these agents was working via a mechanism other than the beta-adrenoceptor.

Parasympathomimetic agents

Parasympathomimetic agents include pilocarpine, carbachol and phospholine iodine. Pilocarpine is the most commonly used in the treatment of glaucoma. It is a muscarinic agonist whose main action is to reduce IOP by increasing outflow via the trabecular meshwork. It contracts the ciliary muscle, pulling the scleral spur and increasing aqueous outflow by mechanical deformation of the trabecular meshwork **(Bill, 1967; Bill, 1971).** Pilocarpine does, however, stop uveoscleral outflow almost completely **(Bill, 1967).**

Muscarinic agents may have some direct effect upon aqueous production **(Bill & Walinder, 1966)** and muscarinic receptors have been identified in the rabbit iris and ciliary body **(Kloog *et al.*, 1979)** and NPE cells of the bovine ciliary body **(Polansky *et al.*, 1985).**

Carbonic anhydrase inhibitors

Carbonic anhydrase has been identified in both pigmented and non-pigmented ciliary epithelial cells of rabbit and human ciliary processes **(Muther *et al.*, 1980; Wistrand *et al.*, 1986).** Oral administration of acetazolamide, a carbonic anhydrase inhibitor, was first shown to be effective at lowering IOP by **Becker *et al.* (1955).** The site of action is intraocular **(Wistrand *et al.*, 1957).** Their action is to inhibit carbonic anhydrase isoenzyme II that catalyses the production of HCO_3^- and H^+ from CO_2 and H_20. This reduction in bicarbonate production, in turn, slows Na^+ and water secretion into the eye **(Maren, 1974, 1976).** Both aqueous Cl^- concentration in primates **(Sears, 1992)** and HCO_3^- concentration in rabbits **(Becker *et al.*, 1955)** are reduced by inhibition of carbonic anhydrase.

A number of hypotheses have been suggested to explain the decrease in active transport of Na by the non-pigmented ciliary epithelial cells when carbonic anhydrase is inhibited **(Caprioli, 1992):**

(1) A reduction in HCO_3^- available for movement with Na^+ into the aqueous to maintain electroneutrality;

(2) Na^+/K^+ ATPase may be inhibited by the change in intracellular pH;

(3) a reduction in the availability of H^+ usually produced by the action of carbonic anhydrase decreases H^+/Na^+ exchange, thus reducing the availability of intracellular Na^+.

They rarely produce systemic acidosis, though do have other systemic side effects, including fatigue, depression, gastrointestinal disturbances and weight loss **(Epstein & Grant, 1977; Flach, 1986).** More recently topical carbonic anhydrase inhibitors have been developed (e.g. dorzolamide), which though lowering IOP and having a minimal side effect profile, are not as potent as oral acetazolamide.

Prostaglandin analogues

Up to 20% of aqueous humour drains from the eye by passing between the ciliary muscles into the episcleral tissue before being reabsorbed by the orbital blood vessels, draining via the conjunctival vessels (uveoscleral outflow). Prostaglandin analogues act by increasing uveoscleral outflow **(Kaufman, 1986; Crawford & Kaufman, 1987).** Studies have suggested that $PGF_{2\alpha}$ relaxes the ciliary muscle, widening the extracellular channels and thus increasing outflow **(Crawford *et al*,. 1987).** Prostaglandins have also been shown to produce changes in extracellular matrix **(Lindsey *et al.*, 1997).**

It appears therefore that two mechanisms may be involved, one due to action on the ciliary muscle resulting in increased aqueous drainage and the other due to remodelling of the extracellular matrix **(Toris *et al.*, 1997).**

Potential ocular hypotensive agents

Nitrates

The topical application of 8-bromo cGMP (an analogue of cGMP) has been shown to lower IOP in rabbits *in vivo* **(Becker *et al.*, 1990).** Azide and atriopeptin act via stimulation of cGMP synthesis. Both produce vasodilatation and both have been shown in the isolated bovine perfused eye to reduce aqueous production and thus in turn lower IOP **(Millar *et al.*, 1997).** Correlations have been established between these effects and the ability of these drugs to affect $[Ca]_i$ in bovine ciliary epithelium **(Shahidullah *et al.*, 1999).**

Bradykinin

Bradykinin (BK) is a vasoactive nonapeptide formed by enzymic action upon kininogens (protein precursors). Once formed it is converted by kininase I to an octapeptide, which is inactivated in the lung by angiotensin-converting enzyme.

Within the plasma there are two forms of kininogen, a high-molecular weight form (M_r 110 000) and a low-molecular weight form (M_r 70 000). Both forms of kininogens may be cleaved to form bradykinin. Prekallikrein, the inactive precursor of kallikrein, is present in the plasma.

Prekallikrein is converted to the active form, kallikrein in a number of ways. Hageman factor (factor XII, in clotting cascade) cleaves prekallikrein to kallikrein during the inflammatory process, which in turn leads to the production of bradykinin from kininogen. Bradykinin is inactived by the kininases.

Kininase II is the same as angiotensin-converting enzyme which is found on the luminal surface of endothelial cells, particularly in the lung. It is also inactivated by kininase I which is a less specific carboxypeptidase found in serum.

Two subtypes of bradykinin receptor are currently recognised, B_1 and B_2. Des-Arg^9-bradykinin and Des-Arg^{10}-kallidin are potent B_1 receptor agonists whereas kallidin and bradykinin are potent B_2 receptor agonists **(Simpson *et al.*, 2000).**

Both B_1 and B_2 receptor have now been cloned and sequenced **(McEachern *et al.*, 1991; McIntyre *et al.*, 1993; Menke *et al.*, 1994).** Despite species variation, all belong to the rhodopsin family of signalling proteins, and as such employ G-proteins in signal transduction **(Marceau, 1995).**

B_1 and B_2 receptors cause phosphoinositol hydrolysis by phospholipase C or arachidonic acid release by phospholipase A_2 **(Hess *et al.*, 1994; Menke *et al.*, 1994; Smith *et al.*, 1995).** In ciliary epithelium it seems likely that phospholipase C releases $InsP_3$ which then mobilises calcium from intracellular stores via a ligand-gated Ca^{2+}-releasing channel.

The previous suggestion of a third type of bradykinin receptor has so far not been shown by molecular cloning. In these initial experiments it is suggested that the B_2 agonist was actually metabolised to a second compound with B_1 agonism **(Regoli *et al.*, 1998; Rhaleb *et al.*, 1991).**

B_1-receptors appear not to be present when cultures are initially set up, but rather synthesised and expressed slowly over several hours **(Marceau, 1995).** Both B_1 and B_2 receptors are thought to play a role in the inflammatory response: the B_2 receptor appears to be important in the early response and the inducible B_1 receptors in the chronic inflammatory response **(Dray *et al.*, 1993; Marceau *et al.*, 1998; Hall, 1997; Haddad, 1999).** It has been suggested that B_1-receptors are involved in persistent inflammatory hyperalgesia **(Ahuwalia *et al.*, 1999).** Potentially bradykinin receptor antagonists may have a role in future, as therapeutic agents, in the treatment of inflammatory disease **(Haddad, 1999).**

Bradykinin causes vasodilatation and increased vascular permeability **(Haddad, 1999).** It expresses its vasodilator action partly by the generation of prostaglandin, partly by the release of nitric oxide, and partly by the production of endothelium-derived hyperpolarising factors (EDHF), as discussed later. It produces pain, an effect that is potentiated by prostaglandin. Bradykinin produces spasm in most types of smooth muscle, other than vascular, namely intestine, uterus and bronchial muscle **(Scherrer *et al.*, 1995; Asano *et al.*, 1997a and b; Griesbacher *et al.*, 1997).**

Bradykinin release is also involved in the control of blood flow to a number of exocrine glands (e.g. pancreas, salivary glands) thus affecting secretions of the glands. Bradykinin has been shown to stimulate ion transport and fluid secretion by various epithelia, including airways and intestine **(Yang *et al.*, 1998).** Bradykinin may play a part in the growth of solid tumour cancers and septic shock, and research is currently underway to look at the potential use of various bradykinin antagonists in the management of these conditions **(Stewart *et al.*, 1999; Shin *et al.*, 1996).**

Bradykinin has previously been shown to cause contraction of isolated rabbit sphincter pupillae muscle and miosis *in vivo* in the rabbit **(Wahlestedt *et al.*, 1984).** This effect appeared to be inhibited by the use of a substance P antagonist, suggesting that bradykinin may have an effect through the release of substance P or by direct action on the substance P receptors.

In addition to causing miosis, bradykinin causes a breakdown of the blood-aqueous barrier, manifest as aqueous flare, when injected *in vivo* at concentrations of 10^{-8}M directly into the anterior chamber of the rabbit eye in **(Bynke *et al.*, 1983).**

Within the cynomolgus monkey eye bradykinin reduces outflow facility, though the mechanism of this is uncertain **(Kaufman *et al.*, 1982).**

In the human eye, specifically the retina, choroid and ciliary body the presence of both B_2 and B_1 receptors have been identified at high levels using the polymerized chain reaction

(Ma *et al.*, 1996). Using in-situ hybridisation techniques, **Ma *et al.* (1996)** have also identified tissue kallikrein, the mRNAs of kininogen and bradykinin B_1 and B_2 receptors in the retina, vascular endothelial cells, lens epithelium and ciliary body.

Bradykinin induces vasodilatation via endothelial B_2 receptors, and this may at least partly be blocked by nitric oxide synthase inhibitors **(Gardiner, *et al.*, 1990; Mombouli, *et al.*, 1992; Bjornstad-Ostensen, *et al.*, 1997).** The component that is not blocked by nitric oxide synthase inhibitors appears to be due to the currently unidentified EDHFs. A number of possible candidates have been put forward including prostacyclin, potassium and products of arachidonic acid metabolism **(Edwards *et al.* 1998; Fisslthaler *et al.* 1999).**

Bradykinin has a vasodilator effect on the ciliary vascular bed of the bovine isolated eye, in a dose-dependent manner **(McNeish *et al.*, 2001).** In the same study it was also found that the response to bradykinin was unaffected by L-NAME, an inhibitor of nitric oxide synthase. This would suggest that nitric oxide does not play a part in the vasodilator responses to bradykinin within the isolated bovine eye. This vasodilator effect of bradykinin was also unaffected by indomethacin, hence it was postulated that bradykinin is acting via EDHF. This contrasts with findings in other species, for example porcine eye, where the bradykinin-induced vasodilator responses were significantly blocked by L-NAME, suggesting an important role for nitric oxide in this species **(Meyer *et al.*, 1993).**

Angiotensin-converting enzyme (ACE) inhibitors have been shown in a number of

studies to potentiate the effect of bradykinin, though currently the exact mechanism by which they have this effect has not been clarified **(Minshall *et al.*, 1997).** However it appears that the effect of ACE inhibitors on the potentiation of the bradykinin effect is independent of their effect on bradykinin metabolism **(Jan Danser *et al.*, 2000).**

Seemingly quite apart from its other effects, bradykinin-induced alterations in intracellular calcium in human non-pigmented ciliary epithelial cells have been reported **(Lee *et al.*, 1979).**

Bradykinin receptor antagonists

HOE 140, is a second generation, long-acting peptide B_2 antagonist. Numerous studies have found it both selective and specific for B_2 receptors. It is a competitive antagonist of bradykinin in humans, but a non-equilibrium antagonist, dissociating slowly in rabbit and guinea pig **(Hock *et al.*, 1991; Wirth *et al.*, 1991).** It has equal activity at rat, guinea pig, rabbit and human receptors **(Regoli *et al.*, 1994).** One author however found HOE 140 to behave as an irreversible antagonist in rabbit jugular vein, though did find it to be a competitive antagonist in human umbilical vein **(Marceau *et al.*, 1994).**

WIN 64338, a non-peptide B_2 receptor antagonist, has been shown to inhibit bradykinin-induced contraction in guinea pig trachea in a non-competitive manner **(Scherrer *et al.*, 1995; Altamura *et al.*, 1999).** Some evidence indicates that it is a competitive antagonist of bradykinin in the guinea pig ileum **(Farmer *et al.*, 1994),** rabbit jugular and human

umbilical veins **(Marceau *et al.*, 1994).** This controversy over WIN 64338's mode of action may be related to the different techniques used. There are number of drawbacks however with this compound as it has a low specificity, with some affinity for muscarinic receptors **(Wirth *et al.*, 1995; Regoli *et al.*, 1996).**

Substance P

Substance P is a peptide, belonging to the family of tachykinins. It has a role as a neurotransmitter, being located in primary afferent terminals **(De Baisi *et al.*, 1988)**. As a systemic hypotensive agent it is effective in producing peripheral vasodilatation **(Pernow, 1983)**. The substance P-induced relaxation of porcine coronary arterioles is mediated by nitric oxide **(Kuo *et al.*, 1991)**. It has previously been suggested **(Sharma *et al.*, 1994)**, that in the porcine coronary artery endothelial cells, substance P induces an increase in intracellular calcium that activates K^+ channels. This results in hyperpolarisation, producing a sustained Ca^{2+} entry required for the endothelial production of nitric oxide.

Substance P is involved in neurogenic inflammation, following its release substance P causes vasodilatation and extravasation, by increasing endothelial permeability via NK1 receptors **(Lembeck *et al.*, 1992)**.

Substance P releases histamine from human mast cells **(Church *et al.*, 1989)** and releases both insulin and glucagon in dogs **(Kaneto *et al.*, 1978)**. This has however previously caused confusion as in contrast it has an inhibitory effect on insulin and glucagon release in rats **(Chiba *et al.*, 1985)**.

Smooth muscle contraction may be elicited in many tissues by substance P, including rabbit pulmonary artery **(D'Orleans-Juste *et al.*, 1986)**, rat portal vein **(Mastrangelo *et al.*, 1987)** and gastrointestinal tract **(Daniel *et al.*, 1989)**.

A number of substance P receptor agonists and antagonists now exist. These include both peptide and non-peptide forms.

The substance P receptor possesses seven membrane-spanning domains with an amino acid sequence similar to the family of GTP-protein-coupled receptors **(Nakanishi, 1991)**.

Substance P has been shown to induce Ca^{2+} entry via activation of cation channels, as well as stored Ca^{2+} mobilization, suggesting that $InsP_3$ regulates both substance P-induced Ca^{2+} mobilization and entry into cells **(Mochizuki-Oda *et al.*, 1994)**.

Substance P is found in all ocular tissues, though the extent to which they are found is species variable **(Elbadri *et al.*, 1991)**. It has been suggested that substance P acts as a neurotransmitter within the bovine retina **(Osborne, 1984)**, and has been found to excite rabbit ganglion and amacrine cells **(Zalutsky *et al.*, 1990)**.

Substance P in the anterior segment is thought to derive from the trigeminal nerve and surgical denervation of the trigeminal nerve decreases the substance P concentration in the anterior segment **(Butler *et al.*, 1980; Miller *et al.*, 1981)**.

A large body of evidence supports that the physiological role for substance P in the eye is control of the pupil size **(Mochizuchi *et al.*, 1981)** and dose dependent contraction under the influence of substance P has been shown **(Tachado *et al.*, 1991)** in a number of species including rabbit, bovine and pig. A dose-dependent vasodilatation of intra-ocular retinal arterioles in dogs to substance P has been identified **(Kitamura *et al.*, 1993)**. This vasodilatation was mediated by nitric oxide synthesis.

Within the rabbit eye, substance P is released into the anterior chamber from substance P-containing nerve endings, located close to ciliary body vasculature and pupillary sphincter muscle **(Hakason *et al.*, 1985)**. It is released in response to noxious stimuli and results in vasodilatation, breakdown of the blood-aqueous barrier and a rise in IOP. Intracameral injection of substance P results in miosis, protein leakage in the aqueous humour and an increase in IOP **(Vaney *et al.*, 1989; Stjernschantz *et al.*, 1981)**. However at much lower doses of substance P (picomolar) though causing significant miosis, does not cause blood-aqueous barrier breakdown or a increase in IOP **(Stjernschantz *et al.*, 1981)**.

Substance P may play a role in both proliferative diabetic retinopathy **(Ziche *et al.*, 1990)** and proliferative vitreoretinopathy **(Kieselbach *et al.*, 1991)**.

Aims of the project

The aim of this project is to investigate the role and mechanism that bradykinin and substance P may have upon aqueous production within the eye.

The effects on aqueous humour formation of bradykinin and substance P (together with certain analogues and antagonists selective for particular receptors) will be measured in the bovine isolated perfused eye.

The mechanisms of any effects of bradykinin are to be investigated using the isolated arterial perfused bovine eye, as previously described by **Wilson *et al* (1993).**

If, as suspected, bradykinin and substance P reduce aqueous humour formation, an inhibitor of nitric oxide synthesis (L-NAME) and an inhibitor of guanylate cyclase (ODQ) shall be used, to demonstrate whether the effect is mediated through nitric oxide (as appears to be the case with azide), or through cyclic GMP.

Much has been written on the probable significant role $[Ca^{2+}]_i$ has on aqueous humour production. It would also therefore be an intention to study the effect both bradykinin and substance P have on movements of intracellular Ca^{2+} in non-pigmented ciliary epithelial cells. Using the above inhibitors, the opportunity will also be taken to classify the receptor and the mechanism by which they produce their effects in these cells.

MATERIALS AND METHODS

Arterially perfused bovine eye model

Using the bovine arterially-perfused eye model (**Wilson, *et al.*, 1993**) enables intraocular pressure and aqueous humour formation to be studied in an *ex vivo* environment under controlled physiological conditions (**Zhu, *et al.*, 1996**). It also removes the influences of neurological and cardiovascular control that affect *in vivo* models. Obviously it also removes the need for experiments on live animals at this stage in investigation.

Bovine eyes were obtained from the abattoir at the time of slaughter. Close proximity of the laboratory to the abattoir was essential to keep non-perfusion time to a minimum. The experimental method used was that as described by **Wilson *et al.* (1993)** using the constant flow method. Bovine eyes were cannulated via a long posterior ciliary artery and perfused with Krebs' solution. The eyes were perfused with a modified Krebs' solution [(mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.5; ascorbate, 0.05](Appendix 3) at a temperature of 37°C via one of the long posterior ciliary arteries within 60 minutes of extraction.

The perfusate was pumped, using a peristaltic pump (Watson-Marlow, Cornwall, England), at a flow rate of 0.3ml.min^{-1} initially at a pressure of <100mmHg, gradually increasing to 2.5ml.min^{-1} over a period of 20 min. The vasculature slowly relaxed so that the perfusion pressure settled at a stable level of 30-45 mmHg. The perfusion pressure was monitored constantly using a pressure transducer attached close to the arterial cannula. Pressure was recorded by either a Grass Polygraph (Figure 6) or a Powerlab

(AD instruments). After a period of about 30 minutes the anterior chamber had reformed and was then cannulated.

The cannula (consisting of a 23G needle shaft) inserted into the anterior chamber was connected to a water manometer, allowing constant monitoring of the IOP. The IOP readings were monitored approximately every 10 minutes during the experiments to ensure active, ongoing aqueous production in a viable eye and also to ensure the IOP remains within the normal physiological range.

Using two additional cannulae (consisting of 23G needle shafts) inserted into the anterior chamber, a separate anterior chamber perfusion system was created. This was a closed system that allowed constant circulation of the aqueous humour (Figure 6). Aqueous humour from the anterior chamber passed (at 0.25 $ml.min^{-1}$) via silicon rubber tubing through a cuvette situated within a fluorescence spectrometer (Perkin Elmer LS-3B), using a peristaltic pump (Watson-Marlow), before returning to the eye. This enabled measurement of fluorescence of the solution.

An initial equilibrating period at the beginning of the experiment was undertaken to allow aqueous humour and the added fluorescein (within an aqueous humour substitute) to mix and equilibrate with the eye's aqueous humour. The aqueous humour substitute consisted of (mM): NaCl, 110; KCl, 3.0; $CaCl_2.6H_2O$, 1.4; $MgCl_2.6H_2O$, 0.5; $NaHCO_3$, 30.0; K_2HPO_4, 0.9; glucose, 6.0; ascorbic acid, 3.0; sodium fluorescein, 0.0186 (Appendix 3). The rate of aqueous humour formation was calculated from the rate of

fluorescein dilution after the initial mixing period (10-15 min). By plotting $\log_e$ of the fluorescein concentration against time, the slope of the regression line was constructed. From this, the rate constant for aqueous humour formation (K_{out} min^{-1}) was calculated.

After a period of steady fluorescence decline during the next 30 min, the drug under investigation was added to the arterial perfusate and the recording of rate of fluorescence decline was continued over a further period of 90 min. The rate of fluorescence decline during 0-30 min was then compared statistically with that during the 90-120 min period using one-way analysis of variance and paired Students' t-test.

The following selection criteria were adhered to: All eyes must be perfused with one hour of slaughter, allowing up to 30 min travel time return from the abattoir reduces the time window to within 30 min of the eyes arriving in the laboratory. Following cannulation, eyes were rejected if: perfusion pressure exceeded 100mmHg; perfusate failed to flow freely from at least two vortex veins; major variations occurred in perfusion pressure that may have been due to an air bubble or blood clot blocking the artery; a stable IOP was not obtained. Failure due to any of these criteria resulted in outright rejection of that eye. The overall rejection rate was approximately 30-40%.

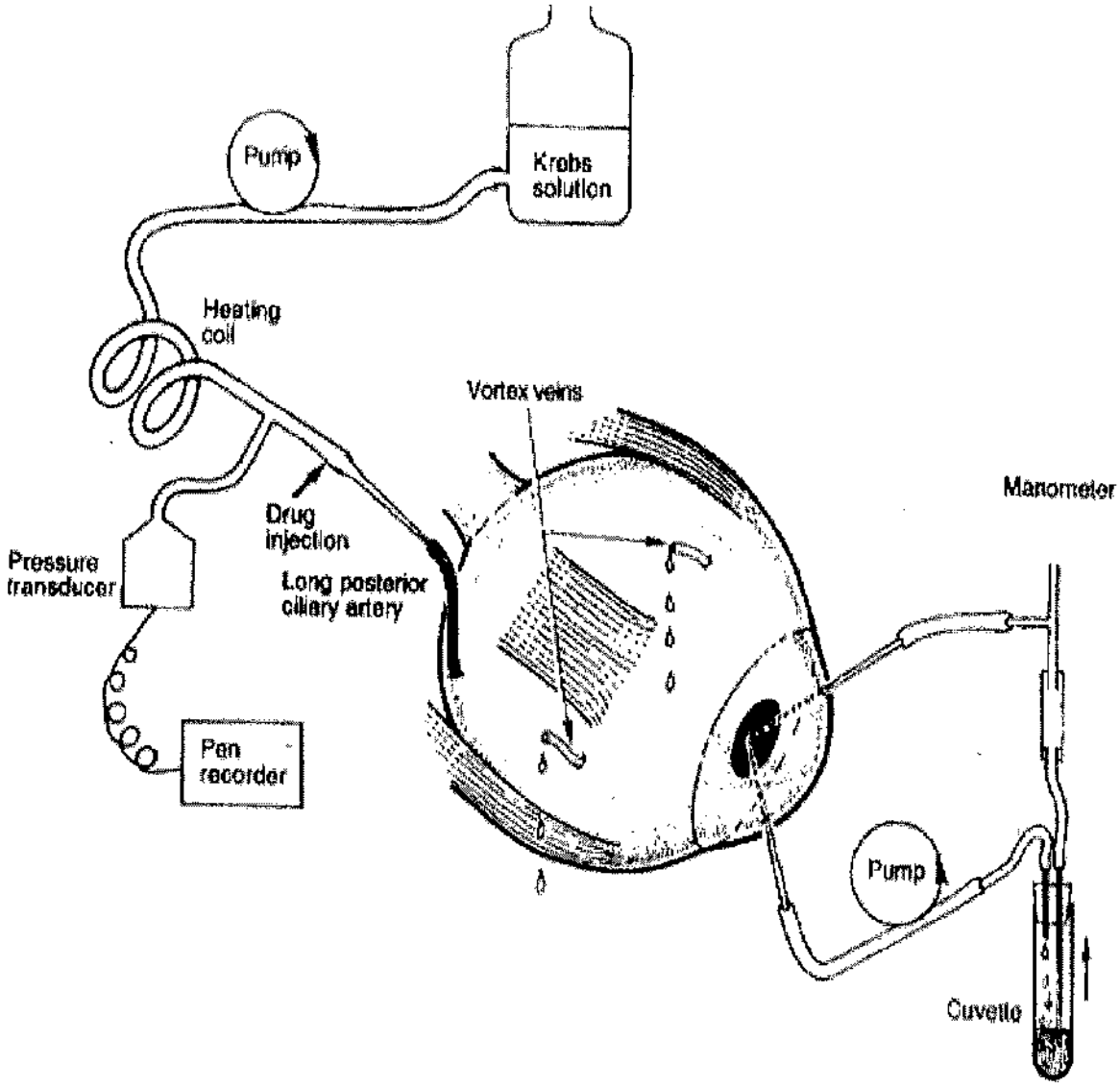

Figure 6

Diagrammatic representation of the isolated bovine eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under conditions of constant flow rate Perfusion pressure, rate of aqueous production and IOP were monitored as shown.
(Wilson *et al.*, 1993).

Administration of drug

As described above, once a stable IOP, required perfusion rate and steady fluorescence decline had all been attained for a period of 30 min then the drug was added. The drug was added to the perfusate reservoir at an exact concentration, being stirred continuously.

Bradykinin

Bradykinin was added to the perfusate at concentrations of 10^{-10}M, $3x10^{-10}$M, 10^{-9}M, $3x10^{-9}$M, 10^{-8}M, $3x10^{-8}$M or 10^{-7}M. Since the flow rate was 2.25ml.min^{-1} a period of 10 min was allowed for the drug to flow from the reservoir via the rubber tubing to the eye, passing through the heating coil *en route*. Fluorescence within the closed anterior chamber perfusion system was monitored continuously by the spectrophotofluorimeter. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

WIN 64338 hydrochloride

WIN 64338 hydrochloride, a non-peptide, competitive bradykinin B_2 receptor antagonist, was added to the perfusate at a concentration of $3x10^{-8}$M. At the same time bradykinin was also added at a concentration of 10^{-8}M. The method was that described previously. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

ODQ

ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), an inhibitor of soluble guanylate cyclase, was added to the perfusate at a concentrations of either 10^{-7}M or $3x10^{-7}$M. At the same time bradykinin was also added at a concentration of either 10^{-8}M or 10^{-9}M. In separate control experiments, ODQ was also added in on its own at a concentration of $3x10^{-7}$M. The method was that described previously. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

L-NAME

L-NAME, a nitric oxide synthase inhibitor, was added to the perfusate at a concentration of 10^{-4}M. Concurrently, bradykinin was also added at a concentration of 10^{-9}M. L-NAME was also added in isolation at a concentration of 10^{-4}M. The method was that described previously. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

Ascorbate

For the majority of experiments ascorbate was added to both the modified Krebs' solution and the aqueous humour substitute, as described by **Wilson *et al.*, (1993)**. In a number of experiments the ascorbate was omitted from either the perfusate alone or both the perfusate and aqueous humour substitute. For these experiments bradykinin was

added in concentrations of either 10^{-9}M or 10^{-7}M, to the perfusate. The method was that described previously. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

No independent controls were performed in the absence of ascorbate. Therefore the only controls used were those of 'same eye controls' over the initial period from 30 min prior to addition of drug until 10 min after.

Substance P

Substance P was added to the perfusate at concentrations of 10^{-8}M, $3x10^{-8}$M or 10^{-7}M. The method was that described previously. The readings were taken every 5 min for 30 min prior to addition of the drug and 90 min following.

Calcium mobilization within ciliary epithelial cells

The effects on intracellular calcium were determined by a fura-2 fluorescence technique in non-transformed cultured ciliary epithelium **(Shahidullah *et al.*, 1999).**

This technique involved dissection of bovine eyes and removal of the ciliary processes. The ciliary processes were prepared to allow dispersion of the ciliary epithelial cells, which were placed onto coverslips to provide a primary explant.

Dissection of bovine eye

A posterior approach was used, initially making a large cruciate incision to one side of the optic nerve, passing anteriorly up to the ora ciliaris retinae. The sclera was then reflected anteriorly, exposing the vitreous body. Using blunt dissection the vitreous was removed *en mass* revealing the posterior lens capsule. Using further blunt dissection it was possible, with care, to remove the posterior lens capsule, lens and anterior lens capsule as one. This exposed the ciliary processes whose tips were individually cut using sharp scissors.

Cell-culture of ciliary epithelial cells

Addition of calcium-free buffer solution

Initially the ciliary processes were placed into a petri dish containing 15ml calcium-free buffer solution containing (mM): NaCl, 142; KCl, 13.41; HEPES, 4.82; EDTA, 0.25. (Appendix 3). The dish was placed in an incubator and gently agitated for 30 min using an automated rotary shaker.

Addition of collagenase solution

The collagenase solution was sterilised using a syringe filter (0.2 μm, Gelman Sciences). The processes were then transferred to collagenase A (0.1%) solution prepared in buffer containing (mM): NaCl, 66.73; KCl, 13.41; HEPES, 3.84; $CaCl_2$ (Appendix 3). The processes were then incubated in the collagenase solution at 37° C for 30 min.

Neutralization of collagenase solution

A mixture of newborn calf serum (NCS) (10%) and fetal calf serum (FCS) (10%) (1:1) was added to the collagenase solution to neutralise the enzymatic effect. The partially digested tips were collected and added to Dulbecco's modification of Eagle's Medium (DMEM). The processes were disrupted by gently drawing up and expelling from a glass Pasteur pipette whose tip had been smoothed by heating. The ciliary processes were removed, leaving the DMEM solution containing suspended ciliary epithelial cells.

Separation by centrifugation and seeding onto coverslips

The test tubes were then centrifuged at 900g.min. The centrifugal pellet was collected and resuspended in 5ml of 'complete' DMEM medium containing a mixture of 10% NCS/ 10% FCS with gentamicin (200 $\mu g.ml^{-1}$) before being seeded onto 13mm sterile coverslips and incubated for 20-30 hours. The cells were incubated at 37°C in a humid incubator, containing 5% CO_2 and 95% air.

The following day the epithelial cells, having adhered to the coverslips, were placed in DMEM (5ml) with bovine serum albumin (1%) and were mixed with fura-2 (2μM), a fluorescent calcium marker and incubated, with continuous mild agitation for 40 min at 37° C, in an environment containing 5% CO_2 and 95% air.

The seeded coverslips were then washed in a modified Krebs' solution comprising (mM): NaCl, 118; KCl, 4.8; $NaHCO_3$, 2.4; $MgSO_4$, 1.0; glucose, 11.0; HEPES, 10.0; $CaCl_2$, 1.8;(Appendix 3) for 20 min prior to putting in position in a coverslip mount within a superfusion bath on the inverted stage of a Nikon Diaphot microscope.

Perfusion with modified Krebs', as above (Appendix 3) was commenced via gravity feed and a thermistor-controlled in-line heater (37°) at an approximate rate of $2.5ml.min^{-1}$ prior to commencement of superfusion with the chemical under investigation.

The use of a Quanticell 500 system (Applied Imaging), high resolution CCD camera and

Nikon Diaphot inverted stage microscope, allowed microfluorimetric imaging of individual ciliary epithelial cells. We know that after prolonged incubation pigmented ciliary epithelial cells lose their pigmentation preventing differentiation between them and non-pigmented ciliary epithelial cells. Using the experimental procedure as described only a short incubation period is required. It was thus possible to identify two populations of cells, those pigmented and those not pigmented using light microscopy.

The system allowed measurement of the fluorescence ratio at two different excitation wavelengths. Switching automatically between 340nm and 380nm filters and the use of fura-2, enabled calculation of the intracellular calcium concentration, using the Grynkiewicz method of dual excitation **(Grynkiewicz *et al.*, 1985).** Fura-2 is a fluorescent indicator with an affinity for Ca^{2+}. Compared to the older dyes (eg. quin2), it has up to 30-fold brighter fluorescence, a significant change in wavelength on Ca^{2+} binding and greater selectivity for Ca^{2+} over other divalent cations **(Grynkiewicz *et al.*, 1985).** It maintains a strong fluorescence while shifting wavelengths in response to calcium. An example of the pseudo-colour allocated to the fluorescence ratio is shown (Figure 7).

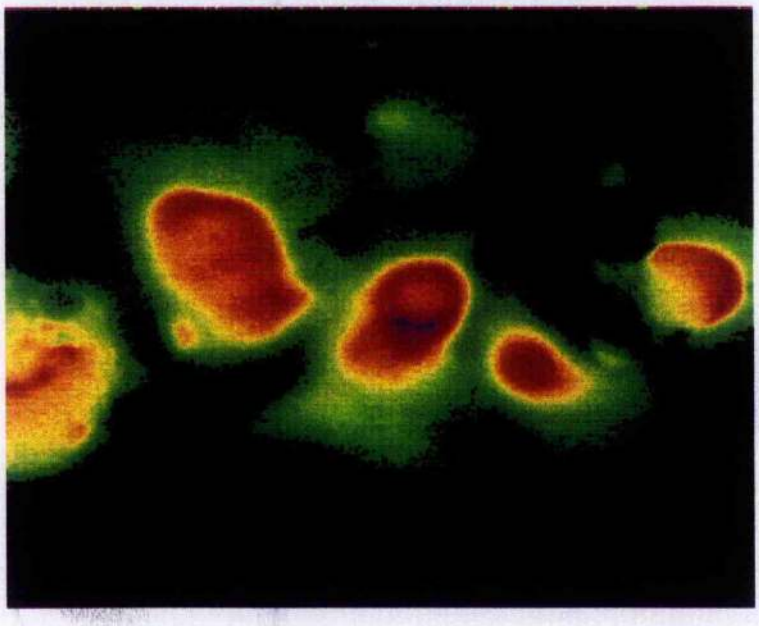

Figure7

An example of the pseudo-colour allocated to the fluorescence ratio.

These images allowed monitoring of the experiment visually, though the data files rather than images were used in final analysis.

Addition of drug under investigation

An initial period of approximately 2 min 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 (Appendix 3). By the use of a switch system we were able change easily between the solutions containing different drugs.

ATP

ATP at a concentration of 10^{-4}M was added to the superfusing solution after an initial 2 minute 'steady-state' period. The fluorescence ratio was observed and recorded for a further 2 minute period.

Bradykinin

Experiments were performed either in the presence of extracellular calcium at a concentration of 1.8 mM, or in a calcium-free environment containing 0.5mM EGTA. Bradykinin was added to the superfusing solution at concentrations of 10^{-10}M, 10^{-9}M, $3x10^{-9}$M, 10^{-8}M or $3x10^{-8}$M after an initial 2 minute 'steady-state' period and superfused for 2 min. A final 2 min period was observed in which only modified Krebs' solution was

superfused, without the presence of any drug. Throughout these experiments the fluorescence ratio was recorded continuously.

Effect of ATP on release of calcium by non-pigmented ciliary epithelial cells previously exposed to a sub-maximal concentration of bradykinin.

At sub-maximal concentrations of bradykinin (10^{-10}M), those producing only approximately 12.5% of the maximal bradykinin-induced release of intracellular calcium, ATP was added later during the experiment. An initial 2 minute 'steady-state' period, was observed. Bradykinin (10^{-10}M) was then added to the superfusing solution for a further 2 min. After this time, having observed the reduced bradykinin response, the bradykinin infusion was stopped and ATP (10^{-4}M) substituted, for a further 2 min. A final 2 minute period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout these experiments the fluorescence ratio was constantly recorded.

WIN 64338

WIN 64338 hydrochloride, a B_2 receptor antagonist (10^{-6}M, $3x10^{-7}$M, 10^{-7}M, 10^{-8}M or 10^{-9}M), was tested in the presence of bradykinin (10^{-8}M). An initial 2 minute 'steady-state' period, was observed. WIN 64338, at the various concentrations recorded above, was then added to the superfusing solution for a further 2 min. After this time, both WIN 64338 and bradykinin (10^{-8}M) were superfused, for a further 2 min. A final 2 min period

was observed in which only modified Krebs' solution was superfused, without the presence of any drug. Throughout these experiments the fluorescence ratio was recorded continuously.

Lys-[des-Arg^9]BK

The effect of Lys-[des-Arg^9]BK, a B_1 receptor agonist, was studied by superfusing at concentrations of $3x10^{-9}$M, 10^{-8}M or $3x10^{-8}$M after an initial 2 minute 'steady-state' period and superfused for 2 min. A final 2 min period was observed in which only modified Krebs' solution was superfused, without the presence of any drug. The fluorescence ratio was observed and recorded for a further 2 minute period.

ODQ

ODQ, an inhibitor of soluble guanylate cyclase (10^{-7}M or 10^{-6}M), was tested in the presence of bradykinin (10^{-8}M). An initial 2 minute 'steady-state' period, was observed. ODQ, at the concentrations above, was then added to the superfusing solution for a further 2 min. After this time, both ODQ (10^{-7}M or 10^{-6}M) and bradykinin (10^{-8}M) were superfused, for a further 2 min. A final 2 min 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.

L-NAME

L-NAME, an inhibitor of nitric oxide synthase (10^{-4}M), was tested in the presence of bradykinin (10^{-8}M). An initial 2 minute 'steady-state' period, was observed. L-NAME was then added to the superfusing solution for a further 2 min. After this time, both L-NAME (10^{-4}M) and bradykinin (10^{-8}M) were superfused, for a further 2 min. A final 2 min 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.

Ascorbate

For all these experiments ascorbate was added to the modified Krebs' solution at a concentration of 3mM, comparable to that found physiologically within the bovine eye. (For all other experiments on intracellular calcium, ascorbate was omitted from the modified Krebs' solution).

Bradykinin was added to the superfusing solution at a concentration of 10^{-8}M after an initial 2 min 'steady-state' period and superfused for 2 min. A final 2 min 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.

Substance P

Substance P was added to the superfusing solution at concentrations of $3x10^{-10}$M, 10^{-10}M, 10^{-9}M, $3x10^{-9}$M, 10^{-8}M, $3x10^{-8}$M and 10^{-7}M after an initial 2 minute 'steady-state' period and superfused for 2 min. A final 2 min 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.

RESULTS

THE EFFECT OF BRADYKININ ON INTRACELLULAR CALCIUM RELEASE FROM NON-PIGMENTED CILIARY EPITHELIAL CELLS

Normalisation of time course

Intracellular calcium concentration was recorded from individual cultured non-pigmented ciliary epithelial cells as the fluorescence ratio. Absolute values of $[Ca^{2+}]_i$ have been calculated for populations of these cells **(Shahidullah *et al.* 1997)** but calibration of a microspectrophotometric system for single cells is very difficult and so the experimental results are shown as the fluorescence ratios (**Suzuki *et al.*, 1997; Shahidullah *et al.*, 1997**). Starting values for the fluorescence ratio were usually in the range 2.2 to 3.2 and release of intracellular calcium by a drug was shown by a rise in the ratio which had an onset between 20 and 40 seconds after the start of drug superfusion (Figure 8).

In some cells baseline ratios showed a very slow but steady increase. There was some variation in the rate of onset of the bradykinin effect in these cells. Hence an arbitrary time interval of 120s prior to the peak of the response was set and from this was measured the starting baseline ratio, in order to prevent bias in the interpretation of the results. Of this 120s delay, approx. 20s was accountable due to the time taken between switching perfusate solutions and arrival of the drug in the chamber.

Calculation of change in fluorescence ratio

For each concentration of a drug, the difference between the peak and steady state ratios was calculated for 8 to 18 experiments and the mean, the standard deviation and the standard error of the mean were calculated. A paired Student t-test was also performed to compare the peak and steady state.

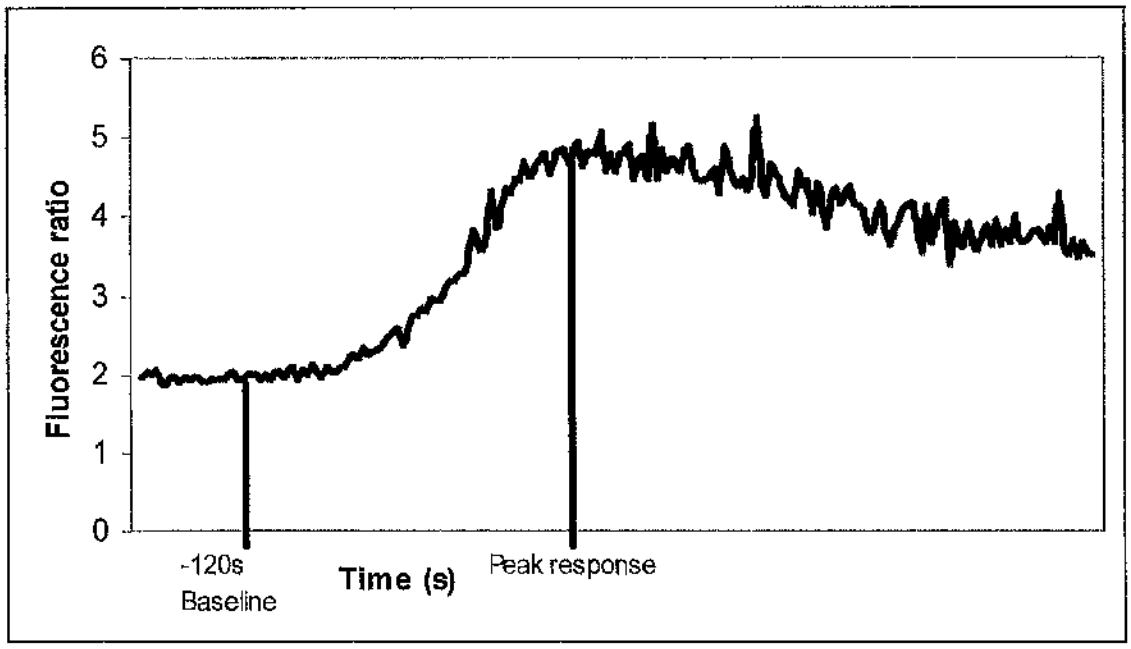

Figure 8

Effect of bradykinin (10^{-8}M) on the intracellular 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 was taken to be an arbitrary time interval of 120 seconds prior to the peak.

The effect of ATP on calcium release in non-pigmented ciliary epithelial cells

Addition of ATP (10^{-4}M) to the solution superfusing the ciliary epithelial cells triggered an almost immediate (10-20s) increase in $[Ca^{2+}]_i$ as indicated by changes in fluorescence ratio (Figure 9). Rapid release of Ca^{2+} from intracellular stores caused a peak within 10s of initial onset (30s from time 0), which at first declined quickly ($t_{1/2}$ <30s), then more slowly.

The effect of bradykinin on calcium release in non-pigmented ciliary epithelial cells

Following the administration of bradykinin to the reservoir of superfusing solution, a time period of approximately 120 seconds passed before the peak response occurred (Figure 10). During this period a gradual increase in the fluorescence ratio was observed. Following the peak, there was an even slower decline toward baseline ($t_{1/2}$ approx.60s).

Bradykinin (10^{-8}M), produced a similar change in fluorescence ratio from baseline to peak in non-pigmented ciliary epithelial cells to that observed with ATP (10^{-4}M). The response to bradykinin (10^{-10}M) occurs at concentrations significantly lower those of ATP (10^{-4}M) required to produce an effect.

By combining data from a number of experiments (n=9 to 24), there appeared to be a concentration-dependent effect of bradykinin upon the release of $[Ca^{2+}]_i$ from non-pigmented ciliary epithelial cells (Correlation coefficient log[bradykinin] (M): Pearson r

=0.9638, p value = 0.008) (Figure 11,12,13), in either the presence or absence of extracellular calcium. All fluorescence ratio changes between baseline and peak response were found to be statistically significant when compared by paired Student t-test ($p< 0.001$).This response was elicited by concentrations of bradykinin in the range 10^{-7}M to 10^{-10}M. The logEC50 was -8.3$\pm$ 0.2 (mean$\pm$ s.e).

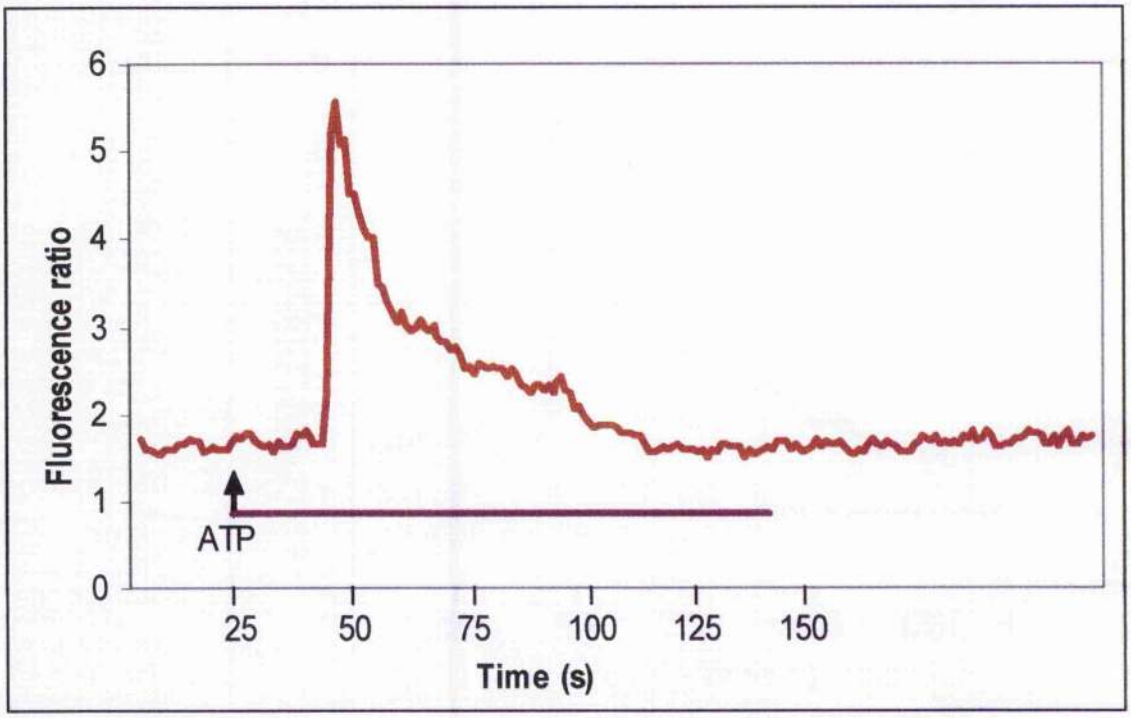

Figure 9

Effect of ATP (10^{-4}M) on the intracellular calcium in a single isolated non-pigmented ciliary epithelial cell

When ATP was added to the superfusing solution, individual non-pigmented ciliary epithelial cells responded with a prompt release of intracellular calcium followed by a rapid return of $[Ca]_i$ to baseline levels. The graph shows the result from an individual non-pigmented ciliary epithelial cell.

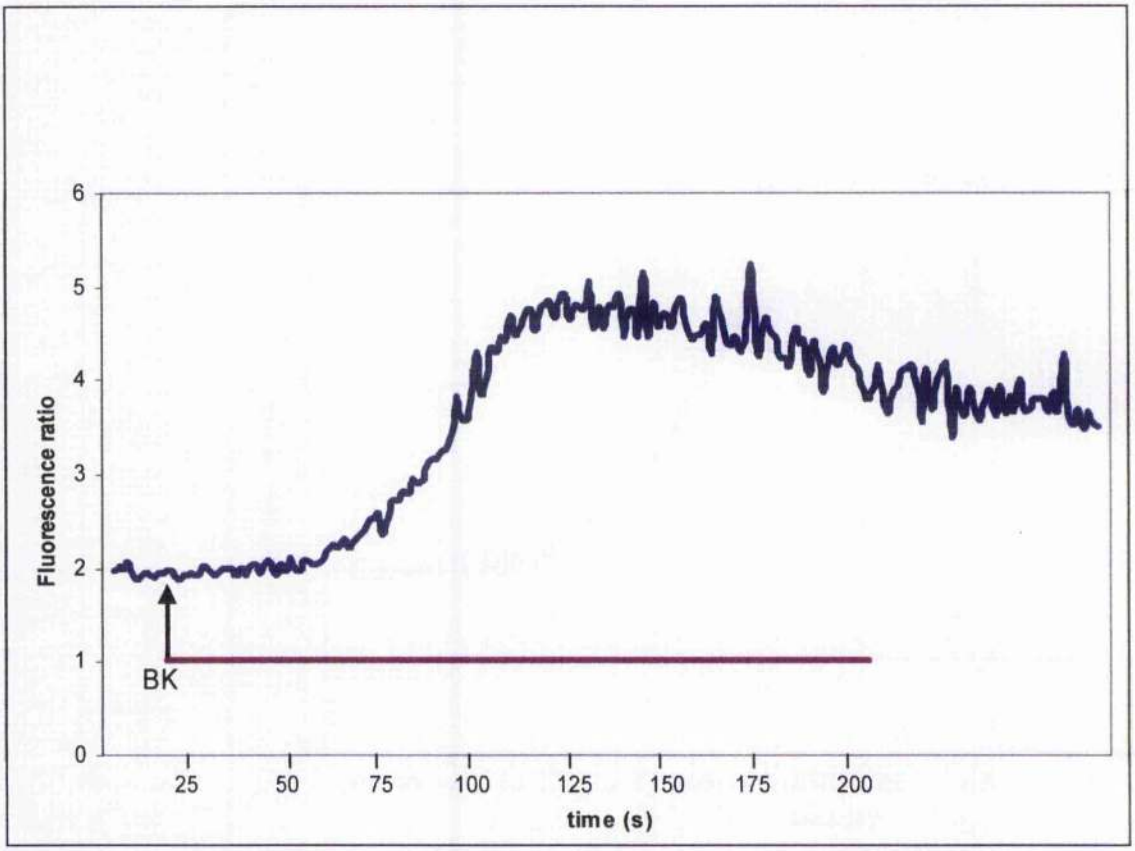

Figure 10

Effect of bradykinin (10^{-8}M) on the intracellular calcium in a single isolated non-pigmented ciliary epithelial cell

When bradykinin (10^{-8}M) was added to the superfusing solution, non-pigmented ciliary epithelial cells responded with a slow release of intracellular calcium followed by a gradual return of $[Ca]_i$ toward baseline levels. The graph shows the result from an individual non-pigmented ciliary epithelial cell (in presence of extracellular calcium).

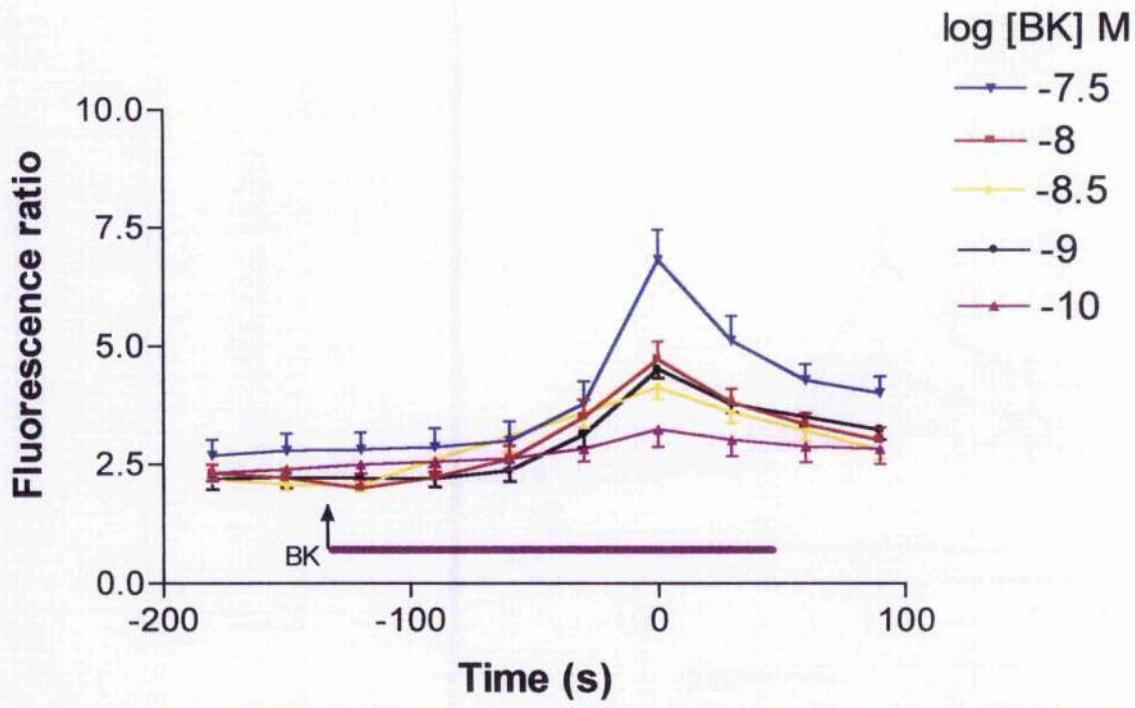

Figure 11

Release of calcium from cultured non-pigmented ciliary epithelial cells (in presence of extracellular calcium); in response to bradykinin
All bradykinin concentrations given as log[bradykinin] (M) values.
Bradykinin at the concentrations shown was added to the medium from –120s.

Each point represents the mean (± s.e. mean) of 9 to 24 experiments.

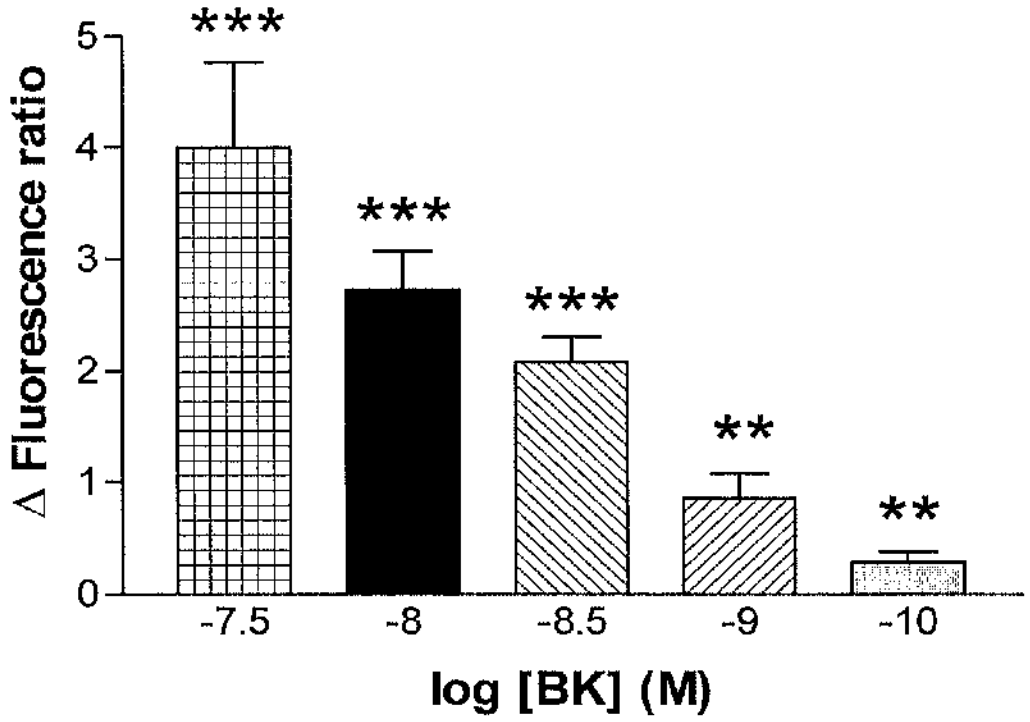

Figure 12

Release of calcium from cultured non-pigmented ciliary epithelial cells (in presence of extracellular calcium); in response to bradykinin
Each column represents the mean (± s.e. mean) of 9 to 24 experiments.

Significance of difference between baseline and peak for each concentration of bradykinin: *p<0.05;**p<0.01;***p<0.001, paired Student t-test.
Correlation coefficient log[bradykinin] (M): Pearson r =0.9638, p value = 0.008.

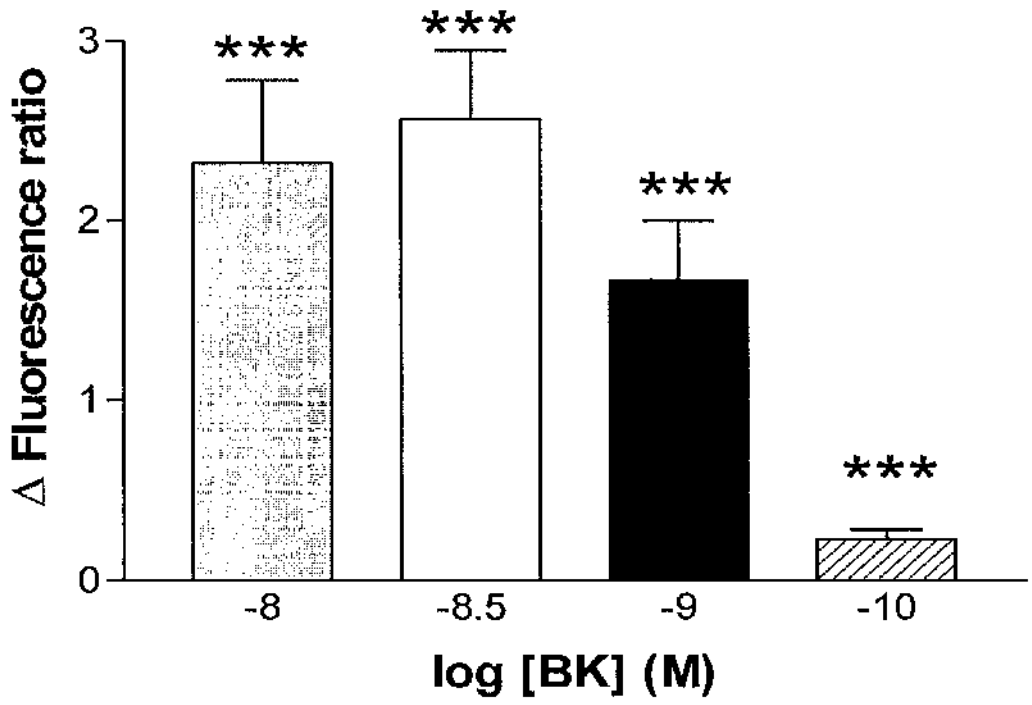

Figure 13

Release of calcium from cultured non-pigmented ciliary epithelial cells (in absence of extracellular calcium); in response to bradykinin
Each column represents the mean (± s.e. mean) of 11 to 14 experiments.

Significance of difference between baseline and peak for each concentration of bradykinin: ***$p<0.001$, Paired Student t-test.
Change in fluorescence ratio found to be statistically significant between 10^{-10}M and all other concentrations (unpaired t-test $p<0.001$). For all other concentrations significant difference was not found when changes in fluorescence ratio compared (unpaired t-test $p>0.1$).

Effect of extracellular calcium on bradykinin-induced response

The initial value of the fluorescence ratio tended to be lower in cells incubated in the absence of normal extracellular Ca^{2+} levels. This allowed the effect of bradykinin to be more clearly observed.

When bradykinin (10^{-10}M) was added to the superfusate, a persistent slow increase was seen in the concentration of intracellular calcium, which was statistically significant (mean$\pm$ s.e.mean: 0.23$\pm$ 0.05; $p<0.01$; paired Student t-test, comparing the baseline line value with that of the peak; n= 11), in the absence of extracellular calcium. In the presence of calcium, though the background starting fluorescence was greater, the effect of bradykinin was comparable (mean$\pm$ s.e.mean: 0.28$\pm$ 0.09; $p<0.01$; paired Student t-test, comparing the baseline line value with that of the peak; n= 16). At higher concentrations of bradykinin (10^{-8}M) (Figure 14), no significant difference in the responses was noted when comparing those in the presence of extracellular calcium with those in its absence (unpaired t-test; p=0.99).

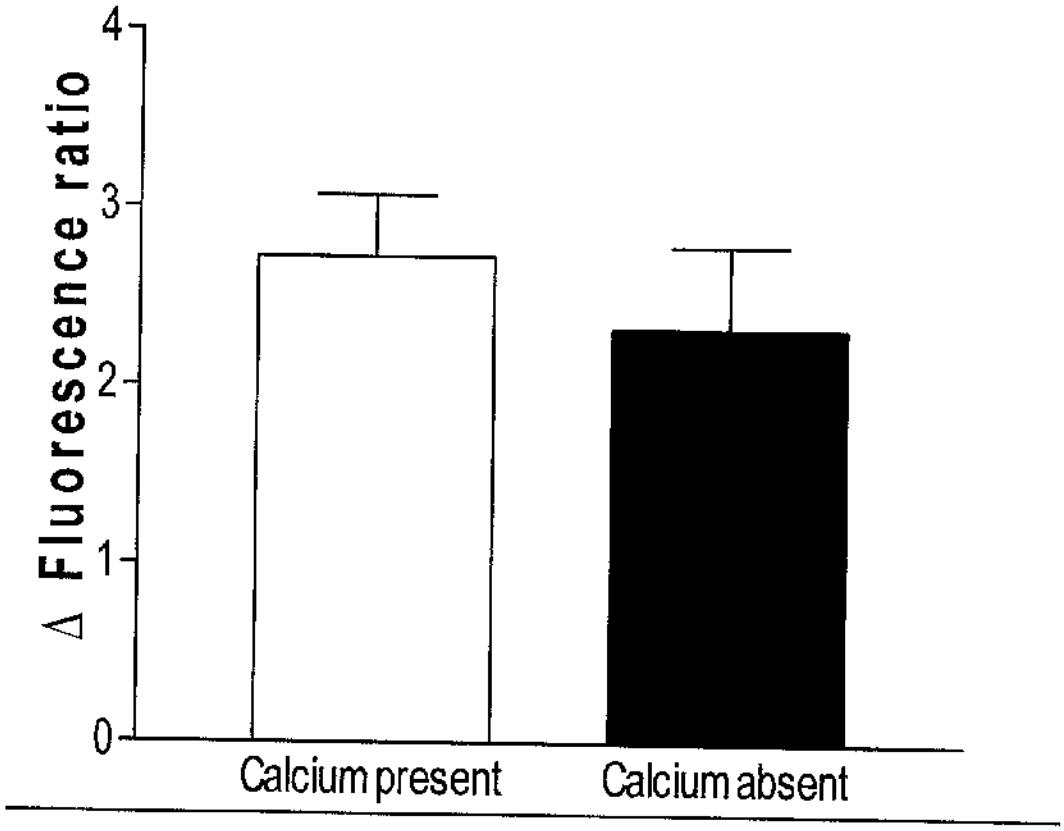

Figure 14

Effect of bradykinin (10^{-8}M) on intracellular calcium in bovine non-pigmented ciliary epithelial cells in the presence and absence of extracellular calcium in the superfusing solution
Each point represents the mean (± s.e. mean) of 12 to 24 experiments.

There was no significant difference the responses seen in the presence or absence of extracellular calcium (unpaired t-test p=0.99).

Effect of ATP on release of calcium from non-pigmented ciliary epithelial cells previously exposed to an effective concentration of bradykinin

At lower concentrations of bradykinin, where it was obvious that there was little or no direct effect of bradykinin, ATP (10^{-4}M) was added later to see if the ATP response was altered in the presence of bradykinin (Figure 15). These experiments showed that a low concentration of bradykinin (10^{-10}M), small enough to produce little effect on the fluorescence ratio, appeared to have an effect on the ATP-induced response. Following addition of ATP to the superfusing solution, the cells responded with the predicted rapid increase in fluorescence ratio. This response however was delayed in onset in the presence of bradykinin when compared to the response elicited by ATP in the absence of bradykinin respectively (59.5 $\pm$ 6.0s compared to 38.6 $\pm$ 6.3s, mean $\pm$ s.e.; unpaired t-test, p=0.03). The change in fluorescence ratio between baseline and peak was also reduced in the presence of bradykinin when compared to effect of ATP alone respectively (2.30$\pm$ 0.28 compared to 3.11$\pm$ 0.24, mean $\pm$ s.e.; unpaired t-test, p=0.03).

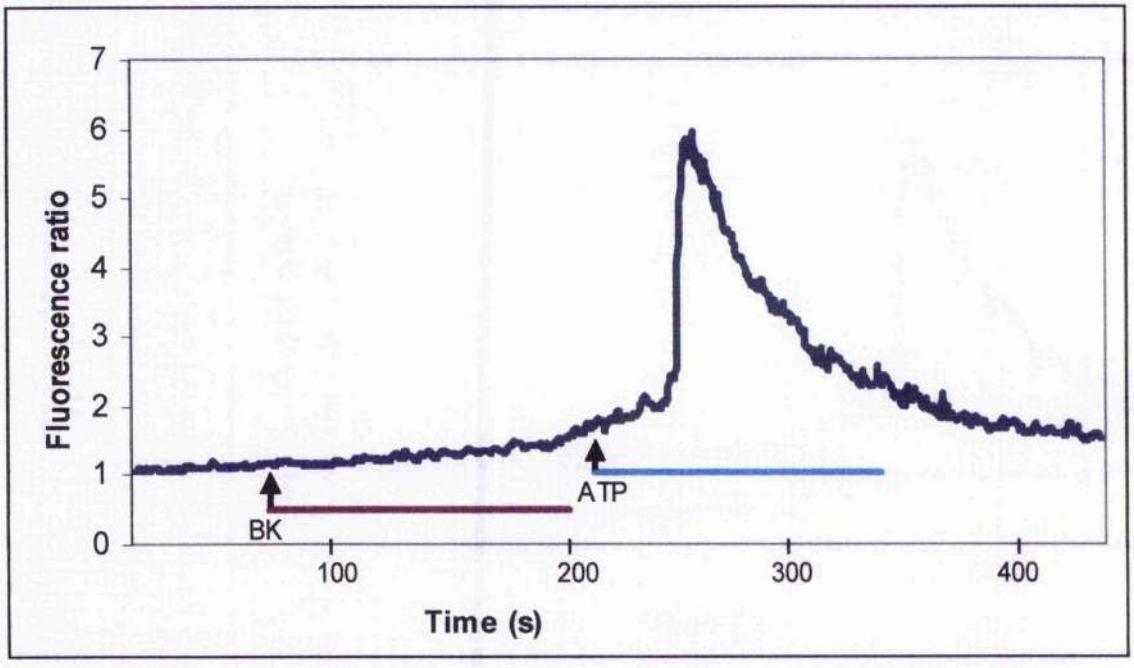

Figure 15

Effect of addition of ATP in the presence of bradykinin
Bradykinin (10^{-10}M) had little effect on the mobilisation of intracellular calcium in non-pigmented ciliary epithelial cells. When ATP (10^{-4}M) was then added to the superfusing solution, non-pigmented ciliary epithelial cells responded with a prompt release of intracellular calcium followed by a rapid return of $[Ca]_i$ to baseline levels. The graph shows the result from an individual non-pigmented ciliary epithelial cell.

Effect of WIN 64338 on bradykinin-induced release of calcium from non-pigmented ciliary epithelial cells

In all experiments comparing the effect of bradykinin alone and in the presence of WIN 64338 hydrochloride, the concentration of bradykinin remained constant (10^{-8}M) (Figure 16).

WIN 64338 hydrochloride, a B_2 receptor antagonist, was tested at concentrations 10^{-6}M, $3x10^{-7}$M, 10^{-7}M, 10^{-8}M and 10^{-9}M. Only at 10^{-6}M concentration was WIN 64338 found to inhibit the bradykinin-induced response when the fluorescence ratio changes were compared with those due to bradykinin alone (unpaired t-test $p<0.001$). At all other concentrations, $3x10^{-7}$M, 10^{-7}M, 10^{-8}M and 10^{-9}M, no statistically significant inhibition of bradykinin was noted (unpaired t-test $p>0.3$).

Effect of Lys-[des-Arg^9]BK on calcium release from non-pigmented ciliary epithelial cells

The effect of Lys-[des-Arg^9]BK, a B_1 receptor agonist, on non-pigmented ciliary epithelial cells was assessed using the concentrations $3x10^{-8}$M, 10^{-8}M, $3x10^{-9}$M (Figure 17). Lys-[des-Arg^9]BK was found to have no effect on calcium release from non-pigmented ciliary epithelial cells (paired t-test $p>0.1$).

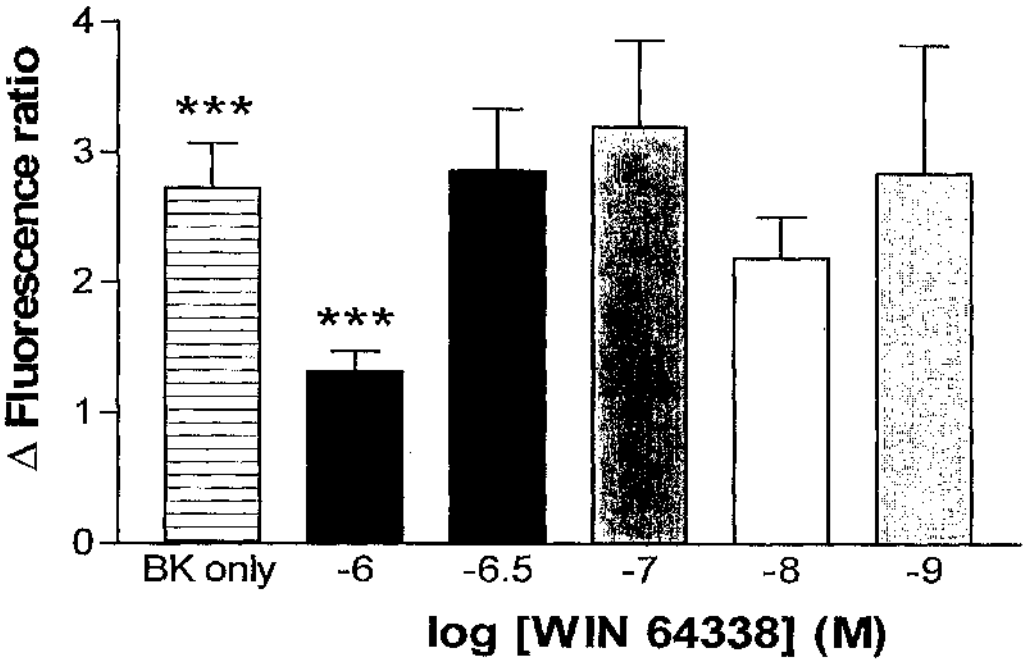

Figure 16

Effect of B_2 receptor antagonist (WIN 64338) on the bradykinin-induced mobilisation of intracellular calcium in bovine non-pigmented ciliary epithelial cells
Each point represents the mean (± s.e. mean) of 6 to 24 experiments.
Bradykinin = 10^{-8}M in all experiments.
All WIN 64338 concentrations given as log[WIN 64338] (M) values.

Only at 10^{-6}M was WIN 64338 hydrochloride found to inhibit the bradykinin-induced response when the fluorescence ratio changes were compared with those due to bradykinin alone (unpaired t-test ***$p<0.001$). At all other concentrations, $3x10^{-7}$M, 10^{-7}M, 10^{-8}M and 10^{-9}M, no statistically significant inhibition of bradykinin was noted (unpaired t-test $p>0.3$).

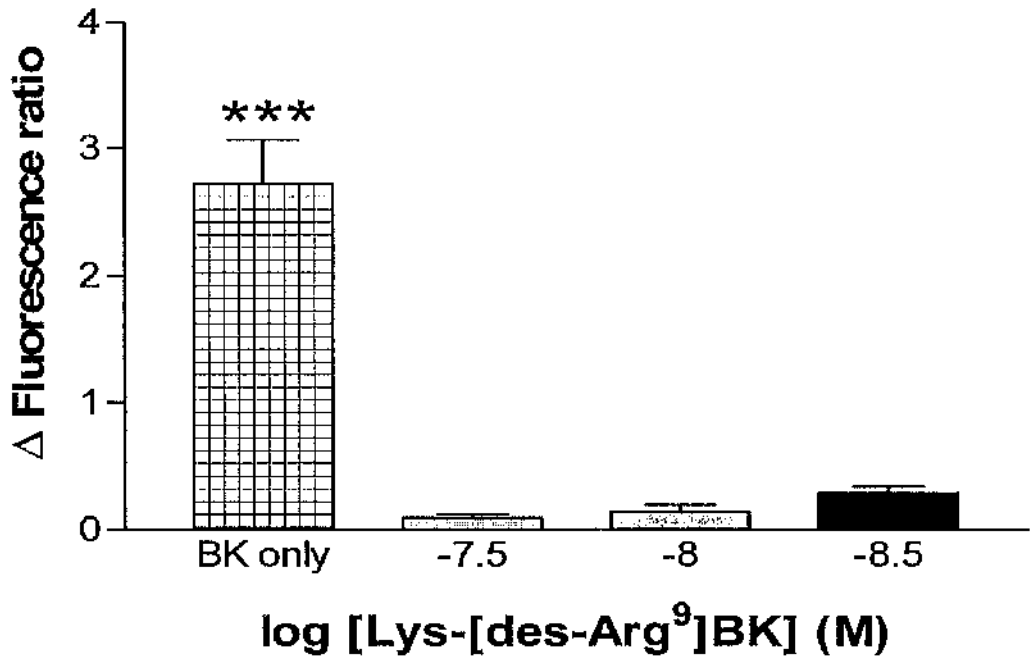

Figure 17

Lack of effect of the B_1 receptor agonist (Lys-[des-Arg^9]BK) on the mobilisation of intracellular calcium in bovine non-pigmented ciliary epithelial cells
Each point represents the mean (± s.e. mean) of 4 to 24 experiments.
First column shows bradykinin (10^{-8}M) for comparison.
Significance of difference between baseline and peak: ***$p<0.001$, Paired Student t-test.

Effect of ODQ and L-NAME on bradykinin-induced release of calcium from non-pigmented ciliary epithelial cells

In all experiments comparing the effect of bradykinin alone and in the presence of either ODQ or L-NAME, the concentration of bradykinin remained constant (10^{-8}M) (Figure 18).

ODQ, a soluble guanylate cyclase inhibitor, at concentrations 10^{-7}M and 10^{-6}M, had no intrinsic effect on fluorescence ratio changes when compared with those of bradykinin alone (unpaired t-test $p>0.7$). L-NAME (10^{-4}M), a nitric oxide synthase inhibitor, had no intrinsic effect on fluorescence ratio changes when compared with those of bradykinin alone (unpaired t-test $p>0.5$).

Effect of ascorbate on bradykinin-induced release of calcium from non-pigmented ciliary epithelial cells

Bradykinin induced calcium release from non-pigmented ciliary epithelial cells was compared in both the presence and absence of ascorbate from the superfusing solution (Figure 19). In both circumstances the response was found to be statistically significant (paired Student t-test $p<0.001$). Ascorbate was found to have no significant effect on the bradykinin-induced release of calcium (unpaired t-test $p>0.7$). When present ascorbate was at a concentration of 3mM.

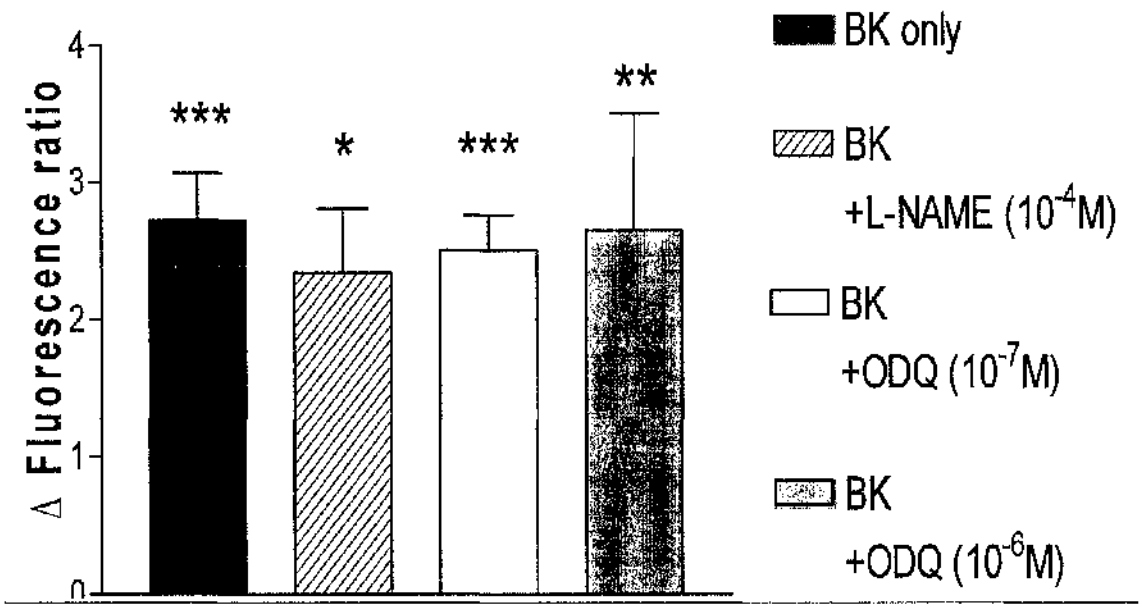

Figure 18

The effect of bradykinin on the mobilisation of intracellular calcium in bovine non-pigmented ciliary epithelial cells in presence of either ODQ or L-NAME
Each point represents the mean (± s.e. mean) of 7 to 24 experiments.
Bradykinin = 10^{-8}M in all experiments.
All concentrations given as log[drug] (M) values.

Significance of difference between baseline and peak: *p<0.05; **p<0.01; ***p<0.001, Paired Student t-test.

ODQ, a soluble guanylate cyclase inhibitor, at concentrations 10^{-7}M and 10^{-6}M had no intrinsic effect on fluorescence ratio changes when compared with those of bradykinin alone (unpaired t-test p>0.7).
L-NAME (10^{-4}M), a nitric oxide synthase inhibitor, had no intrinsic effect on fluorescence ratio changes when compared with those of bradykinin alone (unpaired t-test p>0.5).

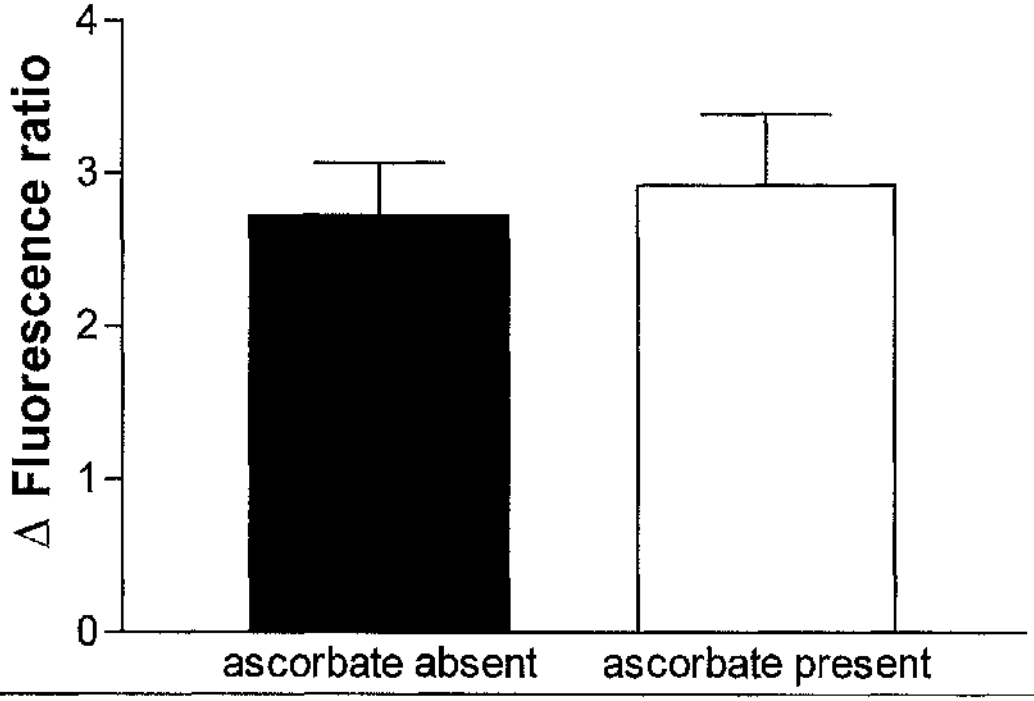

Figure 19

Effect of bradykinin on the mobilisation of intracellular calcium in bovine non-pigmented ciliary epithelial cells in both the presence and absence of ascorbate
Each point represents the mean (± s.e. mean) of 8 to 24 experiments.
Bradykinin = 10^{-8}M in all experiments.
When the fluorescence ratio changes were compared, ascorbate was found to have no significant effect on the bradykinin-inducing release of calcium (unpaired t-test $p>0.7$).
When present ascorbate was in concentration of 3mM.

Effect of substance P on calcium release from non-pigmented ciliary epithelial cells

Addition of substance P (10^{-7}M) to the solution superfusing the non-pigmented ciliary epithelial cells triggered a rapid increase in $[Ca^{2+}]_i$ commencing at approximately 20s, as indicated by changes in the fluorescence ratio (Figure 20). The response peaked within a further 30s, declined quickly ($t_{1/2}$ approx. 40s), then more slowly back to base line.

By comparing data for experiments in which different concentrations of substance P were tested (10^{-7} to 10^{-10}M), there appeared to be a threshold-dependent effect of substance P upon the release of $[Ca^{2+}]_i$ from non-pigmented ciliary epithelial cells (Figure 21). All fluorescence ratio changes between baseline and peak response were found to be statistically significant when compared by paired Student t-test ($p< 0.001$). It appears that there is no difference among the responses to concentrations of substance P >10^{-9}M. The concentration-response curve is very steep (Figure 21). The logEC50 was -9.3$\pm$ 0.5 (mean$\pm$ s.e).

The response to substance P had a similar time course to that produced by ATP and when the delay in onset was compared between that of substance P (10^{-7}M) and ATP (10^{-4}M), no statistically significant difference was found (mean $\pm$ s.e.: 42.4 $\pm$ 3.7s compared to 38.6 $\pm$ 6.3s, respectively; unpaired t-test, p=0.61). The change in fluorescence ratio between baseline and peak produced by substance P (10^{-7}M) and ATP (10^{-4}M) were also comparable (mean $\pm$ s.e.: 3.90 $\pm$ 0.35 compared to 3.11 $\pm$ 0.24, respectively; unpaired t-test, p=0.1). (Figure 22).

When the response elicited by substance P (10^{-7}M) was compared to that of bradykinin (10^{-8}M) a significant difference was found in delay of onset (mean $\pm$ s.e.: 42.4 $\pm$ 3.7s compared to 134.4 $\pm$ 9.4s, respectively; unpaired t-test, $p<0.001$). (Figure 22).

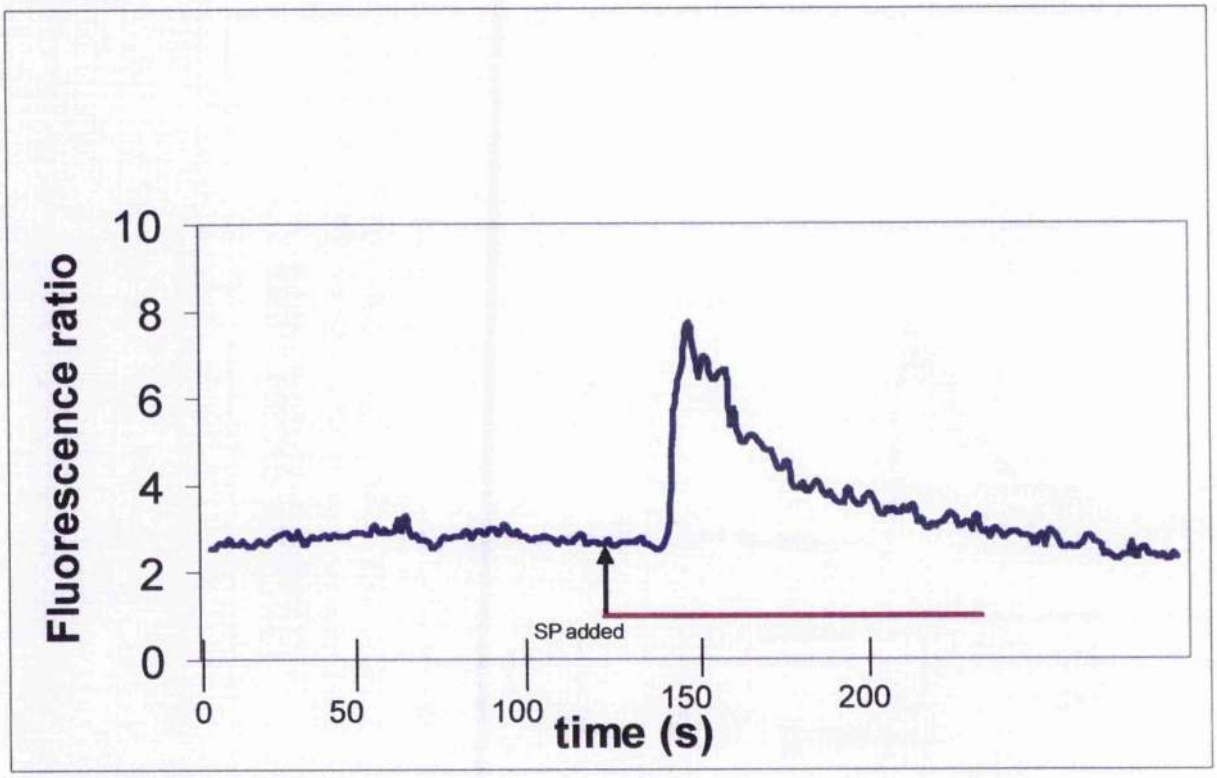

Figure 20

Effect of substance P on calcium release from an individual non-pigmented ciliary epithelial cell

When Substance P (10^{-7}M) was added to the superfusing solution, non-pigmented ciliary epithelial cells responded with a prompt release of intracellular calcium followed by a rapid return of $[Ca]_i$ to baseline levels. The graph shows the result for an individual non-pigmented ciliary epithelial cell.

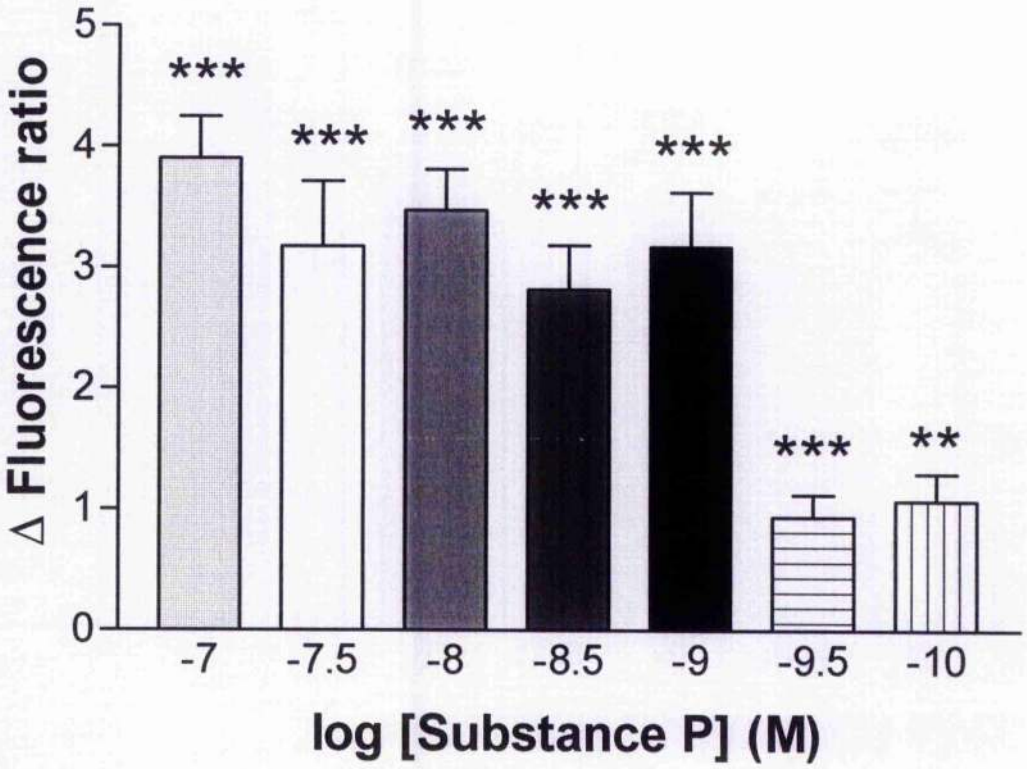

Figure 21

The effect of substance P on the mobilisation of intracellular calcium in bovine non-pigmented ciliary epithelial cells

Each point represents the mean (± s.e. mean) of 7 to 20 experiments.

Significance of difference between baseline and peak: *p<0.05;**p<0.01; ***p<0.001, Paired Student t-test.

When fluorescence ratio changes were compared between concentrations there was only found to be statistical significance between 10^{-10}M, $3x10^{-10}$M and all other concentrations (unpaired t-test p<0.001).

For all other concentrations statistical difference was not found (unpaired t-test p>0.1).

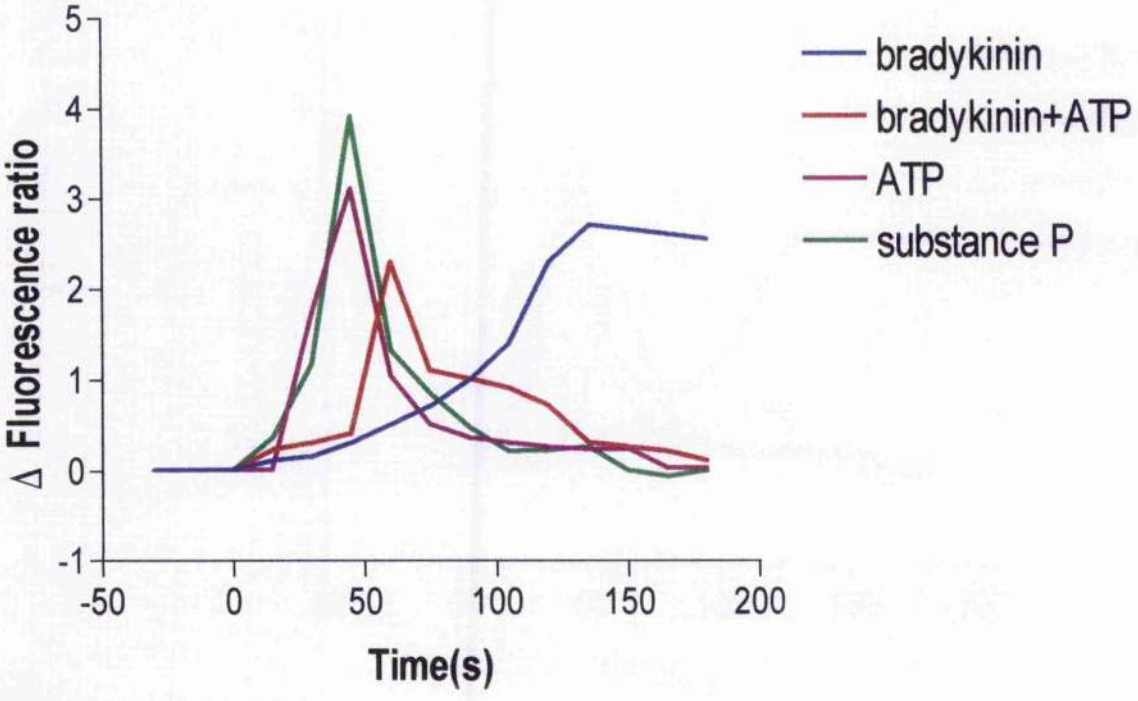

Figure 22

Release of calcium from cultured non-pigmented ciliary epithelial cells (in presence of extracellular calcium); in response to bradykinin, substance P, ATP and ATP following pre-treatment with an effective concentration of bradykinin

Concentrations as follows: Bradykinin alone 10^{-8}M; Bradykinin in the ATP experiments given sub-maximally 10^{-10}M; ATP 10^{-4}M in all experiments; Substance P 10^{-7}M.

Time 0 (zero) time when treatment added and continued for following 2 minutes.

Change in fluorescence ratio between baseline and peak for bradykinin, ATP, ATP in presence of sub-maximal bradykinin, substance P, respectively (mean $\pm$ s.e.: 2.72$\pm$ 0.34; 3.11 $\pm$ 0.24; 2.30$\pm$ 0.28; 3.90 $\pm$ 0.35).
Latency period for bradykinin, ATP, ATP in presence of sub-maximal bradykinin, substance P, respectively (mean $\pm$ s.e.: 134.4 $\pm$ 9.4s; 38.6 $\pm$ 6.3s; 59.5 $\pm$ 6.0s; 42.4 $\pm$ 3.7s).

THE EFFECTS OF BRADYKININ ON AQUEOUS HUMOUR FORMATION IN THE ISOLATED, ARTERIALLY PERFUSED BOVINE EYE

Steady rate aqueous humour formation

The aqueous formation rate was observed for a period of 30 min in order to confirm that there existed steady state fluorescein dilution prior to the addition of the drug. The time taken for newly added drug to traverse the warming coil and reach the eye was approximately 10 min. Hence estimation of aqueous humour formation began 10 min after addition of drug to the perfusate. This continued for a further 80 min with the total duration of the experiment being 120 min. Time zero was the time at which the drug under investigation was added to the reservoir of modified Krebs solution perfusing the long posterior ciliary artery.

Same eye controls

This method allowed each eye to act as its own control, by comparing aqueous humour formation rate over the initial period from 30 min prior to addition of drug until 10 min after, with the two following time intervals (time +10 to +50 min and +50 to +90 min). Therefore the considerable variation in pre-drug (-30 to +10 min) control values amongst different eyes did not militate against detecting significant effects of the drugs used.

Independent eye controls

Independent control experiments ("separate eye" controls) were also undertaken (Table 3). For these experiments no reagents were added to the perfusate for the entire duration of the experiment (120 min).

Effect of bradykinin on aqueous humour formation rate

Analysis of these data showed suppression of aqueous humour formation due to bradykinin over the range 10^{-8}M to 10^{-10}M (Table 3). At higher concentrations (3x 10^{-8}M and 10^{-7}M) aqueous humour formation appeared to increase, though this was not significant. From analysis of the results (Table 3), it appeared that the effect was delayed for up to 40 min after the addition of the drug and for this reason statistical analysis was performed between the 0-40 min period and 90-120 min period. In the results from "separate eye" controls, there were no significant differences among the aqueous humour formation rates for the different time intervals, namely between –30 to +10min and +10 to +50min, nor between –30 to +10min and +50 to +100min (Table 3).

Treatment [BK] (M)	n	Aqueous humour formation rate: (K_{out} min^{-1} x10^{4}) -30 to +10 min	-10 to +50 min	+50 to +90 min	Aqueous humour formation rate: (% decrease of mean) % change between *-30 to +10 min* and *+50 to +90 min* time intervals
(10^{-10}M)	11	33 ± 1.7	27 ± 2.2 **	22 ± 2.0	33***
($3x10^{-10}$M)	14	28 ± 4.5	23 ± 3.3 ns	20 ± 3.4	29 *
(10^{-9}M)	13	36 ± 3.5	31 ± 3.2 ns	21 ± 2.8	42***
($3x10^{-9}$M)	8	37 ± 1.9	28 ± 2.5 **	19 ± 1.6	49***
(10^{-8}M)	8	44 ± 9.4	31 ± 1.5 ns	24 ± 2.4	45**
($3x10^{-8}$M)	6	34 ± 5.4	31 ± 1.5 ns	37 ± 3.6	8 ns
(10^{-7}M)	10	40 ± 2.1	44 ± 5.1 ns	46 ± 10.8	15 ns
Control (0)	8	33 ±1.1	32 ± 1.6 ns	33 ± 2.9	0 ns

Table 3

Effect of bradykinin on aqueous humour formation rate in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min after drug injection.
Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean ± s.e. mean, of the rate of aqueous humour formation, expressed as $K_{out}.min^{-1}$ x10^{-4}M of the number (n) of experiments shown. Control ('separate eye' control) represents values for eyes perfused with Krebs' solution without addition of any drug. Significance of differences from same eye control (time –30 to +10 min) at two separate time intervals (time +10 to +50 min) and (time +50 to +90 min). Significance of difference from initial same eye 'control' period as indicated by paired Student t-test **p<0.01; ***p<0.001; ns =not significant.

Effect of bradykinin on aqueous humour formation in the presence of B_2 receptor antagonist

WIN 64338 hydrochloride, a non-peptide, competitive bradykinin B_2 receptor antagonist ($3x10^{-8}$M), inhibited the aqueous humour formation-reducing effects of bradykinin (10^{-8}M) (Table 4). An unpaired t-test comparing the change in aqueous humour formation rate over the course of experiment for bradykinin alone, with that of bradykinin in the presence of WIN 64338 showed statistically significant inhibition; $p< 0.01$. WIN 64338 was added to the perfusate at the same time as bradykinin.

Effect of bradykinin on aqueous humour formation in the presence of inhibitor of soluble guanylate cyclase

ODQ, an inhibitor of soluble guanylate cyclase, appeared to reduce the effect of bradykinin on aqueous humour formation (Table 5). A number of concentrations of both ODQ and bradykinin were investigated (10^{-7}M ODQ + 10^{-8}M bradykinin; $3x10^{-7}$M ODQ + 10^{-8}M bradykinin ; $3x10^{-7}$M ODQ + 10^{-9}M bradykinin). Significant inhibition of the bradykinin effect only occurred at a concentration of $3x10^{-7}$M ODQ in the presence of 10^{-9}M bradykinin (unpaired t-test, comparing with data for bradykinin (10^{-9}M) alone; $p<0.004$). ODQ on its own, at a concentration of $3x\ 10^{-7}$M, also caused a reduction in aqueous humour formation (paired Student t-test; $p<0.001$). When compared to the 'control eyes' this reduction was found to be statistically significant (unpaired t-test; $p<0.001$).

		Aqueous humour formation rate: (K_{out} min^{-1} $x10^{4}$)			Aqueous humour formation rate: (% decrease of mean)
Treatment	n	-30 to +10 min	+10 to +50 min	+50 to +90 min	% reduction between *-30 to +10 min* and *+50 to +90 min* time intervals
Bradykinin (10^{-8}M)	8	44 ± 9.4	31 ± 1.5	24 ± 2.4	45**
Bradykinin (10^{-8}M) + WIN64338 ($3x10^{-8}$M)	10	35 ± 3.0	39 ± 5.1	35 ± 4.0	0 ns
Control	8	33 ±1.1	32 ± 1.6	33 ± 2.9	0 ns

Table 4

Effect of bradykinin in presence of WIN 64338 hydrochloride, a B_2 receptor antagonist, on aqueous humour formation rate in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min afterwards.
Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean ± s.e. mean of the rate of aqueous humour formation, expressed as $K_{out}.min^{-1}$ $x10^{-4}$M of the number (n) of experiments shown. Control ('separate eye' control) represents values for eyes perfused with Krebs' solution without addition of any drug. The rates of change in aqueous humour formation from same eye control (time –30 to +10 min) and (time +50 to +90 min) were compared for bradykinin (10^{-8}M) alone and in the presence of WIN64338 ($3x10^{-8}$M). Unpaired t-test *$p<0.01$; ns =not significant.

		Aqueous humour formation rate: (K_{out} min^{-1} x10^{4})			Aqueous humour formation rate: (% decrease of mean)
Treatment	n	-30 to +10 min	+10 to +50 min	+50 to +90 min	% reduction between *-30 to +10 min* and *+50 to +90 min* time intervals
Bradykinin (10^{-8}M)	8	44 ± 9.4	31 ± 1.5	24 ± 2.4	45**
Bradykinin (10^{-9}M)	13	36 ± 3.5	31 ± 3.2	21 ±2.8	42***
Bradykinin (10^{-8}M) +ODQ(10^{-7}M)	5	24 ± 3.9	17 ± 2.8	18 ± 3.1	25 ns
Bradykinin (10^{-8}M) +ODQ ($3x10^{-7}$M)	9	27 ± 3.4	16 ± 2.3	19 ± 2.1	30*
Bradykinin (10^{-9}M) +ODQ ($3x10^{-7}$M)	13	28 ± 3.4	28 ± 3.5	26 ± 2.8	4*
ODQ ($3x10^{-7}$M) only	7	40 ± 6.2	33 ± 6.1	19 ± 4.2	53**
Control	8	33 ±1.1	32 ± 1.6	33 ± 2.9	0 ns

Table 5

Effect of bradykinin in presence of ODQ, an inhibitor of soluble guanylate cyclase, on aqueous humour formation rate in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min after drug injection.
Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean ± s.e. mean of the rate of aqueous humour formation, expressed as $K_{out}.min^{-1}$ x10^{-4}M of the number (n) of experiments shown. Control ('separate eye' control) represents values for eyes perfused with Krebs' solution without addition of any drug. The rates of change in aqueous humour formation from same eye control (time –30 to +10 min) and (time +50 to +90 min) were compared for bradykinin (10^{-9}M;10^{-8}M) alone, ODQ ($3x10^{-7}$M) alone and combination of the two, as specified. Unpaired t-test *p<0.01; **p<0.001; ns =not significant.

Effect of bradykinin on aqueous humour formation in the presence of L-NAME

L-NAME, a nitric oxide synthase inhibitor, reduced the usual effect of bradykinin on aqueous humour formation (L-NAME (10^{-4}M) + bradykinin (10^{-9}M)) (Table 6). Thus the bradykinin-induced reduction in aqueous humour formation was inhibited in the presence of L-NAME. This effect was found to be statistically significant when the effect of bradykinin on aqueous humour formation in the presence and absence of L-NAME were compared by unpaired t-test ($p<0.01$). L-NAME in isolation had no effect on aqueous humour formation (paired Student t-test; $p=0.29$). Likewise when compared with the results of the 'control eyes' (unpaired t-test; $p=0.82$).

		Aqueous humour formation rate: (K_{our} min^{-1} $x10^4$)			Aqueous humour formation rate: (% decrease of mean)
Treatment	n	-30 to +10 min	+10 to +50 min	+50 to +90 min	% reduction between *-30 to +10 min* and *+50 to +90 min* time intervals
Bradykinin (10^{-9}M)	13	36 ± 3.5	31 ± 3.2	21 ± 2.8	42***
Bradykinin (10^{-9}M) + L-NAME (10^{-4}M)	11	14 ± 1.8	12 ± 1.6	16 ± 3.0	14*
L-NAME only (10^{-4}M)	10	21 ± 3.0	18 ± 2.4	16 ± 1.8	24ns
Control	8	33 ± 1.1	32 ± 1.6	33 ± 2.9	0 ns

Table 6

Effect of bradykinin in presence of L-NAME, a nitric oxide synthase inhibitor, on aqueous humour formation rate in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min after drug injection.
Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean of the rate of aqueous humour formation as expressed as $K_{out}.min^{-1}$ $x10^{-4}M$ ± s.e. mean of the number (n) of experiments shown. Control ('separate eye' control) represents values for eyes perfused with Krebs' solution without addition of any drug or reagent. The rates of change in aqueous humour formation from same eye control (time –30 to +10 min) and (time +50 to +90 min) were compared for bradykinin (10^{-9}M) alone, L-NAME (10^{-4}M) alone and combination of the two. Unpaired t-test *p<0.01; **p<0.001; ns =not significant.

Effect of bradykinin on aqueous humour formation in the presence/absence of ascorbic acid

The ability of bradykinin to reduce aqueous humour formation was significantly reduced in the absence of ascorbic acid from either the perfusate alone, or from both the perfusate and the aqueous humour substitute (Table 7). At a higher concentration of bradykinin (10^{-7}M) the effect on aqueous humour formation appears to be a small increase rather than a reduction, although this was not statistically significant (unpaired t-test; $p>0.1$). The effect of bradykinin at the lower concentration (10^{-9}M), was significantly inhibited in the absence of ascorbate from the perfusion solution (unpaired t-test; $p<0.01$). In the absence of ascorbate from both the perfusate and the aqueous humour substitute the effect of bradykinin at this lower concentration was also significantly reduced (unpaired t-test; $p=0.004$). No independent controls were performed in the absence of ascorbate. Therefore the only controls used were those of 'same eye controls' over the initial period from 30 min prior to addition of drug until 10 min after.

Effect of substance P on aqueous humour formation

At a concentration of 10^{-8}M, substance P reduced aqueous humour formation (Table 8) when compared to the same eye control (paired Student t-test; $p<0.001$) and when compared to the 'control eyes' (unpaired t-test; $p<0.01$). At all other concentrations its effect was not statistically significant.

		Aqueous humour formation rate: (K_{out} min^{-1} x10^{4})			Aqueous humour formation rate: (% decrease of mean)
Treatment [BK] (M)	n	-30 to +10 min	+10 to +50 min	+50 to +90 min	% reduction between *-30 to +10 min* and *+50 to +90 min* time intervals
(10^{-7}M)	10	40 + 2.1	44 + 5.1	46 + 10.8	15 ns
(10^{-9}M)	13	36 + 3.5	31 + 3.2	21 +2.8	42***
(10^{-7}M) Ascorbate only in AH substitute	10	22 + 3.03	22+ 2.92	18 + 2.47	18 ns
(10^{-6}M) Ascorbate only in AH substitute	10	24 + 3.8	22 + 2.7	22 + 3.6	8 ns
(10^{-9}M) No ascorbate	9	20 + 3.0	20 – 2.7	19 + 2.2	5 ns

Table 7

Effect of bradykinin on aqueous humour formation rate in the absence of ascorbic acid from either the perfusate alone, or from both the perfusate and the aqueous humour substitute in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min after drug injection.
Each eye also acted as its own control, comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean ± s.e. mean of the rate of aqueous humour formation, expressed as $K_{out}.min^{-1}$ x10^{-4}M of the number (n) of experiments shown. The rates of change in aqueous humour formation from same eye control (time –30 to +10 min) and (time +50 to +90 min) were compared for bradykinin (10^{-9}M; 10^{-7}M) in the presence and absence of ascorbate from both the aqueous humour substitute and the modified Krebs' solution, as specified. Unpaired t-test *p<0.01; **p<0.001; ns =not significant.

		Aqueous humour formation rate: (K_{out} min^{-1} $x10^{4}$)			Aqueous humour formation rate: (% decrease of mean)
Treatment [Substance P] (M)	n	-30 to +10 min	+10 to +50 min	+50 to +90 min	% reduction between *-30 to +10 min* and *+50 to +90 min* time intervals
(10^{-7}M)	11	21 ± 3.5	21 ± 3.5 ns	16 ± 2.7	24 ns
($3x10^{-8}$M)	8	19 ± 2.8	16 ± 2.1 ns	19 ± 3.1	0 ns
(10^{-8}M)	10	24 ± 4.7	20 ± 5.5 ns	15 ± 3.5	38**
Control (0)	8	33 ±1.1	32 ± 1.6 ns	33 ± 2.9	0 ns

Table 8

Effect of substance P on aqueous humour formation rate in the isolated arterially perfused bovine eye model

Aqueous humour formation rate was measured during 30 min prior to drug infusion, then for 90 min after drug injection.
Each eye also acted as its own control, by comparing aqueous humour formation rate over the initial period (-30 to +10min) with the two following time intervals (+10 to +50 min and +50 to +90 min). Each value is a mean ± s.e. mean of the rate of aqueous humour formation, expressed as $K_{out}.min^{-1}$ $x10^{-4}$M of the number (n) of experiments shown. Control ('separate eye' control) represents values for eyes perfused with Krebs' solution without reagent. Significance of differences from same eye control (time –30 to +10 min) at two separate time intervals (time +10 to +50 min) and (time +50 to +90 min). Significance of difference from initial same eye 'control' period as indicated by paired Student t-test **p<0.001; ns =not significant.

DISCUSSION

THE EFFECTS OF BRADYKININ ON AQUEOUS HUMOUR FORMATION WITHIN THE ISOLATED, ARTERIALLY PERFUSED BOVINE EYE

The arterially perfused eye has been extensively used by many researchers, using various eye models including rabbit **(Kodama *et al.*, 1983, 1985)**, cat, **(Macri *et al.*, 1978)** and bovine **(Kishida *et al.*, 1985; Wilson *et al.*, 1993).** It allows investigation of aspects of the physiology and pharmacology of the eye (albeit non-human) which cannot be studied so readily *in vivo*.

Though the perfused bovine eye model had previously been rejected by certain investigators as being an unreliable experimental model **(Kishida *et al.*, 1985)**, it has since been shown in numerous experiments to provide reproducible results, consistent with *in vivo* models **(Wilson *et al.*, 1993). Wilson *et al.* (1993)** suggest that the discrepancy between their findings and those of **Kishida *et al.* (1985)**, who had dismissed the perfused bovine eye model as a viable research tool, were related to the delay between the slaughter of the animal and commencement of perfusion. A delay of greater than one hour appeared to significantly reduce the probability of obtaining a stable IOP. Viability has even been shown for up to 5 days if the bovine eye is perfused within 1 hour of slaughter **(de Coo *et al.*, 1993).**

The bovine eye is an ideal model on which to perform arterial perfusion experiments. It is readily available from an obliging abattoir and its size makes it much easier to work with than say the cat eye. **Shahidullah *et al.* (1995, 1999)** have since taken the use of the

model further showing a number of categories of drugs that reduce aqueous production and hence IOP in the bovine arterially perfused eye model. **McNeish *et al.* (2001, 2002)** have used this preparation to characterise the vasodilator effects of bradykinin in the ciliary vascular bed. Confidence in the physiological viability of this model has recently been boosted by the recording of multifocal electroretinograms from bovine arterially perfused eyes **(Shahidullah, *et al.*, 2005).**

Control values for aqueous humour formation in the bovine isolated perfused eye in the present study are similar to, if a little lower than, those previously reported **(Wilson *et al.*, 1993; Shahidullah *et al.*, 1999).** The possible reasons for this variability include the long time period (5 years) over which the present work was carried out and our lack of control on the bovine eyes we were obtaining. Though by being present at the time of slaughter we could be sure of fresh eyes, we are unable to choose the breed, sex and age of the cattle. All these factors may play a part in aqueous humour formation in the bovine eye.

A further potential variable was seasonal variation. It is already known that diurnal variation in IOP occurs and so it is not unreasonable to suppose that seasonal variation in daylight hours may affect aqueous humour formation. Also the hormonal variation that occurs with the seasons may affect aqueous humour formation. Whether any of these factors modulate aqueous humour formation within the bovine eye is currently not known.

Rationale for choice of bradykinin concentrations

Bradykinin agonist concentrations were chosen in an effort to cover the full range of responses from threshold to maximum response. Concentrations chosen for bradykinin were identified from a predicted range extrapolated from bradykinin responses in the vascular tissues of the bovine eye **(McNeish *et al.*, 2003)**, specifically 10^{-10}M to 10^{-7}M. At the low end of the range, the data from 10^{-10}M indicated that a response was present, while at the upper end of the range, the maximum appeared to be about $3x10^{-8}$M, before the response seemed to be opposed by the "barrier breakdown" effect.

The concentration of bradykinin antagonist chosen by trial and error for the calcium experiments, then this same concentration was chosen for the much more labour-intensive perfusion experiments. The result of these choices might have been failure to see the concentration-dependence of an antagonist effect and hence the competitive or non-competitive nature of antagonism would not be seen. The purpose of using the antagonists was however mostly to determine mechanism/receptor identities qualitatively rather than quantitatively.

Bradykinin suppresses aqueous humour formation

Low concentrations of bradykinin (10^{-9} to 10^{-8}M) produced significant suppression of aqueous humour formation (Table 3). At higher concentrations of bradykinin ($3x10^{-8}$M and 10^{-7}M) aqueous production appeared to be increased. This paradoxical effect may be explained as a dual effect: on the one hand the suppression of aqueous humour formation seen at bradykinin concentrations up to 10^{-8}M would reasonably be expected to persist at higher bradykinin concentrations. On the other hand, these higher concentrations seem to induce an increase in aqueous production great enough to overcome the above suppression; such an increase would likely consist of leakage of fluid from the vascular compartment into the chamber of the eye – the well- known phenomenon referred to as breakdown of the blood-aqueous barrier **(Cole *et al.*, 1973; Eakins, 1977).** Injection of bradykinin directly into the anterior chamber has previously been shown to cause breakdown of the blood-aqueous barrier, manifest as aqueous flare, in the rabbit eye **(Bynke *et al.*, 1983).**

Very recent data from our laboratory suggests that perfused eyes treated with bradykinin (10^{-7}M) are more likely to suffer barrier breakdown as measured by penetration of albumin from vascular perfusate to the anterior chamber **(Wilson, W.S. Personal communication)**.

Co-perfusing WIN 64338 ($3 x10^{-8}$M), a B_2 receptor antagonist, initially halved the effect of bradykinin (10^{-8}M), and by the end of the experiment the bradykinin effect was

completely inhibited (Table 4). This would suggest that bradykinin acts via B_2 receptors to inhibit aqueous humour production within the bovine eye. This fits with the conventional view that B_2 receptors are present in many tissues under normal physiological conditions, whereas B_1 receptors require a period of induction **(Dray *et al.*, 1993; Marceau *et al.*, 1998; Hall, 1997; Haddad, 1999).**

L-NAME (10^{-4}M), a nitric oxide synthase inhibitor, significantly reduced the effect of bradykinin on aqueous humour formation (10^{-4}M L-NAME +10^{-9}M bradykinin) (Table 6). L-NAME alone (10^{-4}M) had no significant effect on aqueous humour formation.

Bradykinin is known to release nitric oxide in many blood vessels including the porcine ophthalmic artery **(Yao *et al.*, 1991)** and porcine ciliary artery **(Zhu *et al.*, 1997)**. If bradykinin reduces aqueous production by utilisation of a pathway involving nitric oxide synthase, then one would expect this effect of bradykinin to be abolished by L-NAME. **McNeish *et al.* (2001)** reported a small increase in vascular tone in the isolated bovine eye when L-NAME was perfused.

Lack of effect of L-NAME alone on aqueous humour formation in the present work suggests that there is no tonic influence of endogenous nitric oxide in the bovine ciliary epithelium. Thus nitric oxide release from the bovine vasculature is either of insufficient concentration or does not readily diffuse to the ciliary epithelium in the perfused eye.
In vascular tissue, nitric oxide diffuses into the smooth muscle, where it stimulates the enzyme guanylate cyclase, leading to increased cGMP production and smooth muscle

relaxation (**Ignarro *et al.*, 1985, 1987; Gruetter *et al.*, 1981**). Nitric oxide-sensitive guanylate cyclase is generally accepted as the most important receptor for the signalling molecule nitric oxide. Its activation leads to increased synthesis of cGMP. The nitric oxide/cGMP signalling cascade is important in the cardiovascular and nervous systems, where it controls smooth muscle relaxation (**Lincoln, 1989; Hofmann *et al.*, 2000**), modulation of synaptic transmission (**Garthwaite *et al.*, 1988; O'Dell *et al.*, 1991**) and inhibition of platelet aggregation (**Mellion *et al.*, 1981; Schwarz *et al.*, 2001**). cGMP has previously been shown to be involved in reducing aqueous humour formation within the bovine eye (**Millar *et al.*, 1997; Shahidullah *et al.*, 1999**).

NADPH diaphorase, a marker allowing localisation of the enzymes involved in the production of nitric oxide, has been identified in the posterior ciliary arteries of both pigs and monkeys (**Toda *et al.*, 1997, 1998**). This would suggest that nitric oxide production occurs in the vasculature supplying the ciliary body. The present data suggest that bradykinin suppresses aqueous humour formation via nitric oxide and cGMP since a soluble guanylate cyclase inhibitor (ODQ) appeared to block the bradykinin-induced inhibition of aqueous humour formation (+50 to +90 min,Table 5,rows 2+5).

This conclusion is a little compromised by the fact that ODQ alone caused a reduction in aqueous humour formation; this would suggest that an alternate pathway, that utilises guanylate cyclase, may be also be involved in aqueous humour production. An alternative explanation would be that ODQ may act independently of its well known effect on guanylate cyclase, inhibiting aqueous humour formation by some other

mechanism.

Buckley *et al.* (1998) found no increase in tone of the bovine ciliary artery when exposed to an inhibitor of nitric oxide. This is in contrast to **Su *et al.* (1994)** and **Wiencke *et al.* (1994)** who found that inhibition of nitric oxide production resulted in increased tension within isolated porcine and bovine ciliary arteries, respectively. **Meyer *et al.* (1993)** found that in the isolated porcine eye the nitric oxide synthase inhibitor, L-NAME, reduced perfusion through the eye. Likewise, **McNeish *et al.* (2002)** reported a significant increase in perfusion pressure in the bovine ciliary vasculature when L-NAME was present. This showed that even in the isolated eye basal release of nitric oxide can occur.

It has previously been shown (**Kitamura *et al.*, 1993**) that inhibition of nitric oxide synthase prevents the vasodilator response usually effected by substance P in the retinal arteries of dogs. Inhibition of nitric oxide synthase also prevents the increase in ophthalmic vascular flow usually produced by bradykinin in the perfused porcine eye (**Meyer *et al.*, 1993**).

Nitric oxide has a 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**). These studies have shown that pre-treatment with nitric oxide synthase inhibitors 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**).

In diabetic humans the inhibition of nitric oxide synthase results in less reduction in choroidal blood flow compared to the non-diabetic human eye (**Schmetterer *et al.*, 1997**).

Bovine ciliary muscle and trabecular meshwork have been shown to contract when nitric oxide synthase is inhibited and relax when exposed to nitrovasodilators (**Wiederholt *et al.*, 1994**). **Hessemer *et al.* (1997)** found little reduction in IOP in man and that this minimal reduction may be due to a reduction in systemic blood pressure rather than any local effect. **Grunwald *et al.* (1997)** however found that chronic systemic nitrate therapy produced retinal venous dilatation in glaucomatous patients.

Nitric oxide synthesis inhibition with L-NAME has been shown to cause a dose-dependent reduction in anterior choroidal blood flow in rats (**Koss, 1998**).

It thus appears that there is good evidence with regard to the involvement of nitric oxide in vascular effects within the eye but at present very little is known about its effect on aqueous humour dynamics.

Bradykinin dilates ciliary arterial beds in the bovine eye 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 bradykinin could also release EDHF in the ciliary epithelium, detecting this mechanism using the toxins which selectively inhibit K^+ channels **(McNeish *et al.*, 2001)** would be technically very difficult and expensive.

One remarkable characteristic of the dilatation of bovine ciliary blood vessels largely through the release of EDHF is that this effect is almost completely blocked if ascorbate has been present for more than two hours **(McNeish *et al.*, 2001).**

The possibility that such a mechanism exists also in the secretory cells of the ciliary body was tested in the present work. In the absence of ascorbic acid from either the perfusate alone, or both the perfusate and the aqueous humour substitute, the inhibitory effect of bradykinin on aqueous humour production was reduced.

At a concentration of bradykinin 10^{-7}M (Table 7), the absence of ascorbate from the perfusion solution appears to affect the bradykinin-induced response. The effect of bradykinin at the lower concentration (10^{-9}M), was significantly inhibited in the absence of ascorbate from the perfusion solution (unpaired t-test; $p<0.01$). In the absence of ascorbate from both the perfusate and the aqueous humour substitute the effect of bradykinin at this lower concentration was also significantly reduced (unpaired t-test; $p=0.004$).

It has previously been shown that in the bovine eye ascorbate inhibits the major (EDHF-

mediated) component of bradykinin-induced vasodilatation (**McNeish *et al.*, 2003**). In this current research bradykinin, in the presence of ascorbate, clearly reduces aqueous humour production in the bovine eye. Omission of ascorbate from the perfusate appears to result in the effect of bradykinin on aqueous humour production being inhibited. It is possible that the reduced vasodilator effect of bradykinin in the absence of ascorbate may somehow have countered the effect on aqueous humour formation. However, at present my findings with regard to ascorbate do not easily fit into currently existing data and theories, since analogy with vascular EDHF would have predicted an enhancement of the bradykinin effect on aqueous production when ascorbate was omitted.

Substance P inhibits aqueous humour formation

Substance P (10^{-8}M) appears to inhibit aqueous humour formation. It has previously been suggested that bradykinin either releases substance P or acts via substance P receptors, with the miotic effect of bradykinin in rabbits being inhibited by the use of a substance P antagonist (**Wahlestedt *et al.*, 1984**). It may therefore be possible that we are actually activating the same pathway with substance P as we are with bradykinin. The bradykinin response could possibly be further explored using substance P-specific receptor blockade.

In summary, then it appears that within the perfused bovine eye, bradykinin acting via B_2 receptors, utilising the nitric oxide and cGMP mechanism, inhibits aqueous humour formation in a dose-dependent manner. It is likely that suppression of aqueous humour formation persists at higher bradykinin concentrations but at these concentrations leakage

of fluid from the vascular compartment into the chamber occurs i.e. breakdown of the blood-aqueous barrier.

The omission of ascorbate from the perfusate results in the effect of bradykinin on aqueous humour production being inhibited. These findings do not fit into currently existing data and theories; however, it does appear that an EDHF-mediated pathway is not involved in the bradykinin effect on aqueous humour production.

Substance P (10^{-8}M) appears to inhibit aqueous humour formation, possibly through the same secondary messenger system as bradykinin.

THE EFFECT OF BRADYKININ ON INTRACELLULAR CALCIUM RELEASE FROM NON-PIGMENTED CILIARY EPITHELIAL CELLS

The effects on intracellular calcium were determined by a fura-2 fluorescence technique in non-transformed cultured ciliary epithelium as described previously **(Shahidullah *et al.*, 1997).**

In all cells there appeared to be a very slow but steady increase in the baseline fluorescence ratios. This suggests a constant influx of calcium. We are currently unable to say if this is a natural phenomenon or one resulting from the cell culture technique used.

In order to normalise the data for calcium release, an arbitrary time interval of 120 seconds prior to the peak of the response was chosen due to the variation in the rate of onset of the bradykinin effect. One contributor to this variation is the delivery system of the superfusing solution, in addition to the variation in the response of individual cells. The delivery system was gravity fed and thus the rate varied slightly depending on volume remaining in the feeding reservoir. Despite the delivery system being carefully cleaned after every usage, accumulation of debris in the narrow orifices of the control valve could potentially slow down the flow rate.

The effect of ATP on calcium release in non-pigmented ciliary epithelial cells

Addition of ATP (10^{-4}M) to the solution superfusing the non-pigmented ciliary epithelial cells triggered an almost immediate (10-20s) increase in $[Ca^{2+}]_i$ as indicated by changes in fluorescence ratio (Figure 9). Rapid release of Ca^{2+} from intracellular stores caused a peak within 40s of initial onset, which at first declined quickly ($t_{1/2}$ <30s), then more slowly.

The appearance of this response was comparable to that observed by **Shahidullah *et al.* (1999)**. However their experiments involved populations of bovine cells, rather than individual cells. Absolute values of $[Ca^{2+}]_i$ have been calculated for populations of these cells **(Shahidullah *et al.*, 1997)** but calibration of a microspectrophotometric system for single cells is very difficult and so the experimental results were shown as the fluorescence ratios as is commonly done in the literature **(Suzuki *et al.*, 1997; Shahidullah *et al.*, 1997)**.

Under normal physiological conditions ATP is released from sympathetic neurosecretory vesicles **(Burnstock, 1990)**. It has a role in gut relaxation and bladder contraction **(Rang *et al.*, 2003)**. The pathway by which ATP mobilizes intracellular calcium in non-pigmented ciliary epithelial cells has been postulated, with the backing of substantial experimental data, to involve $P2Y_2$ receptors and their activation of phospholipase C (PLC) causing an increase in intracellular $InsP_3$ **(Shahidullah *et al.*, 1997)**. This then binds to a receptor on the endoplasmic reticulum, leading to the mobilization of

intracellular Ca^{2+}. These findings are consistent with those involving ATP in other species and in other cell-types **(Gibb *et al.*, 1994; O'Conner *et al.*, 1991; O'Conner, 1992). Mitchell *et al.* (1998)** postulated a role for endogenous ATP in the modulation of aqueous humour formation.

The effect of bradykinin on calcium release in non-pigmented ciliary epithelial cells

Bovine non-pigmented ciliary epithelial cells respond to bradykinin and therefore possess bradykinin receptors. Bradykinin (10^{-10}M to $3x10^{-8}$M) was found to have a concentration-dependent effect on $[Ca^{2+}]_i$ (Correlation coefficient log[bradykinin] (M): Pearson r =0.9638, p value = 0.008). Bradykinin (10^{-8}M) produced an increase in $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells comparable in peak height to the effect of ATP (10^{-4}M). ATP (10^{-4}M) produces a near maximal response in these cells **(Shahidullah *et al.*, 1997)**. Production of a similar size of response on addition of bradykinin (10^{-8}M), a concentration ten thousand times less, showed that bradykinin has a much more potent effect on non-pigmented ciliary epithelial cells with regard to the mobilisation of Ca^{2+}, than ATP.

A dose-dependent increase in intracellular calcium has previously been found in human transformed non-pigmented ciliary epithelial cells in cultured cell lines exposed to bradykinin as well as other drugs **(Lee *et al.*, 1989)**.

The time-course of the responses produced by ATP and bradykinin differ (Figure 9,10,22). ATP triggered a rapid release of Ca^{2+} from intracellular stores and caused a peak within 40s, which at first declined quickly ($t_{1/2}$ <30s), then more slowly as the stores were replenished from extracellular Ca^{2+} (Figure 9) (**Shahidullah *et al.*, 1997; Low *et al.*, 1993**). By contrast, the response to bradykinin was much slower than that induced by ATP, taking approximately 2 min to peak (Figure 10). The subsequent decline was also slower ($t_{1/2}$ approx. 60s). These differences in the response may be due to involvement of different receptors and possibly different second messenger pathways with different end-effects.

Bradykinin produced a similar response in both the presence and absence of extracellular calcium (Figures 11,12,13,14). This would strongly suggest that the bradykinin-induced response is utilizing intracellular calcium stores rather than an extracellular influx. The only significant difference between the responses in the calcium-present and calcium-free environments was the lower baseline fluorescence ratios in cells incubated in the absence of normal Ca^{2+} levels. The nature of the system is such that fura-2-labelled extracellular calcium overlying the non-pigmented ciliary epithelial cell or bound to the outside of the membrane might be misinterpreted as intracellular calcium. This would give a high initial reading for the starting fluorescence ratio. Unfortunately with the experimental set-up used we were unable to remove this error. As all experiments were performed under similar conditions this component could be ignored.

Huang *et al.* (2001), used thapsigargin to deplete intracellular stores of Ca^{2+} prior to

instillation of bradykinin, with no effect on the bradykinin-induced increase in $[Ca^{2+}]_i$. This would suggest that the effect of bradykinin on Ca^{2+} mobilization in canine cultured corneal epithelial cells is dependent on extracellular Ca^{2+}, rather than intracellular stores. This contrasts with our finding that the bradykinin-induced response utilizes intracellular Ca^{2+} stores and may be explained as either cell-type or species variation.

At lower concentrations of bradykinin, where there was little direct effect of bradykinin, ATP was added later to see if the ATP response was altered in the presence of bradykinin (Figure 15). The prior treatment with bradykinin delayed the onset of the response elicited by ATP. The change in fluorescence ratio between baseline and peak was also reduced in the presence of bradykinin when compared to effect of ATP alone. Pre-treatment of non-pigmented ciliary epithelial cells with bradykinin therefore affects the ATP-induced response. We are currently unsure as to the mechanism and relevance of this inhibition, but it may simply indicate that ATP and BK release calcium by similar mechanisms.

The bradykinin effect upon $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells appears to be mediated by B_2 receptors (Figure 16). The response was inhibited by WIN 64338, a B_2 receptor antagonist. Direct stimulation with a B_1 agonist, Lys-[des-Arg^9]BK, appeared to have no effect on calcium release in non-pigmented ciliary epithelial cells (Figure 17). This would be consistent with previous findings that when cultures are initially set up B_1 receptors are not present, but rather synthesised and expressed slowly over several hours **(Marceau, 1995).** It has been shown in canine cultured corneal epithelial cells that

bradykinin directly stimulates B_2 receptors resulting in a calcium influx within the cells **(Huang *et al.*, 2001).**

Both ATP via $P2Y_2$ receptors and bradykinin via B_2 receptors usually act through the same G-protein (Gq/11). Activation of this protein leads to receptor-mediated activation of phospholipase C, which catalyses the formation of $InsP_3$ and DAG from phosphatidylinositol 4, 5-bisphosphonate. $InsP_3$ causes release of Ca^{2+} from the endoplasmic reticulum stores, and DAG activates protein kinase C **(Berridge, 1987)** (Figure 23).

ODQ is a potent inhibitor of soluble guanylate cyclase. It has been shown to have an inhibitory action with a threshold at 0.1μM, a concentration of 10 μM abolished any response to all concentrations of glyceryl trinitrate **(De la Lange *et al.*, 1999).**

In bovine non-pigmented ciliary epithelial cells, neither ODQ or L-NAME had any effect upon $[Ca^{2+}]_i$ in their own right. In addition, neither drug appeared to have any effect on the intracellular Ca^{2+} response to bradykinin (10^{-8}M) in these cells (Figure 18). This would suggest, not surprisingly, that neither cyclic GMP nor nitric oxide play a part in the release of Ca^{2+} induced by bradykinin.

Certainly in other systems, nitric oxide has an effect on a separate cell population to those initially acted upon by bradykinin. In porcine ciliary artery, the bradykinin-induced relaxation is mediated by nitric oxide and involves a K^+-channel **(Zhu *et al.*, 1997).** The

relaxation evoked by bradykinin is almost completely abolished by removal of the endothelium. A transient increase in cytosolic free calcium leads to the activation of the K^+-channel, which in turn enhances synthesis of nitric oxide (**Schilling, 1989; Colden-Stanfield *et al.*, 1987**). Other mediators, for example acetylcholine, may also release nitric oxide with the same end result.

As reported previously cGMP inhibits ATP-induced Ca^{2-} release **(Shahidullah *et al.*, 1999).** One may expect that inhibition of cGMP could potentiate bradykinin-induced calcium release. We have shown though that inhibition of cGMP synthesis does not have any effect on the bradykinin-induced release of intracellular calcium in the non-pigmented ciliary epithelial cell. Two possible reasons for this are that in the resting cell there is no cGMP synthesis to inhibit, or that bradykinin and ATP act on different pathways to cause release of intracellular calcium from ciliary epithelial cells.

In vascular tissues bradykinin releases nitric oxide from endothelial cells which then generates cGMP in smooth muscle cells. If bradykinin caused an increase in cGMP in ciliary epithelial cells, it would likely be as one consequence of calcium release, in which case guanylate cyclase inhibition by ODQ might be expected to enhance calcium release. In the environment of the ciliary body it is possible that via a second messenger bradykinin may act upon the pigment epithelial cells.

If the situation was analogous to blood vessels bradykinin would trigger nitric oxide synthesis in a different cell type and nitric oxide might then suppress calcium in non-

pigmented ciliary epithelial cells. However, we have only epithelial cells in the culture. Whole eye preparations appear to contain cells which generate nitric oxide in response to bradykinin, since L-NAME blocks bradykinin suppression of aqueous humour formation. We know that cGMP affects calcium release by ATP **(Shahidullah *et al.*, 1999)** and that a nitric oxide donor (azide) generates cGMP in bovine cultured ciliary epithelium.

The hypothesis would be satisfied if bradykinin receptors in non-pigmented ciliary cells release Ca^{2+} which generates nitric oxide then cGMP, which then inhibits aqueous humour production through an action on another cell; pigmented ciliary epithelial cells would be the obvious candidate (Figure 24).

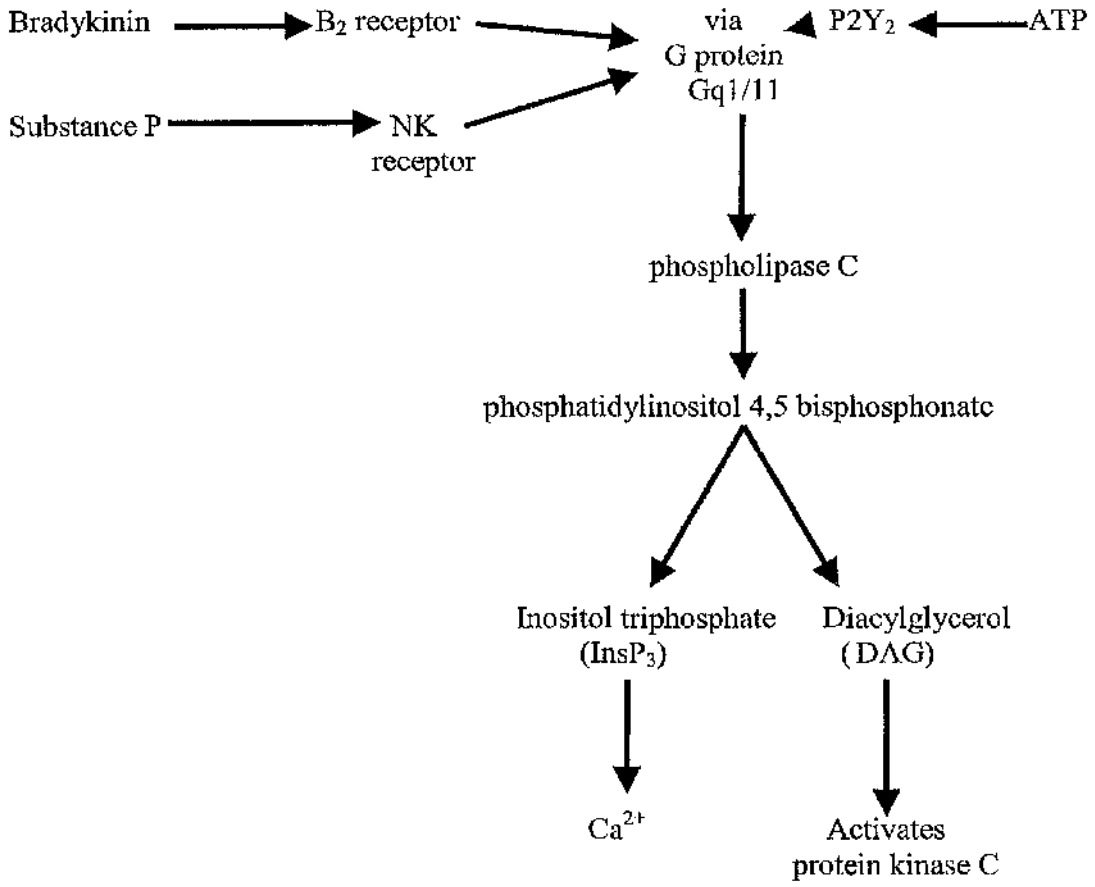

Figure 23

Pathway by which ATP, bradykinin and substance P active the inositol trisphosphate / diacylglycerol pathway

Both ATP, bradykinin and substance P act through the same G-protein (Gq/11). Activation of this protein leads to receptor-mediated activation of phospholipase C, which catalyses the formation of $InsP_3$ and DAG from phosphatidylinositol 4, 5-bisphosphonate. $InsP_3$ causes release of Ca^{2+} from the endoplasmic reticulum stores, and DAG activates protein kinase C **(Adapted from Berridge, 1987).**

Figure 24

Potential pathway by which ATP, bradykinin and substance P acting via the inositol trisphosphate / diacylglycerol pathway results in nitric oxide release which eventually leads to the production of cGMP under the control of guanylate cyclase and the eventual reduction in aqueous humour formation.

(Adapted from Berridge, 1987).

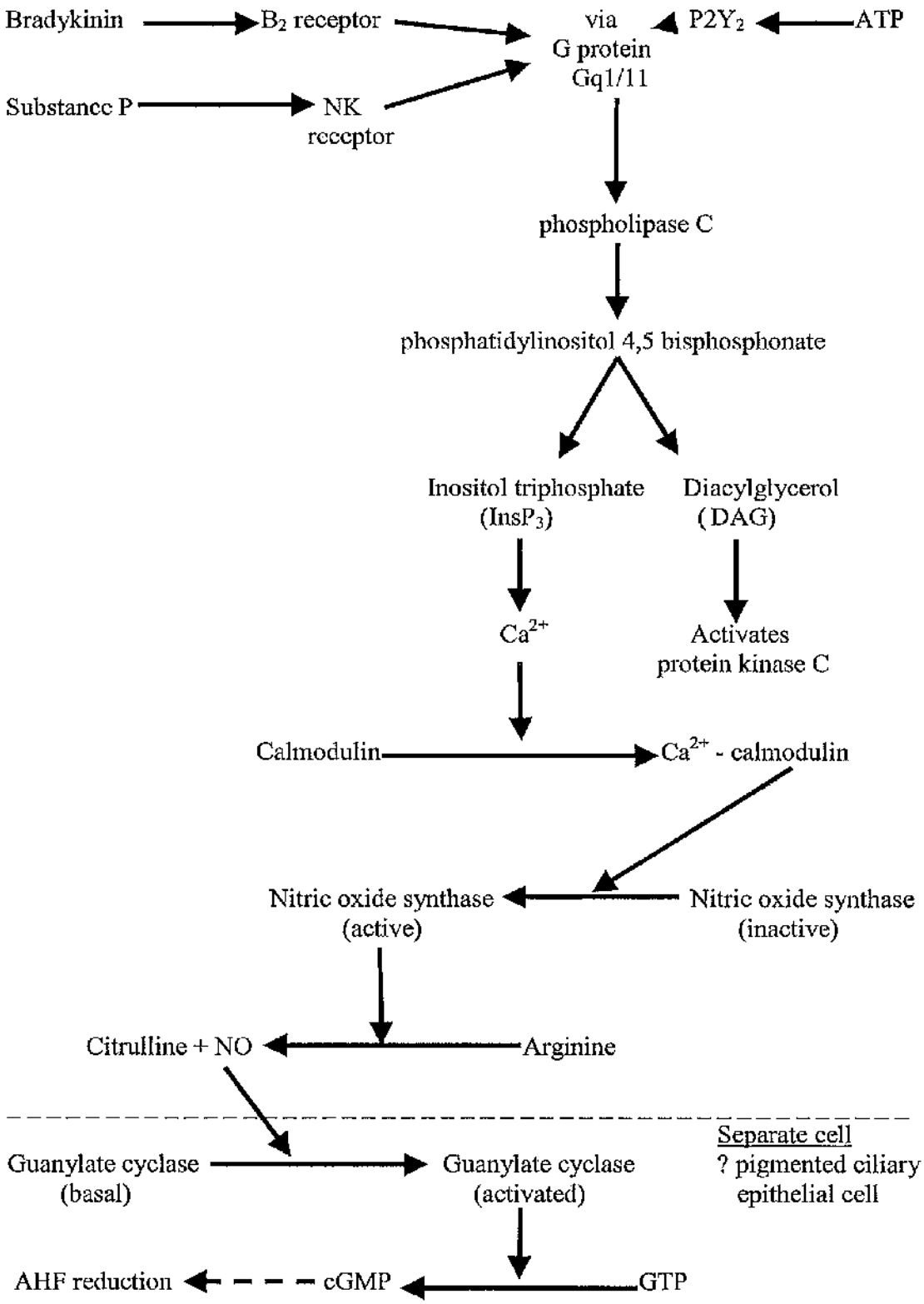
Bradykinin
B_2 receptor
via
$P2Y_2$
ATP
G protein
Gq1/11
Substance P
NK
receptor
phospholipase C
phosphatidylinositol 4,5 bisphosphonate
Inositol triphosphate
($InsP_3$)
Diacylglycerol
(DAG)
Ca^{2+}
Activates
protein kinase C
Calmodulin
Ca^{2+} - calmodulin
Nitric oxide synthase
(active)
Nitric oxide synthase
(inactive)
Citrulline + NO
Arginine
Separate cell
? pigmented ciliary
epithelial cell
Guanylate cyclase
(basal)
Guanylate cyclase
(activated)
AHF reduction
cGMP
GTP

Figure24

Effect of ascorbate on bradykinin-induced increase in calcium within non-pigmented ciliary epithelial cells

Ascorbate is present in high concentrations within aqueous humour. The presence of ascorbate appeared to have no effect on the bradykinin-induced increase in $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells (Figure 19). Within the bovine ciliary artery it has been shown that ascorbate takes two hours to block the bradykinin effect (**McNeish *et al.*, 2003**). It may be that the ciliary epithelial cells require prolonged pre-treatment with ascorbate before any effect on the bradykinin-induced calcium response is observed. Alternatively it may be that ascorbate has no effect on Ca^{2+} release, but rather affects nitric oxide production further along the pathway **(Heller *et al.*, 1999; Shrammel *et al.*, 2000).**

Effect of substance P on calcium release from non-pigmented ciliary epithelial cells

Substance P causes mobilisation of intracellular calcium within non-pigmented ciliary epithelial cells. The response to substance P elicited in the release of intracellular calcium from non-pigmented ciliary epithelial cells appears to be threshold-dependent rather than concentration-dependent, occurring at all concentrations above 10^{-9}M.

The individual cell response appears to be more in keeping with that produced by ATP than by bradykinin (Figures 9,10,20). When compared by unpaired t-test both the delay in onset of calcium release and the size of the response were comparable between substance P (10^{-7}M) and ATP (10^{-4}M). It is possible that ATP and substance P utilize a common pathway and by contrast bradykinin uses an alternative pathway in its mobilization of intracellular calcium.

Both **Yoshimura *et al.* (1995)** and **Lee *et al.* (1989)** found that substance P failed to elicit a Ca^{2+} response from non-pigmented ciliary epithelial cells. However they found species variation between rabbit and human respectively, in the release of calcium by both noradrenaline and carbachol. Their result for substance P would conflict with the current study's findings that this drug does cause a release of calcium from bovine non-pigmented ciliary epithelium. However this may well be due to species variation, as discussed above.

In Conclusion

We have shown that bradykinin has an effect on $[Ca^{2+}]_i$ by mobilization of intracellular calcium stores. Also that low concentrations of bradykinin produced significant suppression of aqueous humour formation. Both of these effects appear to be mediated by B_2 receptors rather than B_1 receptors.

While it is likely that there may be more metabolic loss of bradykinin in the perfused eye than in the cultured cell preparation, it seems likely that its effective concentration in both cases is similar. Calculation of EC50 values is difficult for either of these situations since in both cases the maximum effect is obscured. However the range of concentrations which caused significant reduction in aqueous formation (10^{-8}M to 10^{-10}M) is similar to that where calcium release was observed (3x 10^{-8}M to 10^{-10}M).

There exists a very large difference in the time course of bradykinin's effects on aqueous production and on calcium release. Much of this can be explained by the nature of the isolated eye preparation and the inevitably large inertia involved in altering the rate of fluorescein dilution. The much faster response of single cells in superfusion is to be expected. Direct comparison of the time course of these two responses to bradykinin is thus fruitless. However there may be merit in the following indirect comparison: the calcium response to bradykinin is substantially slower than those to ATP (mean $\pm$ s.e.: bradykinin 136.9 $\pm$ 8.5s compared to ATP 38.6 $\pm$ 6.3s) or to azide **(Shahidullah *et al.*, 1999).** The aqueous formation response to bradykinin is likewise substantially slower; it

was seldom significant within the first 40 min of drug treatment, whereas inhibitions by ATP or azide are reported as significant within 15 min **(Shahidullah *et al.*, 1999).**

Bradykinin-induced calcium release does not appear to be affected by either ODQ or L-NAME. In the whole eye however, L-NAME blocks bradykinin suppression of aqueous humour formation, suggesting the presence of cells which generate nitric oxide in response to bradykinin. If the hypothesis that interfering with calcium movements is the mechanism by which bradykinin suppresses aqueous humour formation is to be upheld, then bradykinin must generate nitric oxide somewhere in the vicinity of the ciliary epithelium (possibly the pigmented epithelial cells) and this nitric oxide could then generate cGMP in pigmented ciliary epithelium.

Omission of ascorbate from the perfusate results in the effect of bradykinin on aqueous humour production being inhibited. However, the presence of ascorbate appeared to have no effect on the bradykinin-induced increase in $[Ca^{2+}]_i$ in non-pigmented ciliary epithelial cells. It may be that the ciliary epithelial cells need prolonged pre-treatment with ascorbate before any effect on the bradykinin-induced calcium response is observed **(McNeish *et al.*, 2003)**. Alternatively as previously suggested it may be that ascorbate has no effect on Ca^{2+} release, but rather has its effect upon nitric oxide production further along the pathway **(Heller *et al.*, 1999; Shrammel *et al.*, 2000).**

Both L-NAME and ODQ blocked the effect of bradykinin on aqueous humour formation. The presence of ascorbate doesn't block the bradykinin effect on aqueous humour

formation. EDHF therefore does not seem to participate in the mechanism of bradykinin in suppressing aqueous humour formation. This contrasts with the bovine ocular vasculature where ascorbate inhibits bradykinin-induced vasodilatation in the bovine eye **(McNeish *et al.*, 2003)** and where bradykinin-induced vasodilatation is largely through the release of EDHF **(McNeish *et al.*, 2001)**.

The potency with which bradykinin exerts these effects may also suggest a physiological role for bradykinin as a modulator in the ciliary epithelium. The close parallel between the very low concentrations of bradykinin required to suppress aqueous humour formation and to affect intracellular calcium movements, supports the hypothesis that intracellular calcium in the ciliary epithelium plays an important role in aqueous humour formation. It has previously been argued **(Shahidullah *et al.*, 1999)** that any process that affecting the normal calcium oscillations in ciliary epithelial cells will upset the normal production of aqueous humour and thus result in a reduction in aqueous humour formation.

Substance P causes mobilisation of intracellular calcium within non-pigmented ciliary epithelial cells and appears to inhibit aqueous humour formation. From the appearance of the individual cell response it may be that ATP and substance P utilize a common pathway and rather bradykinin uses an alternative pathway in its mobilization of intracellular calcium. However, it has previously been suggested that bradykinin either releases substance P or acts via substance P receptors **(Wahlestedt *et al.*, 1984)** and it may therefore be that substance P and bradykinin are actually activating the same

pathway. This needs further investigation but might help to explain the delay which characterised the calcium response to bradykinin.

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APPENDICES

Appendix I

List of abbreviations used in this thesis:

Abbreviation	Definition
α	Alpha
AH	Aqueous Humour
Approx.	Approximately
ATP	Adenosine Triphosphate
β	Beta
BK	Bradykinin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular Calcium Concentration
$CaCl_2$	Calcium Chloride
Cl^-	Chloride
cm	Centimetre
$C0_2$	Carbon Dioxide
°C	Degrees Celsius
dis.	Dissolved
DMSO	Dimethyl Sulphoxide
ed.	Editor
Ed.	Edition
EDTA	Ethylene Diamine Tetraacetic Acid

g	Grammes
H^+	Hydrogen
h	Hours
HCO_3^-	Bicarbonate
H_2O	Water
K^+	Potassium
KCl	Potassium Chloride
KH_2PO_4	PotassiumDihydrogen Orthophosphate
IOP	Intraocular pressure
<	Less than
L	Litre
L-NAME	Inhibitor of nitric oxide synthase
M	Molar
mg	Milligrammes
$MgSO_4$	Magnesium sulphate
min	Minutes
ml	Millilitres
mm	Millimetres
mM	Millimolar
mmH_2O	Millimetres of Water
mol	Moles
Na^+	Sodium

$NaHCO_3$	Sodium Hydrogen Carbonate
NaOH	Sodium Hydroxide
NPE	Non-pigmented Ciliary Epithelium
O_2	Oxygen
ODQ	1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one
PE	Pigmented Ciliary Epithelium
pH	Log concentration of hydrogen ions
%	Percentage
SP	Substance P
µg	Microgrammes
$\mu g.g^{-1}$	Microgrammes per Gramme
$\mu g.ml^{-1}$	Microgrammes per Millilitre
µL	Microlitres

Appendix II

Materials used:

Material:	Source:
Acetic Acid	Fischer Scientific
Adenosine Triphosphate	Sigma Chemical Company
Ascorbate	May and Baker
Bovine Serum Albumin	Sigma Chemical Company
Bradykinin	Sigma Chemical Company
Calcium Chloride	BDH Laboratory Supplies
Collagenase A	Roche
Dulbecco's modification of Eagle's Medium(DMEM)	Gibco
Dimethyl Sulphoxide (DMSO)	Sigma Chemical Company
D-Glucose	Fischer Scientific
EDTA	Sigma Chemical Company
EGTA	Sigma Chemical Company
Fetal Calf Serum	Gibco
Fluorescein Sodium (2%)	Chauvin
FURA 2/AM	Calbiochem
Gentamicin	Gibco
HEPES	Sigma Chemical Company

Hydrochloric Acid	Laboratory Grade
L-NAME	Sigma Chemical Company
Magnesium Chloride	BDH Laboratory Supplies
Magnesium Sulphate	BDH Laboratory Supplies
Newborn Calf Serum	Gibco
ODQ	Sigma Chemical Company
Potassium Chloride	BDH Laboratory Supplies
Potassium Dihydrogen Orthophosphate	BDH Laboratory Supplies
Potassium Hydroxide	BDH Laboratory Supplies
Sodium Carbonate	Fischer Scientific
Sodium Chloride	Fischer Scientific
Sodium Hydrogen Carbonate	Fischer Scientific
Sodium Hydroxide	Fischer Scientific
Substance P	Sigma Chemical Company
WIN 64338 hydrochloride	Tocris Cookson Ltd.

Appendix III

Solution used:

1. Krebs' solution for perfusion of isolated bovine eye

Composition of Modified Krebs' solution

Chemical	Concentration (mM)
NaCl	118
KCl	4.7
KH_2PO_4	1.2
$MgSO_4.7H_2O$	1.2
$NaHCO_3$	25.0
Glucose	11.5
Ascorbate	0.05
$CaCl_2$	2.5

$CaCl_2$ was added once all other chemicals had been dissolved in distilled water.

The solution was bubbled with 95% oxygen and 5% CO_2 for 6 min before use, to adjust the pH to 7.4.

2. Aqueous humour substitute solution for use in the isolated bovine eye

Composition of Aqueous Humour Substitute

Chemical	Molar Concentration (mM)
NaCl	110.0
KCl	3.0
$CaCl_2.6H_2O$	1.4
$MgCl_2.6H_2O$	0.5
$NaHCO_3$	30.0
K_2HPO_4	0.9
Glucose ($C_6H_{12}O_6$)	6.0
Ascorbic acid	3.0

All chemicals were dissolved in distilled water.

The solution was bubbled with 95% oxygen and 5% CO_2 for 6 min before use.

Minims fluorescein (1% w/v) was diluted 1:1000 using AHS, then 150µl was added to 50ml of aqueous humour substitute and used to fill the perfusion circuit and fluorimeter cuvette, prior to insertion of the corneal cannulae.

3. Calcium-free buffer solution for use in preparation of ciliary epithelial cell culture

Ca^{2+}-free buffer solution

Chemical	Concentration (mM)
NaCl	142
KCl	13.41
HEPES	4.82
EDTA	0.25

All chemicals were dissolved in distilled water.

4. Collagenase buffer solution for use in preparation of ciliary epithelial cell culture

Collagenase buffer

Chemical	Concentration (mM)
NaCl	66.73
KCl	13.41
HEPES	3.84
$CaCl_2$	4.8

All chemicals were dissolved in distilled water.

The pH was adjusted to 7.6, using concentrated HCl before addition of HEPES.

The pH was readjusted to 7.6 again after addition of HEPES and $CaCl_2$

5. Modified Krebs' solution for use in ciliary epithelial cell culture experiments

Modified Krebs' solution

Composition	Concentration (mM)
NaCl	118.0
KCl	4.8
$NaHCO_3$	2.4
$MgSO_4$	1.0
Glucose	11.0
HEPES	10.0
$CaCl_2$	1.8

All chemicals are dissolved in distilled water.

pH 7.4

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