Mechanisms of Actions of Drugs Which Alter Intraocular Pressure

A thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Medicine
by

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January 1993

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Declaration

I hereby declare that this thesis embodies the results of my own special work, that was carried out in the Ocular Pharmacology Laboratory within the Department of Pharmacology, The University of Glasgow, and Fisons Pharmaceuticals Research & Development plc, between September 1989 and June 1992.

This thesis does not include work forming part of a thesis presented successfully for a degree in this or another University.

Mechanisms of Actions of Drugs Which Alter Intraocular Pressure

Acknowledgements

This work has been generously supported jointly by the Medical Research Council, and Fisons Pharmaceuticals plc by way of an MRC Industrial Partnership Award. I would like to extend my sincere thanks to Dr. William Wilson, of the Department of Pharmacology, for his expert and patient supervision throughout my period as his postgraduate student, without whose guidance this project would not have been possible. Thanks also to his family, for their much appreciated hospitality over the years. My compliments also to Professor J.S. Gillespie, and Professor Trevor Stone, Head of Department of Pharmacology. My thanks also to Dr. Richard Carr and Mr. Bob Humphries of Fisons plc for their invaluable advice and assistance with the microsphere experimental protocol, to Dr. Paul Leff for providing generous laboratory facilities, to Mr. Glyn Williams for sharing his home, to Ms. Alison Cave for her efficient secretarial assistance, and to all those other people, too numerous to mention who contrived to ensure that my stay at Fisons was a pleasant and productive experience.

My thanks also to John Scott Meats Ltd. of Paisley, Duke Street Abattoir, Glasgow, and Sidney Hackett's abattoir of Nottingham, and in particular to Mr. John Lamb, for a reliable and constant supply of bovine eyes.

Thanks also to Robert Auld of the Department of Pharmacology for his unceasing reserve of technical

assistance and good humour, Trevor Clarke for his metalworking skills and many enjoyable conversations about model steam engines, John Thompson for his many trips out to the abattoir for eyes, and his fascinating reminiscences of the Glasgow tramcars, and Ian Gibson and Adam Ritchie for their camaraderie and readiness to be on hand to assist in the solving of practical problems. I would also like to thank Dr. David Watson, of Strathclyde University, for his kind gifts of laevobunolol and dihydrolaevobunolol, Mrs. Edith MacNab for her expert and patient secretarial skills, and all others in the Department of Pharmacology and Tennent Institute of Ophthalmology who, over the last three years extended to me the hand of friendship, including Dr T.C. Muir, Tricia, Karen, Simon Guild, Jane, Shahid, Dot, Carol, Heather, Mike, Stilianos, Shamas, Jim, Xiaorong, Phil, Paul Skett, Frances Boyle, Billy Martin, David Pollock, Brian Morris, Julian, Duncan, Paul, Bruce, Jim Younger and Irene McLaren.

Thanks also to my Mother, Mary Millar for offering to me her home during the final months of my project work.

Finally, but my no means least, I would like to extend my sincere appreciation to my wife, Anne, for her love and support throughout these last years, and to my children, Gretchen and Brian, for all of their patience and understanding.

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Abstract

The bovine perfused eye model has been validated as an inexpensive and convenient method for the assessment of the ability of drugs to lower intraocular pressure (IOP), free from the complicating effects of the cardiovascular system, the nervous system (including the CNS) and the presence of circulating hormones. This preparation also facilitates the comparison of drug effects on pressure with effects on the uveal vasculature, as well as rapid access to living tissue for biochemical analysis. Using this preparation, the ocular hypotensive effects of several β-adrenoceptor antagonists have been demonstrated. The pressure lowering effect of the β-adrenoceptor antagonists probably does not involve a classical β-adrenoceptor or ciliary cyclic AMP as a signal transduction pathway, and is not dependent upon intact adrenergic innervation. The presence of a ciliary β_2-adrenoceptor population positively coupled via G_s protein to a membrane Ca^{2+} channel is postulated.

Atrial natriuretic factor (Atriopeptin - AP) and the nitrovasodilators sodium nitroprusside (SNP) and sodium azide, all of which are known to activate guanylate cyclase, also reduce IOP. Further, certain L-type Ca^{2+} channel antagonists and the K^+ channel agonist pinacidil reduce IOP in this preparation. A comparative investigation of the pressure lowering and vascular effects of certain of these drugs has been undertaken. It is concluded that the

pressure lowering effect of these drugs cannot be an outcome of their vascular effects, but rather probably results from a direct effect on the ciliary epithelial cells.

The bovine perfused eye may be the first isolated organ to be subjected to analysis of drug induced changes in regional blood flow using a labelled microsphere technique. The effects of these drugs on the uveal vasculature are varied and complex. Timolol at maximal ocular hypotensive dose was found to significantly reduce perfusion in the choroid, whereas at supramaximal dose it was found to significantly reduce perfusion in the iris. By contrast, a maximal ocular hypotensive dose of carteolol significantly reduced perfusion in the iris, ciliary body and choroid. Doses of SNP or verapamil which are known to reduce IOP in this preparation were found to significantly increase perfusion in the iris, ciliary body and choroid.

Doses of AP or of sodium azide which are capable of producing submaximal decreases in IOP in the bovine perfused eye have been shown to produce a significant increase in ciliary cyclic GMP. It is concluded that ciliary cyclic GMP or Ca^{2+} may be involved in the control of IOP.

INTRODUCTION

The Anatomy of The Eye

The Anterior Eye and Retina

The globe of the eyeball consists essentially of three layers - the outermost coat, or cornea and sclera, the middle layer, consisting of the choroid, ciliary body and iris, and the innermost layer, the retina (Fig.1). The choroid is the vascular layer, through which arteries run to the anterior eye and in which is located a network of small vessels which supply part of the requirements of the retina. The choroid is separated from the retina by two membranes, termed the membrane of Bruch, and the pigment epithelium. Arteries of the choroid (Fig.2) continue into the ciliary body, and further extensions of these vessels project into the iris. The ciliary body (Fig.3) consists of a smooth muscle ring (comprising both circular and tangential fibres), and functions to enable the lens to focus incident light rays onto the retina. It achieves this by stretching the lens, causing it to become flatter and decreasing its refractive properties, or by releasing tension upon it and allowing it to return naturally to its more convex form, by way of its inherent elasticity. The iris functions as a diaphragm, controlling the amount of light which enters the eye and it consists of circular and radial smooth muscle fibres. The retina comprises all of the nervous tissue responsible for the reception of light and its transmission

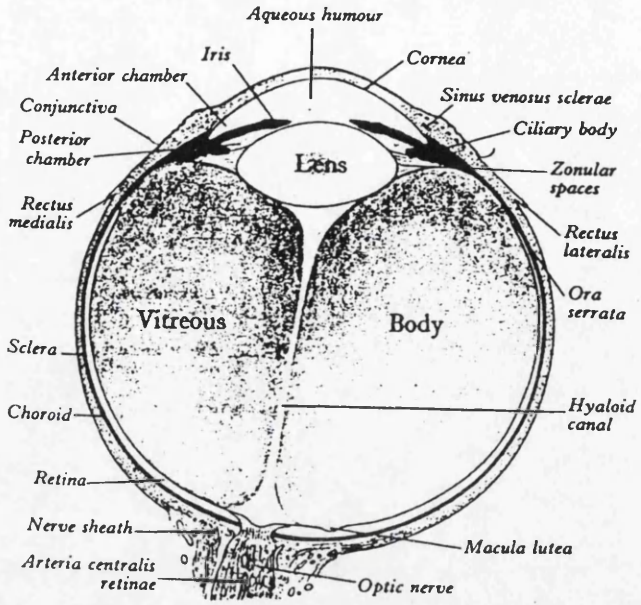


Fig.1 Horizontal section of the eye. Superior Aspect. (Gray's Anatomy).

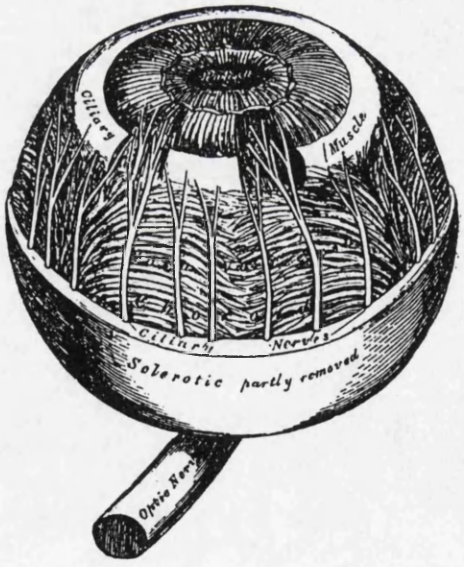


Fig.2 The choroid and iris. (Gray's Anatomy, Masterclass edition).

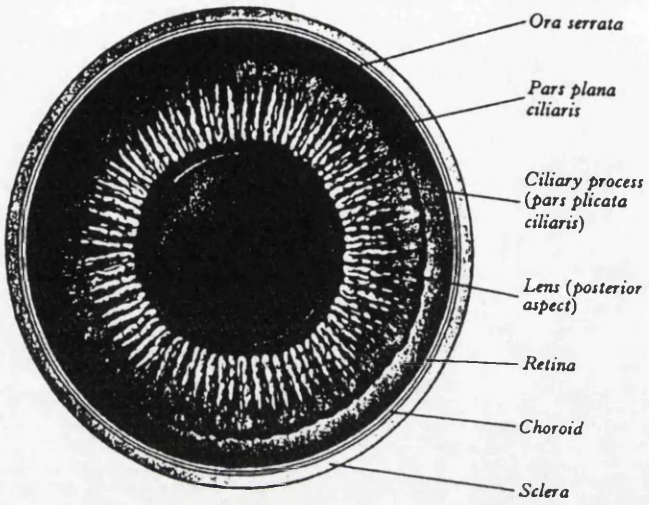


Fig.3 The interior aspect of the anterior half of the eyeball, showing the lens, ciliary body, corona ciliaris and ora serrata. (Gray's Anatomy).

to the brain by way of the optic nerve; this nervous tissue being an extension of the central nervous system (CNS). The retina has its own blood supply, arising as a separate arterial branch from the ophthalmic artery. The retina terminates at the ciliary body, forming the ora serrata, however a forward extension of the retina forms a double-layered epithelium which covers the ciliary body, termed the ciliary epithelium. Internally, the eye has two spaces (Fig.1), the larger forming the space between the lens and retina, termed the vitreous, and the smaller, between the internal surface of the cornea and lens, the aqueous chamber. The aqueous chamber is itself divided into the posterior chamber (between the posterior surface of the iris and the anterior surface of the lens), and the anterior chamber (between the anterior surface of the iris and the internal surface of the cornea). The vitreous is filled with a large mass of hydrated connective tissue, the vitreous body or humour, which is bounded by the hyaloid membrane. Forward extensions of the hyaloid membrane form the suspensory ligaments supporting the lens, known as the zonule. The outer edge of the zonule attaches to seventy processes extending from the ciliary body; the ciliary processes. Viewed posteriorly, these processes appear as radial ridges to which the name corona ciliaris has been given (Fig.3). The posterior and anterior chambers are filled with the aqueous humour, secreted from the region of the ciliary epithelium covering the ciliary

processes, on that part of the ciliary body known as the pars plicata.

The blood vessels of the choroid supply many of the internal structures of the eye. The choroid, ciliary body and iris are supplied by the ciliary system of arteries, comprising the medial and lateral long posterior ciliary arteries, the short ciliary arteries and the anterior ciliary arteries (Figs.4 & 6). These ciliary blood vessels are often referred to as the uveal vessels. They arise from the main arterial supply to the eye, the ophthalmic artery, which is itself derived from a branch of the internal carotid artery, in the human. Venous blood from the uvea (Figs.4 & 7) drains into the episcleral veins, fine veins running through the sclera, and from there into the four vortex veins, finally leaving the eye by way of the superior and inferior ophthalmic veins. The nervous components of the retina are supplied from the central retinal artery, a branch of the ophthalmic artery arising proximal to the ciliary arteries (Figs.4 & 5). The venous blood from the retina drains into the retinal veins and hence into the ophthalmic veins.

The cornea and the lens are nourished by a process of diffusion from the vitreous and aqueous humours of dissolved oxygen and nutrients, derived from the various capillaries. Additionally, the central cornea absorbs oxygen directly from the atmosphere by a process of diffusion.

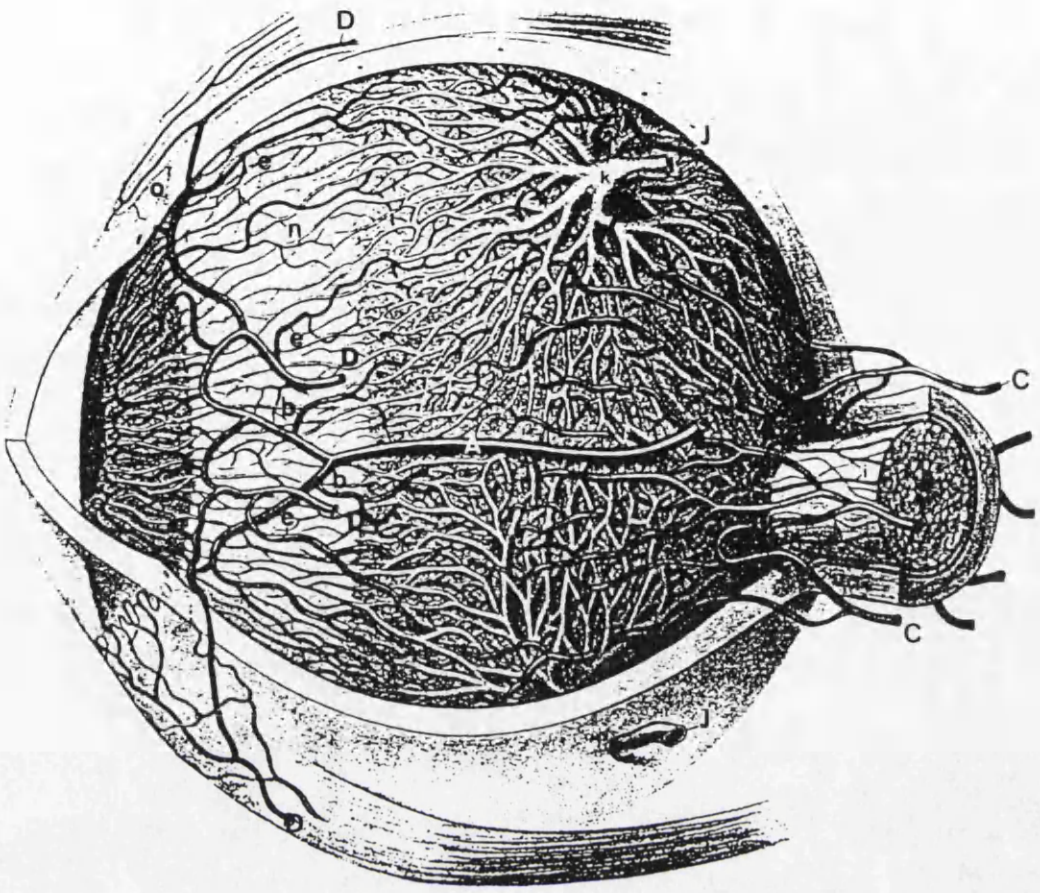


Fig.4 The vascular arrangements of the uveal tract. The long posterior ciliary arteries, one of which is visible (A), branch at the ora serrata (bb) and supply the capillaries of the anterior part of the choroid. Short posterior ciliary arteries (CC) divide rapidly to form the posterior part of the choriocapillaris. Anterior ciliary arteries (DD) send recurrent branches to the choriocapillaris (ee) and anterior rami to the major arterial circle (ff). Branches from the circle extend into the iris (g) and to the limbus. Branches of the short posterior ciliary arteries (CC) form an anastomotic circle (h)(of Zinn) around the optic disk, and branches from this (i) join an arterial network on the optic nerve. The vortex veins (J) are formed by the junctions (k) of the suprachoroidal tributaries. Smaller tributaries are also shown (m,n). The veins draining the canal of Schlemm (o) join anterior ciliary veins and vortex tributaries. (From Gray's Anatomy, 35th Edition).

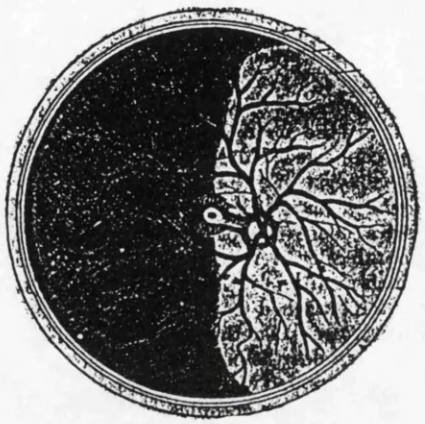


Fig.5 Anterior view of the retina, showing the central retinal artery. Anterior section of globe removed. (Gray's Anatomy, Masterclass edition).

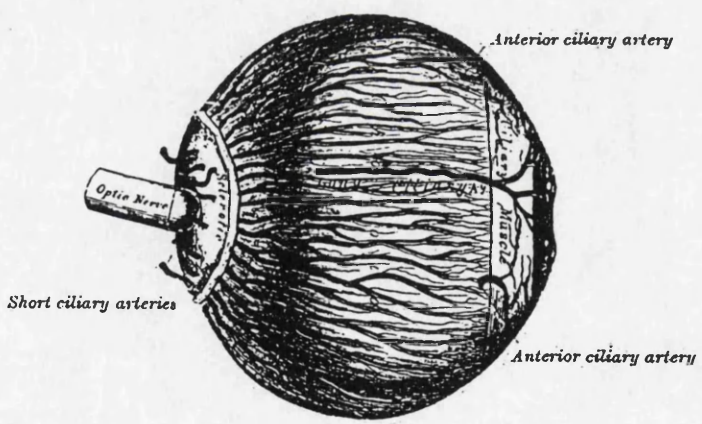


Fig.6 The arteries of the choroid and iris. Sclera has been mostly removed. (Gray's Anatomy, Masterclass edition).

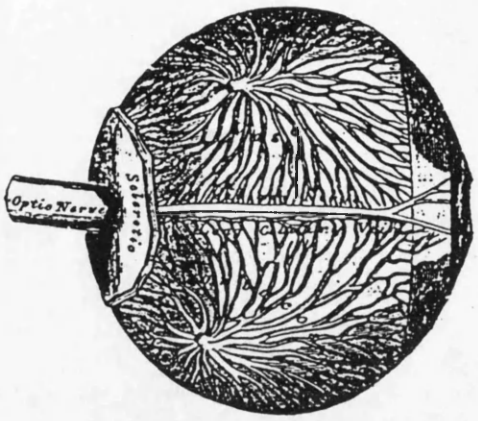


Fig.7 The veins of the choroid. Sclera has been mostly removed. (Gray's Anatomy, Masterclass edition).

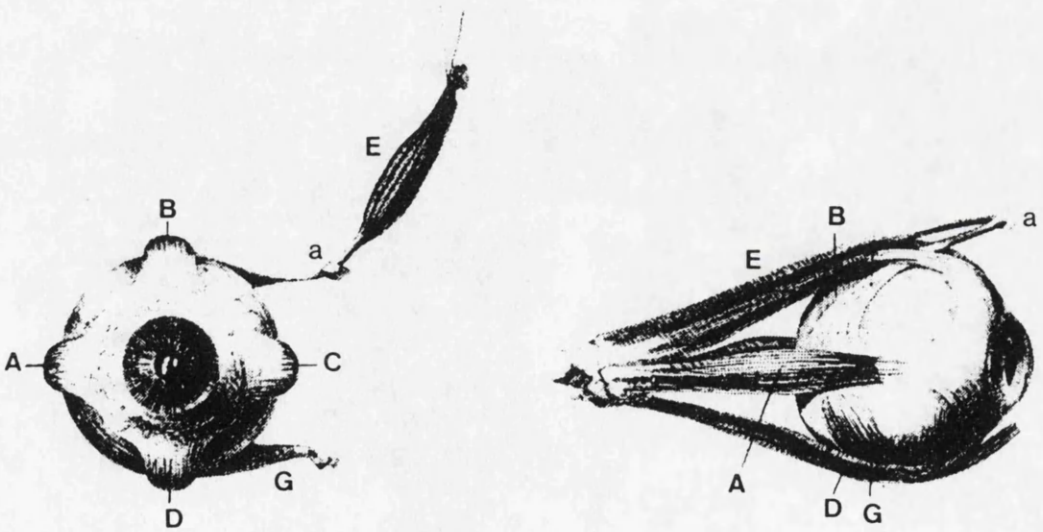


Fig.8 The extraocular muscles of the human right eye.
A; lateral rectus, B; superior rectus,
C; medial rectus, D; inferior rectus,
E; superior oblique, acting via the trochlea, a;
G; inferior oblique. (Bell, 1823).

The anterior portion of the sclera and the cornea are covered by epithelia. The conjunctival epithelium continues forward on to the inner surface of the eyelids, thus creating the conjunctival sac. The remainder of the sclera is enveloped by Tenon's capsule.

Extra-Ocular Muscle

The six extra-ocular muscles (Fig.8), comprising two pairs of rectus muscles: superior and inferior, and medial (internal) and lateral (external), respectively; and the superior and inferior oblique muscles, control movement of the eye within the orbit, which is essentially rotation about a fixed point in space. The six muscles form a cone, with the eyeball itself located at the base of the cone, the apex of the cone located posteriorly in the orbital cavity. The space between the eyeball and the orbit not filled with muscle is filled with orbital fat, and the lacrimal gland, responsible in part for the production of tear fluid.

Nerve Supply

The eye has a rich supply of autonomic nerves, but these nerves are distributed only within the uvea and the extraocular parts of the retinal blood vessels (Bill & Sperber, 1990). Sympathetic nerves originate in the superior cervical ganglion, and parasympathetic nerves in the pterygopalatine and ciliary ganglia. The precise role of the nerves is not very well understood.

With the exception of the visual apparatus, sensory impulses are conveyed through the long and short ciliary nerves (all part of the autonomic nervous system - Fig.9). The long ciliary nerves are composed mainly of axons of nerve cells in the Gasserian ganglion - the ganglion of the trigeminal nerve. These nerves convey impulses from the iris, ciliary body and cornea. The short ciliary nerves also contain axons of the trigeminal; they pass through the ciliary ganglion into the naso-ciliary nerve. These fibres carry impulses from all parts of the eyeball, but principally from the cornea. Preganglionic parasympathetic motor fibres to the ciliary muscle and iris run through the lower division of the oculomotor as the motor root of the ciliary ganglion; the postganglionic fibres supplying the muscles are contained in the short ciliary nerves.

Sympathetic fibres from the superior cervical ganglion enter the orbit as the sympathetic root of the ciliary ganglion and run into the short ciliary nerves to supply the

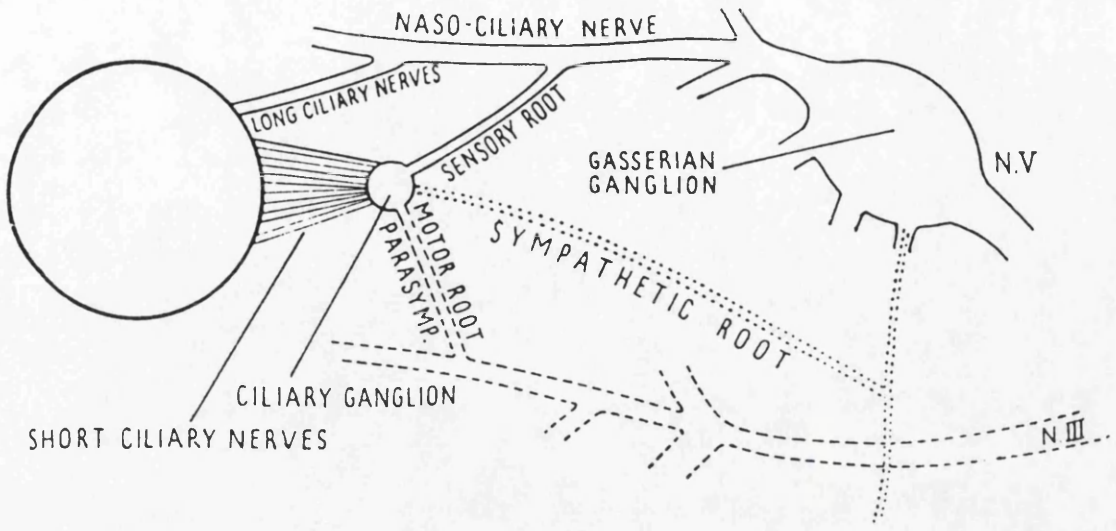


Fig.9 Nervous supply to the eyeball. (Davson, Physiology of the Eye).

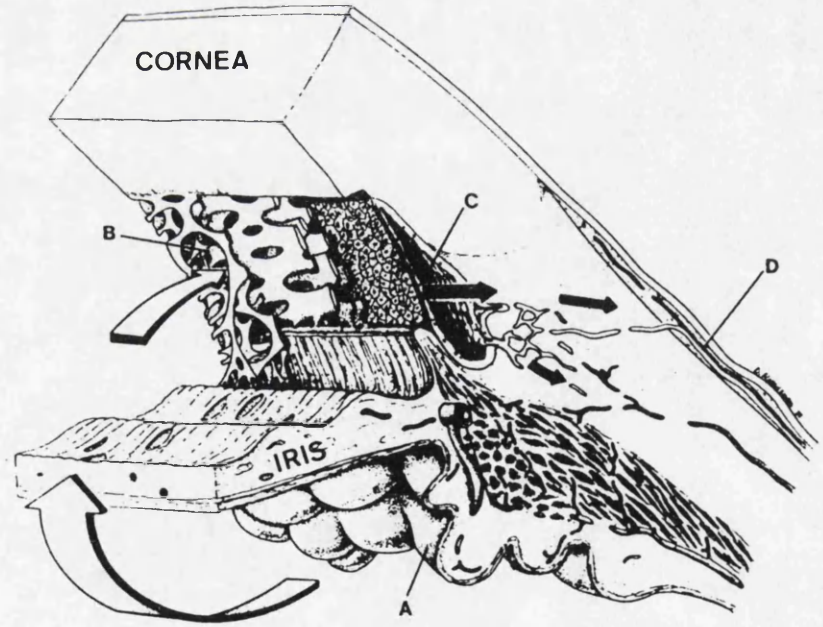


Fig.10 Cross-section through the anterior segment of the eye illustrating the chamber angle. Under steady-state conditions intraocular pressure is maintained by a balance between the formation of aqueous humour by active secretion by the ciliary processes (A), and its drainage by outflow pathways, in particular the trabecular meshwork (B) and Schlemm's canal (C), into aqueous veins (D). (Karnezis & Murphy, 1988)

vessels of the globe and the dilator pupillae fibres of the iris. Other sympathetic fibres avoid the ciliary ganglion, passing through the Gasserian ganglion and entering the globe in the long ciliary nerves, whilst still others enter the globe in the tunica adventitia of the ciliary arteries.

The Aqueous Humour and Intraocular Pressure

The aqueous humour is a transparent colourless fluid, formed continuously in the posterior chamber by the ciliary processes (Fig.10). It passes through the pupil into the anterior chamber, from where it is drained into the venous system in the angle of the anterior chamber by way of Schlemm's canal, in the primate eye. Nonprimate eyes possess a structure equivalent to Schlemm's canal (Fig.11), known as the angular aqueous plexus (Tripathi, 1974). As in primates, the plexus forms a circumferential structure in lower mammals, birds and many reptiles, but is more localized in amphibians and fishes. Schlemm's canal is a circular canal in the corneo-sclera of the limbus (Figs.12 & 13), which comes into relation with the aqueous humour on the one hand and the intrascleral venous plexus on the other. It is essentially an endothelium-lined vessel. On the outside it rests on the scleral tissue whilst on the inside, nearest the anterior chamber it is covered by a meshwork of endothelium covered trabeculae: the trabecular

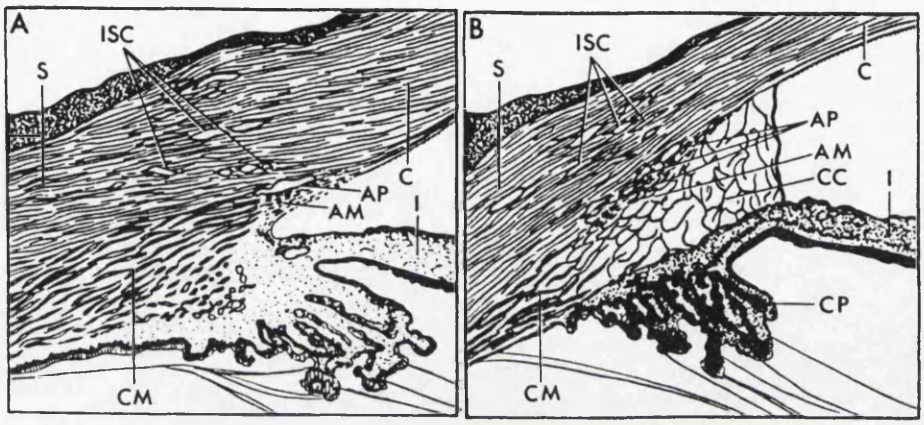


Fig.11 Diagrammatic representation of the angular region of a primate (A) and a lower placental mammal (B) showing comparative morphological organizations. S: sclera; C: cornea; I: iris; AM: angular meshwork; AP: angular aqueous plexus; CM: ciliary muscle; CC: ciliary cleft; CP: ciliary processes; ISC: intrascleral collector channels. (Tripathi, The Eye).

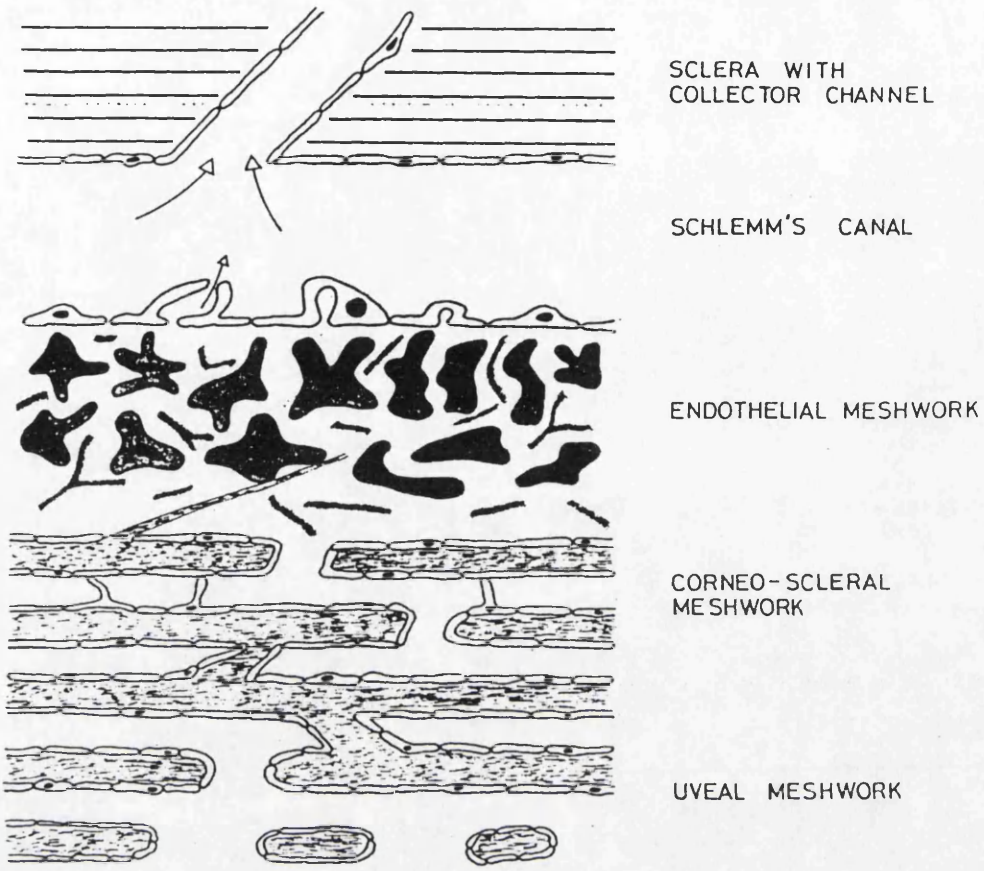


Fig.12. Schematic representation of a section through chamber-angle tissue. (Bill, Physiol. Rev.)

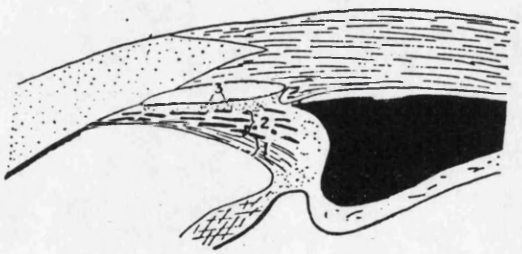


Fig.13. Schematic drawing of the chamber-angle in primates, showing the three distinct parts of the trabecular meshwork. 1. Uveal Meshwork. 2. Corneo-Scleral Meshwork. 3. Endothelial Meshwork. (Rohen, Structure of the Eye).

meshwork or pectinate ligament. The meshwork has been divided histologically into three parts with characteristically different ultrastructures; the innermost portion is the uveal meshwork, the middle portion is the corneoscleral meshwork, and the third portion, lying immediately adjacent to the canal is referred to as the endothelial meshwork.

Although most of the outflow of aqueous is via the trabecular meshwork and Schlemm's canal, or the angular aqueous plexus in the nonprimate eye, a small proportion (approximately 20%) leaves the anterior chamber via the uveoscleral pathway; here the aqueous flows through the ciliary body into the suprachoroidal space, to be drained by the venous circulation in the ciliary body, choroid and sclera.

As a circulating fluid, the aqueous humour is a medium whereby the lens and cornea receive their nutrient materials. The ciliary epithelium, as previously related consists of a double layer of cells. The layer immediately adjacent to the aqueous is non-pigmented, whilst the outer layer is heavily pigmented and represents the forward continuation of the retinal pigment epithelium. The non-pigmented layer represents the forward continuation of the neuroepithelium from which the retinal cells are derived. The ciliary epithelial cells have been subjected to intensive study histologically, and with the electron microscope. A striking feature of the cells is the

interdigitation of the lateral surfaces of adjacent cells, and the basal infoldings (Fig.14), which is a characteristic feature of secretory epithelia concerned with fluid transport (Bill, 1975). The relation of the two epithelial cell layers is of importance, since as the secreted aqueous humour is derived from the ultrafiltrate of blood in the stroma of the ciliary body. transport must occur across both layers. The cells of the two layers face each other apex-to-apex, as a result of the invagination of the neuroepithelial layer during embryogenesis. Thus the basal aspects of the non-pigmented cells face the aqueous humour (Fig.15).

IOP is largely determined by the rate of formation of aqueous humour. the rate of outflow (by at least two routes), and episcleral venous pressure.

The Blood-Aqueous Barrier

A restraint in the free passage of many solutes from the blood vessels of the ciliary stroma into the aqueous humour exists. This has come to be known as the blood-aqueous barrier, the anatomical correlate of which are the tight junctions in the non-pigmented layer. The i.v. injection of horseradish peroxidase into the monkey has been shown to lead to a rapid filling of the ciliary stroma by the enzyme (Vegge, 1971). The enzyme passes through the

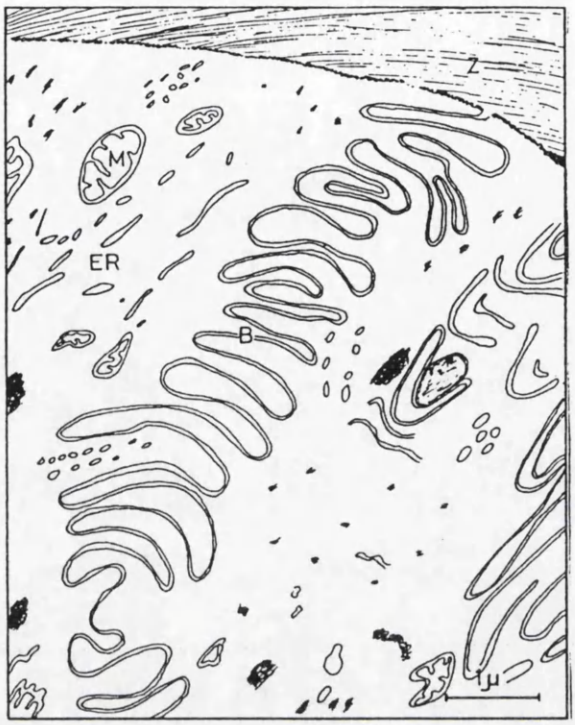


Fig.14 A section of portions of two adjacent ciliary epithelial cells of the rabbit showing complex interdigitations of their boundaries (B). Zonular fibres (Z) can be seen in close approximation to the surface of these cells. Endoplasmic reticulum (ER) and mitochondria (M) also visible. (After an electron micrograph by Pappas & Smelser).

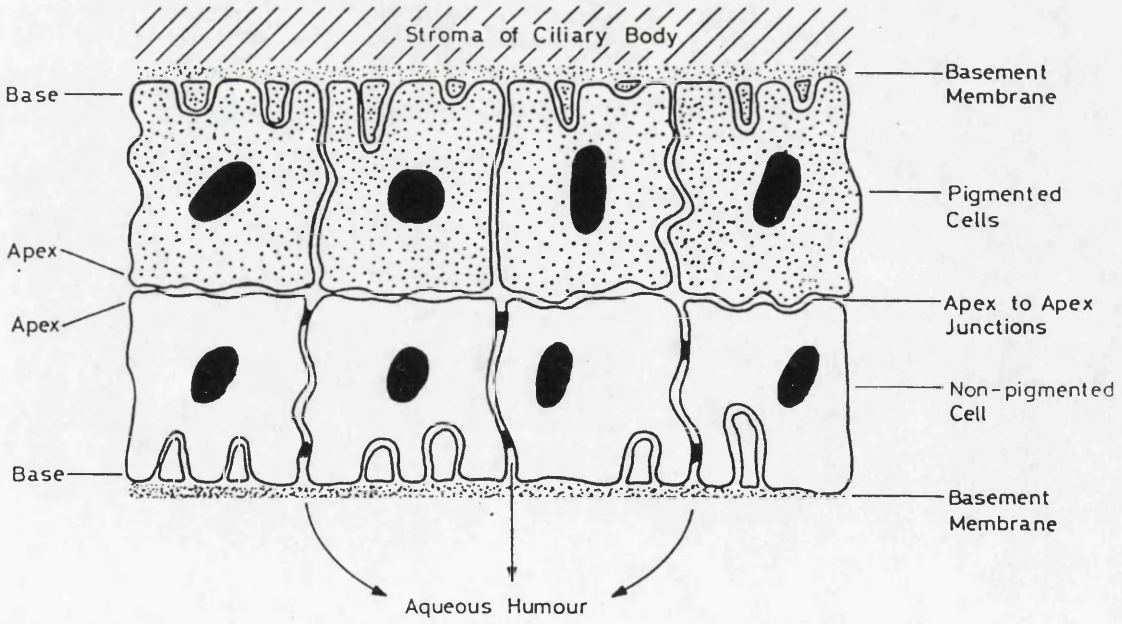


Fig.15 The apex-to-apex relation of the two layers of cells of the ciliary epithelium. (Davson, Physiology of the Eye).

many fenestrations present in the blood capillaries of the ciliary body, and also has been found to fill the spaces between the pigmented cells. However, it is prevented from entering the aqueous by the tight junctions of the non-pigmented layer, between the cell apices. Comparable results have been reported in the mouse (Shiose, 1970) and in the rabbit (Uusitalo et al., 1970).

A similar barrier to the passage of solutes exists in the retinal pigment epithelium, thus forming a blood-vitreous barrier between the vitreous and the blood capillaries of the choroid. Blood capillaries with tight junctions in their endothelia form further physiological barriers (functionally similar to the blood-brain barrier) in the iris and retina.

Secretion and Composition of Aqueous Humour

Aqueous humour is derived from an ultrafiltrate of plasma in the ciliary stroma, and as such has a chemical composition quite similar to that of plasma. However it differs from that of plasma in that it is richer with respect to chloride, bicarbonate, lactate, pyruvate, ascorbate and total amino acids. The concentration of sodium, potassium, calcium and magnesium ions, urea and glucose are all lower than in plasma. There are also small quantities of all of the plasma proteins present in the

aqueous, albeit at concentrations less than 1% those of plasma. The table below shows the comparitive chemical composition of aqueous humour and plasma in the rabbit.

Component	Aqueous Humour	Plasma
Na^+	143.5	151.5
K^+	5.25	5.5
Ca^{2+}	1.7	2.6
Mg^{2+}	0.78	1.0
Cl^-	109.5	108.0
HCO_3^-	33.6	27.4
Lactate	7.4	4.3
Pyruvate	0.66	0.22
Ascorbate	0.96	0.02
Urea	7.0	9.1
Reducing Value (as glucose)	6.9	8.3
Total Amino Acids	0.17	0.12

Table 1. Chemical composition of aqueous humour and blood plasma of the rabbit. Concentrations expressed in mmol/kg of water. (After Davson, 1969).

Because the concentration of electrolytes in the secreted aqueous is not equal to that of a simple ultrafiltrate of plasma, aqueous humour must be actively secreted by the ciliary epithelium into the posterior chamber. A consideration of Gibbs-Donnan equilibrium demonstrates this. If the aqueous were simply an ultrafiltrate of plasma, then in accordance with Gibbs-Donnan equilibrium, the concentrations of positive ions such as potassium and sodium would be greater in the plasma than

in the aqueous. The opposite would be the case with the respective concentrations negative ions, such as bicarbonate and chloride. Further, the actual values of the ratios of the concentrations of each respective ion in aqueous to plasma would conform to the values obtained experimentally when plasma is dialysed against its own filtrate. As Table 2 indicates, however, this is not the case. Therefore the aqueous is actively secreted.

Component	$\frac{\text{Conc. in Aqueous}}{\text{Conc. in Plasma}}$	$\frac{\text{Conc. in Dialysate}}{\text{Conc. in Plasma}}$
Na^+	0.96	0.945
K^+	0.955	0.96
Ca^{2+}	0.58	0.65
Mg^{2+}	0.78	0.80
HCO_3^-	1.26	1.04
Cl^-	1.015	1.04
Urea	0.87	1.00

Table 2. Values of the two distribution ratios. Ratio of concentration in aqueous to concentration in plasma; ratio of concentration in dialysate to concentration in plasma. (Davson, Physiology of the Ocular and Cerebrospinal Fluids).

Such active secretion by the ciliary processes is accomplished by the transport of one or more ions from the blood side of the system to the aqueous side. The osmotic gradient caused by the transport of the ion or ions leads to a flow of water, and, depending upon the permeability

characteristics of the limiting membranes across which the transport occurs, the fluid may be ultimately isotonic, hypertonic or hypotonic. Thus the process in the eye takes place across the ciliary epithelium (Fig.16); in the ciliary stroma the blood is filtered by the capillaries to produce an ultrafiltrate comparable with tissue fluid in other parts of the body. Because of the large fenestrations in the ciliary capillary endothelia, this ultrafiltrate still bears a considerable amount of protein. The non-pigmented epithelium, however, acts as a diffusional barrier to this protein and to most of the other constituents of the ultrafiltrate, so that the transport is slowed and becomes selective. The essential hypothesis of aqueous secretion, (Cole, 1977), is that the non-pigmented cells absorb selectively sodium ions from the stroma and transport them into the intracellular clefts which are closed at the stromal side by tight junctions but are open on the aqueous humour side. The development of hyperosmolarity in the clefts leads to osmotic flow of water from the stroma and thus to a continuous flow of water along the clefts. The passage of other ions may also be governed by independent active processes, for example, chloride, potassium and bicarbonate ions. Others may diffuse passively down concentration gradients established by the primary process. The inhibition of active transport of sodium by the cardiac glycosides is related to the inhibition of Na^{+}-K^{+}-Mg^{2+}-ATPase present in the membranes of the non-pigmented cells.

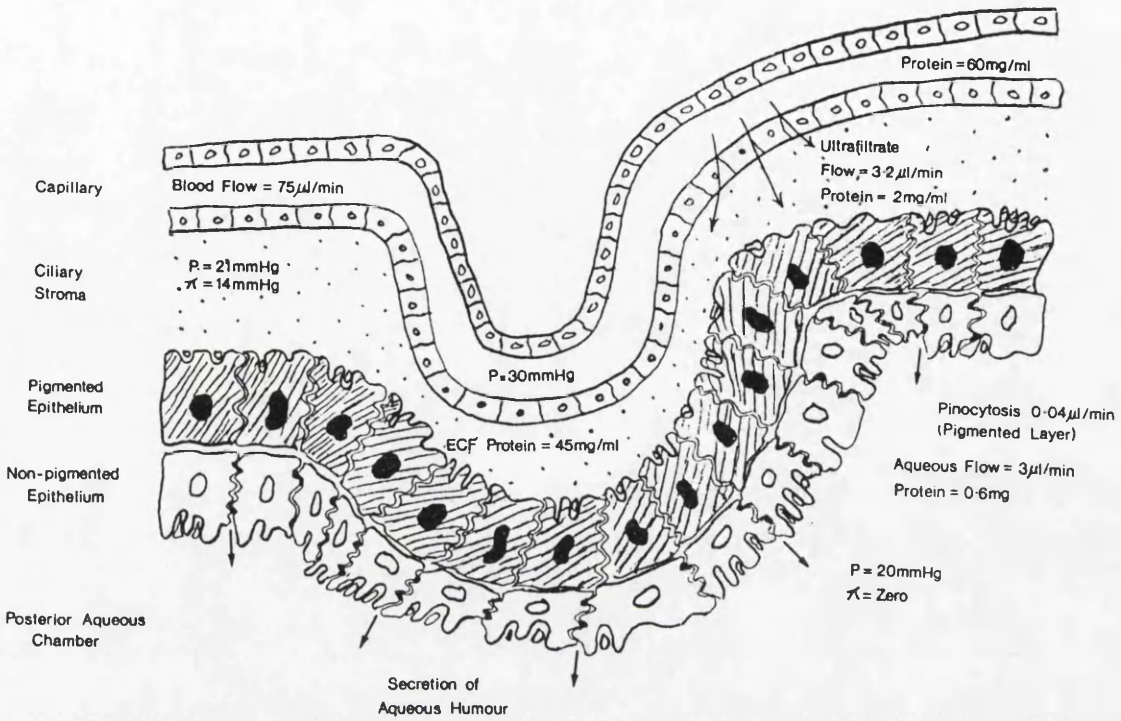


Fig.16. Secretion of aqueous humour by the ciliary processes. (After Bill).

Cole (1977) reported that stropanthin G reduced influx of sodium into the anterior chamber by more than 50%. Bonting and Becker (1964) reported that when ouabain was injected into the vitreous humour, there was a fall in IOP, due to a reduction in the rate of secretion of aqueous. The effects of ouabain are strongly suggestive of the existence of a primary active transport of sodium ion across the ciliary processes. A probable linkage of this with an active transport of bicarbonate is suggested by the prominent action of acetazolamide, which reportedly lowers IOP and reduces the rate of secretion of aqueous by approximately 50% (Garg & Oppelt, 1970). The drug appears to affect an ion-secreting mechanism. As acetazolamide inhibits the enzyme carbonic anhydrase, it is probable that its effect upon aqueous secretion is mediated by the inhibition of synthesis of bicarbonate by the secretory cells.

Passive filtration may contribute to aqueous secretion also. Bill and Bárány (1966) reported that an artificially-induced rise in IOP caused a reduction in secretion. This has been confirmed in the monkey (Bill, 1968). An intracameral injection of erythrocytes yielded a partial blockade of the trabecular meshwork, and hence an elevation in IOP. This led to a suppression of aqueous secretion, corresponding to 0.06μl/min/mmHg rise in pressure. Therefore with a normal secretion rate of 1.24μl/min, a rise in IOP of approximately 20mmHg should theoretically suppress aqueous secretion altogether. This does not in fact occur,

however. It is well established that aqueous secretion will continue even against very high pressures. But the observation does indicate that when IOP reaches a critical level, the influx by secretion is just balanced by an outflow through the drainage channels.

Glaucoma

The clinical condition of glaucoma is a common disease which afflicts people mainly from middle age onwards. Approximately two million people in the United States suffer from some form of the disease, the numbers in other Western countries including the United Kingdom being proportionally similar. The boundaries between glaucoma, low tension glaucoma and ocular hypertension are indistinct, however glaucoma is often associated with an IOP of greater than 21mmHg. The disease consists of pathological changes in the optic disk and axons of the optic nerve, and a chronic loss of visual field over a period of years, in most cases. Low tension glaucoma is defined as the condition whereby the pathological changes associated with glaucoma and loss of visual field are present, but with a normal IOP. Ocular hypertension is defined as the condition whereby IOP is greater than 21mmHg, but there are no pathological changes present associated with glaucoma, although this condition often heralds the development of glaucoma. Jay (1992)

suggests that an eye found to be in such a condition should be treated as though glaucoma were present. The disease may be caused by a number of different factors, such as the bowing of the iris over the drainage angle (Narrow Angle Glaucoma), thus partially occluding the passage of aqueous through the trabecular meshwork into the canal of Schlemm. This leads to an elevated IOP which stretches the scleral layer, and further leads to cupping of the optic disk. IOP may rise as high as diastolic pressure in the retinal vessels, thus giving rise to progressive retinal ischaemia and detachment, with concomitant increasing loss of visual field (scotoma). In such cases topical application of a muscarinic agonist such as pilocarpine, instilled in the form of eyedrops will reduce IOP via a reduction in outflow resistance, both by mediating a miosis (contraction) of the iris, and by improved flow through the trabecular meshwork, which is brought about by contraction of the ciliary muscle and consequent pull on the scleral spur, leading to an alteration in the configuration of the meshwork fibres (Havener, 1983). When IOP is only moderately elevated such drug treatment is often all that is necessary to return it to its normal value of 10 to 15mmHg above episcleral venous pressure. In more extreme cases however, when drug treatment alone fails to control the condition, surgery to form an opening in the iris through which the aqueous humour may drain (iridectomy) will be performed. This procedure may be carried out as a non-invasive technique using an N-YAG

laser; termed N-YAG laser iridotomy. Chronic open-angle glaucoma (COAG) consists of a partial blockage of the trabecular meshwork, perhaps by the build-up of cellular debris, or by glycosaminoglycan deposits, which decreases the rate of outflow of aqueous humour, also leading to an elevated IOP. This is the most common form of glaucoma and may be treated via the instillation of eye drops containing a drug (such as a β-adrenoceptor antagonist) which will reduce the rate of secretion of aqueous from the ciliary processes, thus mediating a reduction in IOP. As with Narrow Angle Glaucoma, when drug treatment fails to adequately control the condition, the disease may be treated surgically by trabeculectomy or Scheie's thermosclerostomy, or via N-YAG laser trabeculoplasty. In each case, the procedure involves the creation of a fistula through which the aqueous can flow. In trabeculectomy, the fistula so formed is 'guarded' by a superficial scleral flap, wheras in Scheie's procedure, the fistula passes through the entire thickness of the sclera.

Drugs Which Lower Intraocular Pressure

β-Adrenoceptor Antagonists

Since 1967, when Phillips et al. discovered that oral propranolol will reduce IOP in patients with ocular hypertension, many β-adrenoceptor antagonists have been evaluated clinically for topical ocular hypotensive activity, and several members are in current clinical use. The effect of this group of drugs upon aqueous humour dynamics has been well established, both clinically (Zimmerman et al., 1977; Keates & Stone, 1984; Sugrue et al., 1985) and experimentally in several species in vivo (Nathanson, 1981; Potter, 1981; Liu et al., 1983). The (-)-isomer of timolol is a potent, non-selective β-adrenoceptor antagonist (Scriabine et al., 1973), often prescribed in cases of COAG to lower IOP. The effect of the drug is known to be due to a reduction in the rate of formation of aqueous humour. Vareilles et al. (1977) first reported the ability of topically administered timolol to lower IOP; this group demonstrated that the instillation of 0.5% and 1.5% solutions of (-)-timolol significantly lowered IOP in rabbits with an experimentally-induced form of OAG, utilising α-chymotrypsin. This form of induced OAG is most likely a consequence of impaired outflow facility (Sears & Sears, 1974). The pathological changes occurring in the α-chymotrypsin pretreated rabbit eye somewhat resemble those

observed in human glaucoma, those being principally the progressive cupping of the optic disk and atrophy of the optic nerve head (Best et al., 1975). In contrast to ocular hypertensive, α-chymotrypsin pretreated rabbits, the effect of topical timolol on the IOP of normotensive rabbits is less well defined. Significant and reproducible falls in IOP have been observed in some studies (Radius et al., 1978; Bar-Ilan, 1984; Alkondon et al., 1986), but not in others (Vareilles et al., 1977; Bartels et al., 1980; Boas et al., 1981; Watkins et al., 1985; Woodward et al., 1986). The IOP in ocular normotensive dogs and cats is lowered by topical timolol in a dose-dependent manner (Svec & Strosberg, 1986; Colasanti & Trotter, 1981). Seidehamel and Dungan (1974) reported that the oral administration of a large volume of water (60 to 100 ml/kg) to conscious rabbits elicits a marked and rapid rise in IOP, which returned however to its baseline value in less than 2h. The effect of this water loading upon IOP is due to a reduction in the osmolality of the blood (van Loenen et al., 1984) and hence the establishment of an osmotic gradient between the aqueous and the plasma, leading to increased aqueous secretion. Once again, the effect of timolol on this model is contradictory, an antagonism of the water load-induced increase in IOP being observed by some workers (Vareilles et al., 1977; Liu et al., 1984), but not others (Rowland & Potter, 1980; Palkama et al., 1985).

In contrast to results obtained in ocular normotensive rabbits, the instillation of timolol to normal human volunteers invariably lowers IOP (Katz et al., 1976; Coakes & Brubaker, 1978; Yablonski et al., 1978). Topical timolol is also very effective in lowering IOP in patients with elevated IOP, such as in COAG (Radius et al., 1978; Zimmerman & Boger, 1979). Fluorophotometric studies in human volunteers indicate that the ocular hypotensive effect of timolol is mediated by its ability to reduce the rate of secretion of aqueous (Coakes & Brubaker, 1978; Yablonski et al., 1978; Schenker et al., 1981; Dailey et al., 1982). In general, topically applied timolol has been observed to have little or no effect on the facility of outflow in humans (Zimmerman et al., 1977; Sonntag et al., 1978), although a small increase in outflow facility has been reported in some patients in response to topical timolol (Thomas & Epstein, 1981). The ability of timolol to reduce aqueous secretion has also been observed in the rabbit (Vareilles & Lotti, 1981), the cat (Liu & Chiou, 1981) and the monkey (Miichi & Nagataki, 1983). The site of action of topically applied timolol is believed to be local in humans because the reduction in IOP is greater in the treated than in the untreated contralateral eye, following unilateral administration of the drug (Katz et al., 1976; Zimmerman & Kaufman, 1977). Why timolol should lower IOP in the contralateral eye is unknown, however it has been proposed that the effect may be due to sufficient timolol entering

the systemic circulation and thus locally affecting the ciliary processes of the contralateral eye. The drug may also act at a site either within or via the central nervous system (Neufeld et al., 1983). It has further been suggested that timolol may act to reduce aqueous secretion by reducing the levels of melatonin in the iris and ciliary body (Rhode et al., 1985). Indeed, orally administered melatonin reportedly lowers the IOP of healthy volunteers (Samples et al., 1988). However, levels of melatonin in the iris and ciliary body of rabbits and chicks are unaltered by topical timolol (Rhode & Chiou, 1987).

The presence of β-adrenergic receptors in ciliary tissue has been demonstrated from results of radioligand binding and adenylate cyclase activation studies (Neufeld & Page, 1977; Page & Neufeld, 1978; Dafna et al., 1979; Nathanson, 1980; Bhargava et al., 1980; Bromberg et al., 1980; Cepelik & Cernohorsky, 1981). Many of these authors have inferred the involvement of β-adrenergic receptors in aqueous humour formation. It appears that these ocular receptors are primarily of the β_2 subtype (Potter & Rowland, 1978; Nathanson, 1980, 1981; Cepelik & Cernohorsky, 1981; Colasanti & Trotter, 1981; Schmitt et al., 1984). In the rabbit eye β-adrenoceptors are present mainly in the ciliary epithelium, but also in the walls of the episcleral vessels (Dafna et al., 1979).

Controversy surrounds the nature of the mechanism of action of timolol as an ocular hypotensive agent. It is

tempting to assume that blockade of β-adrenoceptors in the ciliary processes is the basis of action of the drug. However, various sources of evidence suggest otherwise (Lotti et al., 1984). To date, considerable bodies of evidence have been amassed, both to support the hypothesis that β-adrenoceptor antagonists lower IOP via antagonism of a classical β-adrenoceptor, and also to support the hypothesis that β-adrenoceptor antagonists do not lower IOP via antagonism of a classical β-adrenoceptor. Each of these hypotheses are considered in the following sections.

Experimental Evidence that β-Adrenoceptor Antagonists Lower IOP via Antagonism of a Classical β-Adrenoceptor

Numerous studies on the secretion of aqueous humour suggest that the process is modulated by ciliary β_2 adrenoceptors (reviewed by Potter, 1981: Potter & Rowland, 1981). The ocular structures associated with aqueous humour dynamics are rich in β_2 adrenoceptors, and most β-adrenoceptor antagonists are known to lower IOP in vivo, including a number of structurally unrelated agents having β-adrenergic blocking properties. The β-adrenoceptor agonist terbutaline is reported to increase aqueous formation in primates (Nilsson et al., 1990). Timolol is a potent β_1 and β_2 adrenoceptor antagonist, which is not, however noted for any other pharmacologic activities shared

by a number of β-adrenergic antagonists, such as intrinsic sympathomimetic activity, membrane stabilizing activity or local anaesthetic activity. However timolol is one of the most effective and widely studied agents known to affect aqueous secretion and IOP. Wax and Molinoff (1987) demonstrated that timolol effectively displaces radioligand binding to β_2-adrenergic receptor binding sites in membrane fractions of both iris and ciliary body, obtained from the human eye. A similar result was obtained in the rabbit (Neufeld & Page, 1977; Schmitt et al., 1984), and also with ciliary processes obtained from rabbit (Bromberg et al., 1980), sheep (Trope & Clark, 1982) and bovine (Elena et al., 1984) eyes. Comparable activity was observed for inhibition of adenylate cyclase in ciliary processes obtained from rabbits (Nathanson, 1980, 1985) and humans (Nathanson, 1981), and in non-pigmented epithelial cells of the bovine ciliary processes (Elena et al., 1984).

For a compound to be active as a pharmacological antagonist, there must be tonic agonist-induced stimulation to block. Thus, the ability of timolol to lower IOP implies that an endogenous adrenergic tone stimulating β-adrenoceptors exists in the eye. Aqueous humour formation in human volunteers is decreased by approximately 45% during sleep (Reiss et al., 1984), and in contrast to daytime, 0.5% timolol is devoid of any ocular hypotensive effect (Topper & Brubaker, 1985). These observations suggest that endogenous adrenergic activity during the day stimulates aqueous humour

formation and that this stimulus subsides during sleep. Supportive evidence for this comes from experiments in which the topical application of isoprenaline failed to alter aqueous humour formation in volunteers during the day, but significantly increased it during sleep (Larson & Brubaker, 1988). In contrast to this, however, experiments in rabbits have indicated that prolonged sympathetic nerve stimulation decreases aqueous humour formation (Belmonte et al., 1987).

Nathanson (1981) showed that isoprenaline stimulates cyclic AMP synthesis and increases aqueous formation in the monkey eye, while timolol blocks these effects. This lends support to the classical hypothesis that control of aqueous secretion is via a classical β-adrenoceptor. Linner and Prijot (1955) noted that cervical sympathetic ganglionectomy resulted in reduced aqueous flow.

Experimental Evidence that β-Adrenoceptor Antagonists do not Lower IOP via Antagonism of a Classical β-Adrenoceptor

Timolol and other β-adrenoceptor antagonists inhibit the production of intracellular cyclic AMP. However, agents such as cholera toxin (Gregory et al., 1981) and forskolin (Caprioli & Sears, 1983; Smith et al., 1984; Bartels et al., 1987; Shibata et al., 1988) stimulate the production of intracellular cyclic AMP, yet they also lower IOP by reducing the rate of formation of aqueous. Aqueous outflow

facility remains unaltered (Caprioli et al., 1984; Leeet al., 1984). Topically applied timolol can block β-adrenoceptors in the eye as evidenced by the attenuation of the ability of topical isoprenaline to blunt both the increase in the IOP of rabbits following water loading, and the enhancement of levels of cyclic AMP in rabbit aqueous humour by this β-adrenergic agonist (Vareilles et al., 1977: Schmitt et al., 1981b). The doses of timolol required to block both responses to isoprenaline are much less than those required for ocular hypotensive activity, thus implying that no relationship exists between the two phenomena. Additionally, the effectiveness of various β-adrenoceptor antagonists in lowering IOP in rabbit models of experimental ocular hypertension reportedly does not correlate with their ability to block β-adrenoceptors (Bonomi et al., 1979). However, in this study the criterion used for β-adrenoceptor antagonism was isoprenaline-induced tachycardia. This is a β_1 mediated response and experimental evidence indicates that mainly β_2 receptors exist in the ciliary processes (Cavero et al., 1983). A lack of correlation between β_2 adrenoceptor antagonism and ocular hypotensive activity is emphasised by comparing timolol with the β-adrenoceptor antagonist betaxolol. The drug exhibits an *in vitro* pA_2 value for guinea-pig cardiac β_1-adrenoceptors of 8.5 to 8.8, but an *in vitro* pA_2 value for guinea-pig tracheal β_2-adrenoceptors of only 5.4 to 7.0 (Schmitt et al., 1984: Manoury et al., 1987). Thus

betaxolol is a highly β_1 selective antagonist, exhibiting approx. a 850:1 ratio of binding of β_1 to β_2 adrenoceptors, at extraocular sites. The ocular response in humans to both drugs however is not markedly different (Levy et al., 1985; Stewart et al., 1986). More recent studies, however suggest that the ocular hypotensive effect of betaxolol is less than that of timolol (Allen et al., 1986; Allen & Epstein, 1986), but both drugs are in common clinical use as potent ocular hypotensive agents. The elevated intraocular pressure of glaucoma patients is lowered by the twice-daily instillation of 0.125% (Radius, 1983) and 0.25% (Levy & Boone, 1983; Caldwell et al., 1984) betaxolol.

The β-adrenoceptor antagonist sotalol reportedly inhibits the production of intracellular cyclic AMP, as do timolol and the other β-adrenoceptor antagonists. Sotalol, however, when applied topically at 2% concentration to patients with COAG has no effect upon IOP (Merté & Stryz, 1983). This suggests that the observed effect of timolol upon IOP may be independent of levels of ciliary cyclic AMP.

The fact that topically applied (+)-timolol can lower IOP of both rabbits (Share et al., 1984) and humans (Keates & Stone, 1984; Mills et al., 1988) has also been used to support the contention that the ocular hypotensive effect of timolol is not mediated via a β-adrenoceptor (Liu & Chiou, 1981; Chiou et al., 1985). Both timolol and its (+)-enantiomer were found to be equally active in reducing aqueous humour production when perfused intracamerally in

the anaesthetised cat. However, only one concentration (0.005%) of each agent was employed (Liu & Chiou, 1981). Both enantiomers were also found to be equipotent after topical administration in a model which indirectly measures aqueous humour secretion in conscious rabbits (Chiou, 1983). The ability of topically applied (+)-timolol to lower the elevated IOP in α-chymotrypsin- pretreated rabbits is approximately one-third that of timolol (Share et al., 1984). Additionally, the instillation of solutions of (+)-timolol ranging from 0.25% to 2% has been found to lower IOP in glaucoma patients, however all of the concentrations of (+)-timolol were less effective than 0.25% (-)-timolol (Mills et al., 1988). (+)-timolol at 1% concentration has some extraocular β-adrenoceptor blocking acivity in human volunteers, although it is reportedly 13 times less potent than (-)-timolol at human bronchial β_2-adrenoceptors, and additionally is 49 times less potent than (-)-timolol at extraocular β_2-adrenoceptors in animal studies (Richards & Tattersfield, 1985; 1987). This observation is further supported by the finding that (+)-timolol has only 3% of the potency of (-)-timolol in blocking the isoprenaline-induced synthesis of cyclic AMP in ICB preparations (Liu et al., 1981; 1983). (+)-timolol is about one-third as potent as timolol in displacing ^{3}H-dihydroalprenolol binding to iris-ciliary body tissue, reducing aqueous formation and lowering IOP of α-chymotrypsin hypertensive eyes (Share et al., 1984). The (+)-enantiomer is fifty to ninety times less

potent than the (-)-enantiomer in antagonising the effects of isoprenaline on pulmonary and atrial β-adrenergic receptors (Share et al., 1984), and may be effective in lowering IOP at concentrations which may reduce the systemic side effects associated with peripheral β-adrenergic blockade. The inhibitory effect of both enantiomers of timolol upon isoprenaline-induced stimulation of adenylate cyclase in the rabbit ciliary processes is comparable (Nathanson, 1988). In water-loaded pigmented rabbits both enantiomers blunt the peak increase in intraocular pressure, the (-)-enantiomer is only slightly more effective (Liu et al., 1983).

Third neurone Horner's syndrome can occur spontaneously in humans or secondary to an underlying disorder, for example, migrainous neuralgia, or a tumour (such as a lymphoma), and is the clinical equivalent of superior cervical ganglionectomy. Aqueous humour secretion in such patients is normal however. The suppression of aqueous secretion following 0.5% timolol is the same in such individuals as in the normal population (Wentworth & Brubaker, 1981). This suggests a postsynaptic locus of action of timolol and the lack of a need for intact adrenergic innervation. However, the results of this study contrast with preclinical findings. The ability of 4% timolol to lower the IOP of cats is completely prevented by superior cervical ganglionectomy (Colasanti & Trotter, 1981,1983). This observation implies that an intact

adrenergic innervation is essential for the ocular hypotensive action of the drug. Adrenergic innervation is also required for timolol to block the increase in IOP following the water-loading of ocular normotensive rabbits, as indicated by the lack of effect in denervated eyes (Liu et al., 1984). Thus a paradox exists between the clinical and preclinical findings.

Treatment of the eye with various β-adrenoceptor agonists will also lower IOP, although the literature is much confused in this area of study. There exists fairly widespread agreement that topical administration of adrenaline to the human eye leads to a decrease in IOP, and indeed the IOP lowering effects of adrenaline and timolol are additive in the human. However, the mechanism of the lowering of IOP with adrenaline appears to be different to that associated with the β-adrenoceptor antagonists. Topical administration of adrenaline leads to an increase in aqueous humour production with a concomitant initial rise in IOP (Townsend & Brubaker, 1980; Schenker et al., 1981), followed within thirty minutes by an increased facility of outflow (Sonntag et al., 1979; Townsend & Brubaker, 1980; Kaufman & Bárány, 1981; Kaufman & Rentzhog, 1983; Kaufman, 1986) thus mediating finally a reduction in IOP. Anderson & Wilson (1990) reported that the topical application of adrenaline lowers IOP in the rabbit, although these authors also indicate that this is largely due to an increase in the facility of outflow. Further to these observations, however

the specific β-adrenoceptor agonist isoprenaline reduces IOP in the human eye via a reduction in aqueous formation. In addition, topical application of the β_2-adrenoceptor agonist salbutamol to the human, feline or rabbit eye leads to a reduction in aqueous secretion (Langham & Diggs, 1974; Colasanti & Trotter, 1981). The effect can be blocked by pretreatment with propranolol or timolol, however. Gaasterland et al. (1973) reported that topical isoprenaline lowered both IOP and secretion rate in normal human volunteers, but had no effect upon the facility of outflow. In rabbits, topical isoprenaline was reported to decrease outflow facility in one case (Norton & Vierstein, 1972) and increase it in another (Langham & Diggs, 1974). Eakins (1963) injected isoprenaline into the vitreous and found that aqueous humour formation was depressed. When isoprenaline was perfused through the anterior chamber of monkey eyes, it was found to increase aqueous secretion and outflow facility, including uveoscleral drainage (Bill, 1970).

Timolol, and other β-adrenoceptor antagonists, as will be seen lower IOP in the bovine arterially perfused eye, a preparation which lacks intact adrenergic innervation and therefore has no endogenous sympathetic tone for a β-adrenoceptor antagonist to block, further suggesting that the mechanism of action of timolol upon IOP is not mediated via a classical β-adrenoceptor.

Another β-adrenoceptor antagonist, carteolol, which is also used to lower IOP in patients with COAG, reportedly has 1.67 times the potency of timolol for its extraocular β-adrenoceptor binding response (Ishizaki et al., 1983). However, carteolol is only 0.25 times as potent as timolol as an ocular hypotensive agent in the human eye (Horie et al., 1982).

If a β-adrenoceptor is involved in the ocular response to IOP lowering drugs, it is likely to be atypical, perhaps both in structure and its post-receptor mechanism.

Vascular Effects of β-Adrenoceptor Antagonists

The direct IOP-lowering effect of ocular β-adrenoceptor antagonists is well documented. However, reducing IOP is poorly correlated with protecting against visual field loss (Vogel et al., 1990; Schulzer et al., 1991). It is now considered that other factors, such as ocular vascular effects play an important role (Chauhan et al., 1989). Unlike non-selective β-adrenoceptor antagonists such as timolol, betaxolol has been shown to minimise long-term arteriolar constriction in the ciliary body and retina, thus permitting potentially normal ocular vasoregulation and perfusion (van Buskirk et al., 1990). Recent clinical evidence (Messmer et al., 1991) suggests that betaxolol is superior to timolol in preserving the visual field in

glaucoma patients. However several workers have suggested that timolol and other β-adrenoceptor antagonists actually reduce aqueous secretion via a modification of blood flow to specific structures within the uveal tract. In this respect, the effect of topically administered timolol on ocular blood flow has been studied in both the rabbit and human eye. A pretreatment for 1h with 0.25% timolol has been observed to decrease blood flow in the iris root-ciliary body and the choroid of the rabbit whilst flow in the iris and retina was unchanged (Watanabe & Chiou, 1983). However, in a later study, 0.25% solutions of (−)-timolol or (+)-timolol did not affect regional blood flow in the rabbit eye (Chiou & Yan, 1986). A similar result was observed in a study in which 0.5% timolol was applied as eight drops, every 7.5 min. to the rabbit eye in the hour immediately preceding ocular blood flow determinations (Jay et al., 1984). Additionally, the three-times daily instillation of 0.5% timolol for 5 to 6 weeks had no effect on regional ocular blood flow in the rabbit (Green & Hatchett, 1987). Conversely, Ernest & Goldstick (1983) found that timolol increased flow to the choroid in the human eye. A radiolabelled microsphere technique was utilised in all of these studies of ocular blood flow. Labelled microspheres offer the advantage to the investigator of being able to differentiate the responses of various subdivisions of a specific ocular vascular bed to the drugs under investigation. In addition to the labelled microsphere

technique, unlabelled microspheres have also been used in an attempt to assess ocular blood flow (Geijer & Bill, 1979).

The techniques of Laser Doppler Velocimetry (Riva et al., 1985; Robinson et al., 1986), Oculo-Oscillo-Dynamography (Urlich & Urlich, 1985), Ocular Pneumoplethsysmography (Gee et al., 1976), Compression Ophthalmodynamometry (Grunwald & Furubayashi, 1989) and Colour Doppler Ultrasound (Baxter et al., 1992) have also been used in attempts to measure ocular blood flow.

Other β-Adrenoceptor Antagonists Used to Lower Intraocular Pressure

The non-selective β-adrenoceptor antagonist carteolol is in common clinical use in the treatment of COAG. Topical doses required to lower IOP with this drug however are higher than with those discussed previously with timolol; the twice-daily instillation of 1% and 2% solutions of carteolol are used to lower IOP in glaucoma patients. As with the other β-adrenoceptor antagonists, carteolol mediates a reduction in IOP via a reduction in aqueous secretion (Araie & Takase, 1985). The drug is not considered to affect outflow facility (Gorgone et al., 1983). Unlike the other β-adrenoceptor antagonists used in the treatment of ocular hypertension, however, carteolol

also possesses intrinsic sympathomimetic activity (Koch, 1983).

Laevobunolol is a β_1 and β_2 adrenergic antagonist which was originally developed for the treatment of systemic hypertension and ischaemic heart disease (Arce-Gomez et al., 1976; Bray, 1977; Shapiro & Park, 1978; Partamian et al., 1983). It is a potent β-antagonist, structurally related to propranolol. The agent has no intrinsic sympathomimetic or local anaesthetic activity (Novack, 1986), and has a potency comparable to that of timolol. The drug is metabolised in the anterior segment of the eye to dihydrolaevobunolol (DHL) (Chen et al., 1987; Tang-Liu et al., 1987); the ability of DHL to block ocular β-adrenoceptors is comparable to that of laevobunolol (Woodward et al., 1987). As with timolol, levels of laevobunolol after topical instillation are greater in the ICB of pigmented rabbit eyes as opposed to non-pigmented eyes (Chen et al., 1988). Partamian et al. (1983) noted a significant decrease in the IOP of glaucoma patients after the topical instillation of a single dose of 0.03%, 0.3%, 0.6%, 1% or 2% laevobunolol, lasting for up to twelve hours after administration in the case of the 1% and 2% concentrations. No side effects were noted, nor were any substantial changes in visual acuity, pupil diameter, pulse rate or blood pressure recorded. Repeated instillation of lower concentrations of laevobunolol is also reported as significantly lowering IOP in glaucoma patients. Bensinger et al. (1985) reported a mean reduction in IOP of 9.0 mmHg

compared with placebo treated control after the twice-daily instillation of 0.5% or 1% laevobunolol, over a three-month period. Similarly, Boozman et al. (1988) reported a mean reduction in IOP of 5.1 mmHg in response to the twice-daily instillation of 0.25% or 0.5% laevobunolol, over a period of one year. Boozman also compared these concentrations of topical laevobunolol with the same concentrations of topical timolol; in the case of timolol, a similar mean reduction in IOP of 4.6 mmHg was found, over the same one-year period. Further studies upon the relative efficacy of laevobunolol and timolol have shown that the long-term, twice-daily instillation of 0.5% solutions of laevobunolol or timolol elicits a comparable reduction in the IOP of glaucoma patients (Berson et al., 1985; Long et al., 1985). In contrast, 0.25% and 0.5% solutions of laevobunolol are more effective than 0.5% betaxolol on long-term administration (Long et al., 1988). The results of one study indicate that once-daily 0.5% laevobunolol is more effective than similarly administered timolol in controlling the IOP of glaucoma patients (Wandel et al., 1986). Surprisingly, laevobunolol has been found to be more effective than timolol in preventing post-operative ocular hypertension after cataract extraction (West et al., 1988). In this respect, betaxolol was found to be ineffective. As with timolol, laevobunolol reduces IOP of glaucoma patients via a decrease in aqueous humour secretion (Yablonski et al., 1987).

Metipranolol is a non-selective β-adrenoceptor antagonist a topical preparation of which has been licensed in the United Kingdom for the treatment of glaucoma since 1986, although it has been in widespread use in Europe for many years, and in 1991 was marketed in the United States. As with the other β-adrenoceptor antagonists, the drug reduces IOP via a reduction in aqueous secretion. Topically applied metipranolol does not, however, mediate an ocular hypotensive effect upon the normal healthy eye (Sugrue et al., 1985). The affinity profile of metipranolol as a β-adrenoceptor antagonist is similar to that of propranolol (Sugrue et al., 1985). Mills and Wright (1986) observed similar reductions in IOP after 1 month twice-daily instillations of 0.3% metipranolol or 0.25% timolol. Topical metipranolol is reported to yield a modest reduction in heart rate and blood pressure (Krieglstein et al., 1987). Some authors believe, however, that metipranolol is less well tolerated than other topical β-adrenoceptor antagonists, with a high incidence of side-effects (Kruse, 1983; Krieglstein et al., 1987). Metipranolol has since been conclusively shown to cause granulomatous anterior uveitis, blepharoconjunctivitis and elevation in IOP (Akingbehin & Villada, 1992; Akingbehin et al., 1992), adverse effects never previously reported with any other of the ophthalmic β-adrenoceptor antagonists. In consequence, the drug has now been withdrawn in the United Kingdom for the treatment of glaucoma.

Other β-adrenoceptor antagonists reported to possess ocular hypotensive activity include: oxprenolol and metoprolol (Lotti et al., 1984), befunolol (Main & Tucker, 1985), bupranolol (Novack, 1987), pindolol (Bonomi & Steindler, 1975), labetalol (Murray et al., 1979), falintolol (Bouzoubaa et al., 1984) and spirendolol (Nathanson, 1985b).

Inhibitors of Carbonic Anhydrase

Carbonic anhydrase inhibitors have been used in the treatment of open-angle glaucoma since the 1950's. Carbonic anhydrase has been shown to be present in both the pigmented and the non-pigmented ciliary epithelial cells of the rabbit (Muther & Friedland, 1980; Lutjen-Drecoll & Lonnerholm, 1981). Becker (1954) noted that oral administration of the carbonic anhydrase inhibitor acetazolamide resulted in a reduction in aqueous secretion. Since this discovery, several members of this group of drugs have been utilised in the treatment of COAG, those being: acetazolamide, methazolamide, ethoxzolamide and dichlorphenamide. The drugs are given systemically, and in some cases lead to unpleasant side-effects, such as nausea and vomiting, or headache.

Vasodilator Drugs Which Lower Intraocular Pressure

In addition to β-adrenoceptor antagonists, it has also been reported, somewhat surprisingly, that the vasorelaxant cardiac peptide atrial natriuretic factor (atriopeptin - AP), which inhibits cyclic AMP synthesis, will lower IOP in the rabbit (Sugrue & Viader, 1986) and human eye when topically applied, probably by slowing the rate of aqueous formation. AP is synthesised, stored and released by cardiac myocytes (deBold, 1985; Needleman & Greenwald, 1986). Extra-atrial sites of AP synthesis have been described (McKenzie et al., 1985; Gardner et al., 1986), however no mRNA transcripts of AP have been localised in ocular tissue (Stone, 1987). These peptides have direct natriuretic, diuretic and vasodilatory properties (Currie et al., 1983; Maak et al., 1986). The biochemical mechanism by which AP achieves a reduction in aqueous humour formation appears to be very different from that of the β-adrenoceptor antagonists. AP promotes a rise in ciliary cyclic GMP (Fawcett & Wilson, 1989; Korenfeld & Becker, 1989), and indeed topical application of 8-bromo cyclic GMP (an analogue of cyclic GMP more resistant to hydrolysis by phosphodiesterases than cyclic GMP) reportedly lowers IOP in the rabbit (Becker, 1990). The effect is reportedly mediated by AP receptors (Nathanson, 1986), which have been detected in rabbit ciliary processes in high density by both autoradiography (Bianchi et al., 1986) and by guanylate

cyclase stimulation studies (Mittag et al., 1987; Nathanson, 1987). There is a lower density of AP receptors present in the choroid, ciliary musculature and retina. Additionally, intracameral and intravitreal injection of AP markedly lowers IOP of normotensive albino rabbits (Mittag et al., 1987: Nathanson. 1987). The AP receptor is coupled to a membrane-bound guanylate cyclase molecule (Waldman et al., 1984: Winquist et al., 1984: Hamet et al., 1986); recent evidence suggests that the AP receptor is more heterogeneous than was previously thought (Takayanagi et al., 1987; Budzik et al., 1987: Shinjo et al., 1988). The evidence for the effects of other vasodilator drugs upon IOP is much less abundant.

However. there are also reports in the literature of the ocular effects of other classes of vasorelaxant drugs which mediate an increase in intracellular cyclic GMP concentration via activation of soluble guanylate cyclase; for example. several nitrovasodilators. These reports are conflicting. however. Topical instillation of a 0.03% solution of nitroglycerine activates ciliary guanylate cyclase in the rabbit eye. mediating a reduction in aqueous secretion and IOP (Nathanson. 1988). Further. i.v. infusion of SNP into patients resulted in both a decrease in systemic blood pressure and IOP (Karnezis & Murphy. 1988). Conversely. topical administration of 1% or 2% solutions of sodium azide or SNP to rabbits were found to increase IOP in a dose-dependent manner (Krupin et al., 1977). Aqueous

outflow facility, as measured by tonography was unchanged, as was systemic blood pressure and heart rate, suggesting an increase in aqueous flow as the mechanism for the increase in IOP. However, systemic pretreatment with the α-adrenergic blocker phenoxybenzamine was found to prevent the rise in IOP, but not to affect the rise in aqueous humour cyclic GMP in response to these drugs, suggesting that an intact α-adrenergic pathway is required for increased IOP following sodium azide or SNP.

Sodium azide is thought to directly stimulate an intracellular soluble guanylate cyclase, and also promotes the release of endothelial derived relaxing factor - EDRF (Furchgott & Zawadzki, 1980) from blood vessel endothelia, since shown to be nitric oxide (Palmer et al., 1987). SNP also directly stimulates a soluble guanylate cyclase enzyme, but has no effect upon secretion of EDRF. ACh promotes the production and release of EDRF from vascular endothelium, by activation of the enzyme nitric oxide synthase, the enzyme responsible for the production of EDRF from L-arginine. It does so via binding to endothelial muscarinic receptors. EDRF is then taken up by vascular smooth muscle cells, whereupon it mediates vascular relaxation by stimulation of a soluble guanylate cyclase, leading to an increase in sarcoplasmic cyclic GMP which activates a cyclic GMP dependent protein kinase. This in turn elicits an inhibition of release of Ca^{2+} from the sarcoplasmic reticulum, increases the rate of uptake of Ca^{2+} by the

sarcoplasmic reticulum, and increases the rate of extrusion of Ca^{2+} from the cell across the plasma membrane (Murad, 1986). This promotes vascular smooth muscle relaxation and vasodilation. The ocular vascular endothelium may produce EDRF. Since EDRF stimulates intracellular soluble guanylate cyclase, it may be involved in the control of aqueous secretion.

There are reports of similar ocular effects of other classes of vasorelaxant compounds, for example, a variety of L-type Ca^{2+} channel antagonists. Topical nifedipine (0.01%, 0.1%, 0.5% & 1%), topical verapamil (0.01%, 0.05%, 0.1% & 0.25%) and topical diltiazem (0.1%, 0.5% & 1.0%) had no significant effect upon IOP in rabbits (Payne et al., 1990), but when given systemically, verapamil (0.1 mg/kg) and nifedipine (0.33 mg/kg) yielded a significant reduction in IOP between 2 and 6h after administration, the nifedipine response, however following a significant increase in IOP after 30 min. Intravenous diltiazem (1 mg/kg), given three times daily for three days, produced no change in IOP. The ocular hypotensive effect of topical nifedipine (Kelly & Walley, 1988) and verapamil (Abelson et al., 1988) upon the human eye have been demonstrated, however. Conversely, Beatty et al. (1984) reported an increase in IOP in response to topical diltiazem and verapamil in human volunteers.

Certain vasodilator drugs which mediate a decrease in intracellular free potassium ion via the opening of plasma-membrane ATP-sensitive potassium channels, are also reported

as producing a concomitant decrease in intracellular free calcium ion, in several examples of vascular smooth muscle, for example, rabbit aortic smooth muscle (Gelband et al., 1988; Bray et al., 1991), rabbit superior mesenteric artery smooth muscle (Meisheri et al., 1991) and rat portal vein smooth muscle (Hamilton et al., 1986; Hu et al., 1990). There is little published in the literature to date concerning their ocular effects, however certain drugs of this class are known to reduce IOP in the bovine perfused eye preparation (Millar & Wilson, 1992).

It has been suggested that dopaminergic mechanisms may play a role in the ocular hypotensive effect of timolol (Watanabe & Chiou, 1983). Dopamine is a known neurotransmitter in the retina, but its actions elsewhere in the eye have not been recognised until recently, when studies in animals and humans have indicated that it may be involved in control of IOP (Kramer, 1971). The topical administration of the DA_2 agonists bromocriptine, lergotrile or pergolide to rabbits results in a reduction in IOP, with pergolide exhibiting the greatest ocular hypotensive activity. Of these three compounds, however, bromocriptine does not lower the IOP of normal monkeys. The ocular hypotensive action of these compounds in rabbits is mediated via a reduction in aqueous secretion (Leopold & Duzman, 1986). Moreover, their ability to lower IOP in the rabbit is blocked by superior cervical ganglionectomy, or by pretreatment with the DA_2 antagonist domperidone (Potter et

al., 1984). Domperidone itself has no reported effect upon IOP. Pergolide has also been shown to lower the IOP of cynomolgous monkeys (Siegel et al., 1987). Both orally and topically administered bromocriptine lowers the IOP of human volunteers (Mekki et al., 1984). and oral bromocriptine lowers the IOP of glaucoma patients (Lustig, 1983; Geyer et al., 1987). In contrast, the ocular hypotensive effect of timolol in healthy human individuals is unaltered by a previous intravenous injection of the DA_2 antagonist metoclopramide (Mekki & Turner, 1988). thus indicating that the ocular hypotensive effect of timolol is probably not mediated via a dopaminergic mechanism.

Turner and Mekki (1985) suggest that the mechanism of action of dopamine agonists in the modulation of IOP may involve presynaptic DA_2 receptor stimulation of noradrenergic nerve endings in the ciliary body. However, subsequent binding assays for ocular DA_2 receptors have been performed in the rabbit using ^3H-spiroperidol (Mallorga & Sugrue, 1987), but none have been found, either in the ciliary tissue or elsewhere in the eye. Experimental evidence suggests the existence of DA_1 receptors in human and bovine ciliary tissue (De Vries et al., 1986). In one study, infusion of the selective DA_1 receptor agonist fenoldopam ($0.5\mu gkg^{-1}.min^{-1}$) in normal human volunteers elevated IOP by 24% without altering systemic blood pressure (Karnezis et al., 1987). Comparison of the effects of fenoldopam with the vasodilator SNP in patients showed that,

whilst both drugs reduced blood pressure, fenoldopam increased, wheras SNP decreased, IOP. In another study (De Vries et al., 1986), fenoldopam induced a dose-dependent increase in intracellular cyclic AMP content in bovine and human ciliary processes. Pretreatment with the selective DA_1 receptor antagonist SK&F-83566 inhibited the latter response. The recent isolation from the non-pigmented ciliary epithelium of the regulatory phosphoprotein of cyclic AMP, DARPP-32, a protein previously found only in cells bearing large numbers of DA_1 receptors, further supports the presence of these receptors in the ciliary processes.

Other Agents Which Lower Intraocular Pressure

There are a wide variety of other agents which reportedly lower IOP. These include directly acting cholinergic agonists, such as pilocarpine, carbachol and aceclidine (Havener, 1983) as well as the reversible anticholinesterases physostigmine, demecarium, ecothiophate and isofluorophate (Leopold, 1984). α-adrenoceptor agonists such as clonidine (Harrison & Kaufmann, 1977; Hodapp et al., 1981) and apraclonidine (Abrams et al., 1987), and antagonists such as prazosin (Krupin et al., 1980), yohimbine (Mittag et al., 1985), dibenamine, phentolamine, phenoxybenzamine and thymoxamine (Mittag, 1983) reportedly

lower IOP in animal experiments. Selected prostaglandins (Camras et al., 1977), ACE inhibitors such as captopril, enalapril and SCH 33861 (Watkins et al., 1987), the 5-HT antagonist ketanserin (Chang et al., 1985; Krootila et al., 1987), glucocorticoids (Becker, 1965), H_1-antihistamines such as antazoline and pyrilamine (Krupin et al., 1980), colchicine (Bhattacherjee & Eakins, 1978) and a component of marijuana, $\triangle^9$-tetrahydrocannabinol (Purnell & Gregg, 1975), also are reported to possess ocular hypotensive activity.

The Isolated Arterially Perfused Eye

To date, most studies concerning the effects of drugs upon aqueous humour dynamics and IOP have been conducted in vivo upon a number of different animal species, and in the human. Such studies are complicated by the influence of the central nervous system (CNS), the endocrine system and the cardiovascular system (CVS) upon secretion and/or drainage of aqueous. However, several workers, in order to obviate such complications in the interpretation of data, have used as an experimental model, the isolated arterially perfused eye from a number of species, including rabbit (Kodama et al., 1983, 1985), cat (Macri & Cevario, 1973, 1974; Helal et al., 1979; Macri et al., 1980; van Alphen & Macri, 1981; van Alphen et al., 1982) and bovine (Kishida et al., 1985; Wilson, 1988). The isolated perfused eye lends itself to

the direct correlation of drug effects on IOP, aqueous humour formation, the uveal vasculature and the patency of the blood-aqueous barrier. Additionally, it permits experimental manipulation of perfusion media. Wilson describes the bovine arterially perfused eye as an experimental model upon which timolol will reduce IOP. Isolation of the eye from the influence of the CNS and hence sympathetic and parasympathetic tone is particularly important when studying the ocular effects of the β-adrenoceptor antagonists (as opposed to agonists). Isolation of the eye from the effects of the CVS is also important since changes in systemic blood pressure (and hence systemic effects of drugs under investigation) can affect aqueous secretion rate and therefore IOP. Isolation of the eye from the endocrine system is also important, as certain hormones are known to have an effect upon aqueous humour dynamics.

There is a long history in the literature on the study of the ocular cyclic AMP response to various β-adrenoceptor agonists, antagonists and other compounds, as measured in ciliary tissue and the aqueous (Bhargava et al., 1980; Nathanson, 1980; Boas et al., 1981; Cepelik & Cernohorsky, 1981; Liu et al., 1983; Elena et al., 1984a; Mittag & Tormay, 1985; Bartels et al., 1987; Kintz et al., 1988). Other work with AP (Sugrue & Viader, 1986; Mittag et al., 1987; Nathanson, 1987; Fawcett & Wilson, 1989; Korenfeld & Becker, 1989) and nitrovasodilators (Krupin et al., 1977)

has indicated a possible involvement of cyclic GMP as another candidate for an intracellular second messenger involved in the ocular response. β-adrenergic receptors are positively coupled to the generation of intracellular cyclic AMP via a membrane bound G_s protein. associated with adenyl cyclase. also bound to the cell membrane (Fig.17). When an agonist binds to the receptor. the agonist-receptor complex so formed causes a conformational change in the adjacent G_s protein. which promotes the binding of the G_s protein to intracellular GTP. The G_s protein-GTP complex then activates the adenyl cyclase molecule. leading to an increase in the concentration of intracellular cyclic AMP. formed from the hydrolysis of cytoplasmic ATP. The cyclic AMP formed then binds to cytoplasmic cyclic AMP dependent protein kinases. thus activating them and promoting various intracellular phosphorylation reactions leading to many different effects. depending upon the cell type. Cyclic AMP may also directly decrease the release of Ca^{2+} from intracellular stores - this effect is especially seen in muscle cells. and the resultant effect here is inhibition of muscle cell contraction. In this context. Ca^{2+} also acts as a second messenger. This is how the binding of the β-adrenergic receptor by a molecule of agonist is translated into a cellular response. The intracellular cyclic AMP formed is hydrolysed by cytoplasmic cyclic AMP phosphodiesterase (PDE). thus limiting its effects. Receptors coupled to guanylate cyclase (such as AP

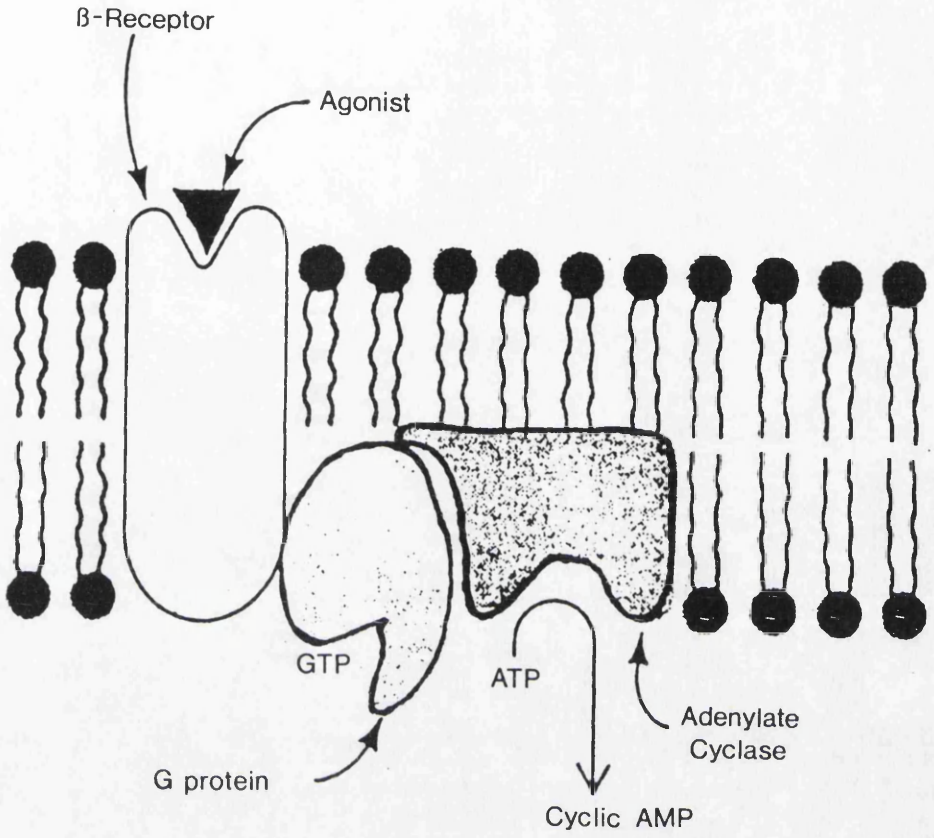


Fig.17. Formation of intracellular cyclic AMP as a consequence of agonist - receptor interaction. (After Stryer, Biochemistry, 2nd Edition).

receptors) mediate a rise in intracellular cyclic GMP in a similar fashion. The role of cyclic GMP in certain physiological processes has only become apparent since 1978 (Murad & Aurbach, 1978; Murad et al., 1979; Mittal & Murad, 1982; Rapoport & Murad. 1983). This is probably a consequence of the low concentration of the nucleotide in tissues, the complex and insensitive methods available during the early studies, and the biases many investigators had regarding its possible functions. The latter was undoubtedly influenced by the many similarities of the cyclic GMP system with the cyclic AMP system, and the attenuation cyclic AMP has received during this period. Whilst analogies and similarities between these two cyclic nucleotide systems do exist, the cyclic GMP system presents more complexities due to the existence of several isozymes responsible for its synthesis.

Certain other receptor types. such as α_1-adrenoceptors. are coupled to the production of intracellular inositol trisphosphate (IP_3) and membrane diacylglycerol (DAG). from the membrane phospholipid precursor phosphatidyl inositol bisphosphate (PIP_2). The other intracellular second messengers, Ca^{2+} and PIP_2, which represent almost universal intracellular second messengers in many tissues, do not appear to have been investigated at all with regard to the IOP lowering effect of ocular hypotensive drugs. It seems that an investigation of some of these other possibilities would now be appropriate. The

bovine perfused eye preparation offers the opportunity to do this, more especially since the preparation facilitates the rapid access to substantial amounts of living tissue, thus enabling the investigator to relate physiological responses to biochemical events.

The isolated arterially perfused eye was chosen as an experimental model for the reasons related above; the bovine species was selected primarily for reasons of availability and cost. Additionally, the bovine eye is large and thus relatively straightforward to set up experimentally as an isolated perfused eye preparation: smaller eyes from other species are in practice much more difficult due to the small size and inaccessibility of the blood vessels.

Aims of Project

There is a great need for an *in vitro* animal model for use in studies on aqueous humour dynamics, and its potential pharmacological manipulation, particularly as the anti-animal experimentation lobby continues to gain momentum. Since there will be progressively more restrictions applied to the sacrificing of animals for experimental purposes, such *in vitro* models will almost certainly become more important in the future, especially in such an emotive area as ocular research. The aims of this project were to develop and improve the method of Wilson (1988), so that the

isolated perfused bovine eye preparation could be utilised for the routine screening and comparison of drugs for possible IOP lowering effect. Further, an investigation of the pharmacological mechanisms governing aqueous secretion was undertaken, by way of a study of the effect of a number of β-adrenoceptor antagonists of upon IOP in the isolated perfused bovine eye, which reportedly reduce the rate of secretion of aqueous humour but by an anomalous mechanism. The finding that the vasoactive drug AP lowered IOP in this model, probably via a novel biochemical mechanism prompted closer analysis of this effect to determine whether the effect on IOP was a primary one or secondary to the vascular changes induced by the drug, especially with a view to determining, if vasodilatation is not involved in the lowering of IOP, whether the cellular mechanism is the same in blood vessels as in the ciliary processes. The isolated perfused bovine eye provides an ideal experimental model for such investigation. Additionally, in view of the conflict in the literature, an investigation of the ocular effects of other classes of vasodilators was undertaken, specifically, selected nitrovasodilators, and drugs reported to decrease intracellular Ca^{2+}, in order to investigate the possibility that a novel IOP lowering mechanism might be found. The effect of selected ocular hypotensive drugs upon ciliary cyclic GMP was investigated.

An attempt was also made to differentiate the vascular effects of some of these drugs with respect to the various

subdivisions of the ciliary vascular bed in the isolated perfused bovine eye by the use of radiolabelled microspheres. The technique of utilising labelled microspheres for the estimation of blood flow through a tissue or organ is not new. Numerous studies have been carried out, including estimations of cardiac output distribution in the foetal sheep and goat (Rudolph & Heymann, 1967), rabbits (Neutze et al., 1968), dogs (Kaihara et al., 1968) and rats (Sasaki & Wagner, 1971). More recently, there have been studies to measure intrarenal blood flow in the dog (Katz et al., 1971), cochlear blood flow in the rat (Hillerdal et al., 1987), and coronary flow in the cat and rabbit (Hof et al., 1981), dog (Hale et al., 1988) and rat (Wicker & Healy, 1989). Ocular blood flow measurements have also been undertaken utilising this technique, for example, in the rabbit (Morgan et al., 1981; Watanabe & Chiou, 1983; Chiou & Yan, 1986), the monkey (Caprioli et al., 1984), the dog (Roy et al., 1989) and the cat (Oksala, 1988). However, in all of the previous studies, blood flow was measured in vivo. Several authors deal with the theoretical and practical considerations when utilising labelled microspheres, and potential sources of error (Buckberg et al., 1974; Warren & Ledingham, 1974), however the use of labelled microspheres to estimate flow to various parts of an organ perfused in vitro does not appear to have been described elsewhere.

A comparison of the effects of the various ocular hypotensive drugs investigated upon the divisions of the uveal vasculature of the isolated perfused eye, with the results reported *in vivo* was sought. The isolated perfused bovine eye offers the opportunity to develop this area of investigation.

METHODS

The Bovine Perfused Eye Preparation

Bovine eyes obtained from a local abattoir were maintained at room temperature and transported to the laboratory. They were not placed on ice, since the resulting solidity of the orbital fatty tissues seriously hampered dissection and cannulation. It was also felt that the return of the eyes' core temperature to 37°C would be slowed considerably by cooling the whole eye in ice. Ambient temperatures during transportation were in the range 2 to 17°C, depending on seasonal variation. Within 1h of slaughter they were trimmed of adnexal tissue, taking care not to damage the blood vessels of the retrobulbar vasculature. A few mm of each extraocular muscle was left attached to the globe. One of the long posterior ciliary arteries (medial or lateral) was cleared of connective tissue and cannulated distal to the point at which the retinal artery leaves the ophthalmic artery but proximal to the heavy pigmentation which appears in the arterial wall before it enters the sclera (Fig.18). The eye was placed on a jacketed holder maintained at 37°C and covered with a polycarbonate cup to prevent drying. The long posterior ciliary artery and thus the blood vessels of the choroid. ciliary body and iris were then perfused with a Krebs solution comprising (mM): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 11.5. The solution was bubbled with a mixture of O_2:CO_2 (95:5), which

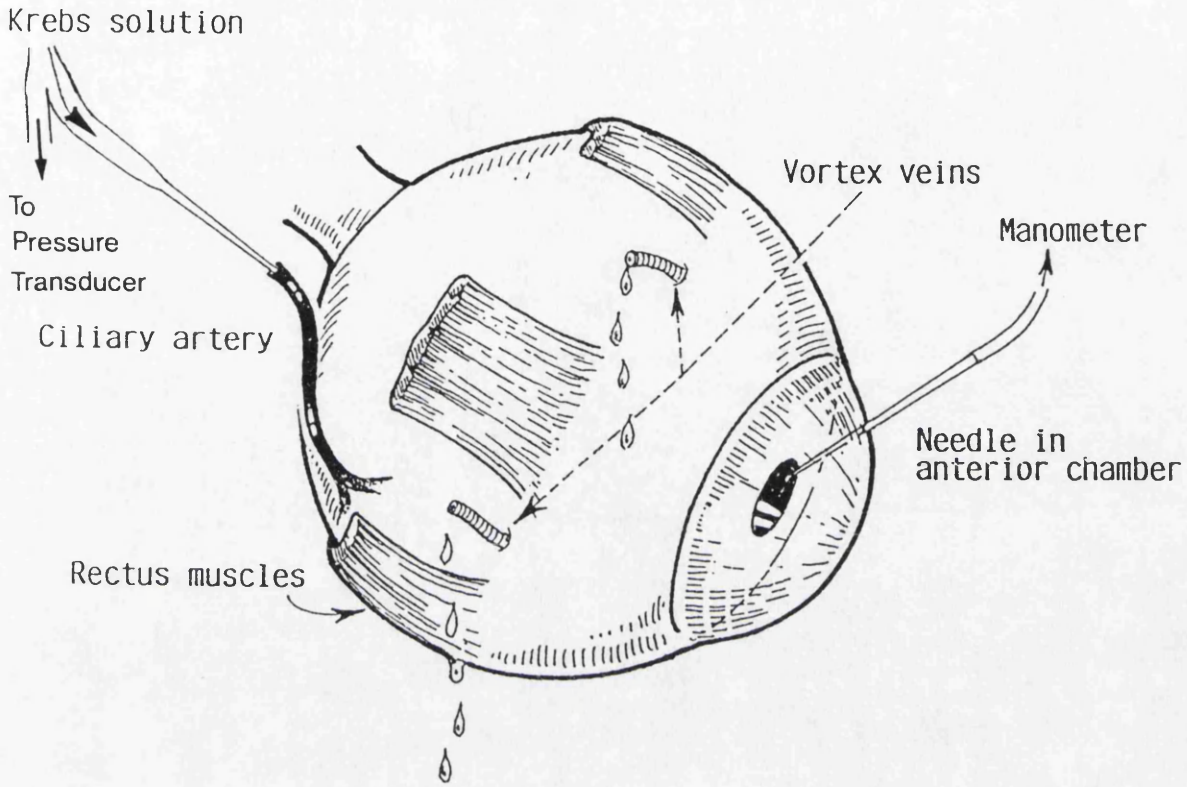


Fig.18. The Bovine Arterially Perfused Eye Preparation.

brought the pH to 7.4. Flow was induced in this solution by a Watson-Marlow peristaltic perfusion pump fitted with a 502S pumphead. Arterial perfusion pressure was recorded via a pressure transducer and Linseis pen recorder, and flow rate was increased in 0.225 $ml min^{-1}$ increments until reaching 2.25 $ml.min^{-1}$, over a period of approx. 50 min. During this period, perfusion pressure often fluctuated but the flow rate was adjusted to prevent the pressure from exceeding 100 mmHg. Successful perfusion was signalled by the appearance of small amounts of blood from the cut ends of the vortex veins: on occasion it was necessary to cut away the remnants of the extraocular muscles in order to encourage visible flow. After approx. 50 min, when aqueous humour secretion had started and the globe had become firm, the anterior chamber was cannulated with a 23 gauge steel needle, and connected via a fine silicon tube of internal diameter 1 mm to a water manometer. Observations of IOP were usually made at 5 min intervals throughout the remainder of the experiment. Only eyes maintaining a stable IOP within the range 95 to 150 mmH_2O (7 to 11 mmHg), and a stable arterial perfusion pressure within the range 20 to 60 mmHg, after an equilibration period of a further hour were accepted for study. At this point, bolus doses of drugs or vehicle solution could be injected immediately proximal to the arterial cannula, and their effects upon both IOP and arterial perfusion pressure could be measured and recorded.

Effects of β-Adrenoceptor Antagonists on IOP

In order to confirm that the bovine isolated perfused eye preparation is able to demonstrate significant and reproducible ocular effects of specific drugs, a selection of β-adrenoceptor antagonists, including some of those utilised in the treatment of COAG, were tested in this preparation. Isolated perfused eye preparations were set up and, after equilibration of arterial perfusion pressure and IOP, the latter was recorded every 5 min for a period of 15 min, to ensure that it did not fluctuate by more than 0.5 mmH_2O. Upon establishing that IOP was thus stable, a bolus injection of 3nmol (3μl of 10^{-3} M) of a solution of a β-adrenoceptor antagonist dissolved in Krebs solution was injected into the arterial perfusate, proximal to the arterial cannula through self sealing tubing, using a Hamilton Microsyringe. The time of injection was designated time zero: IOP measurements were recorded every 5 min thereafter for a period of 20 min. At 20 min, a 10nmol (10μl of 10^{-3} M) bolus injection was given; continued measurements of IOP were made every 5 min until 40 min from time zero, whereupon a further bolus dose of 30nmol (30μl of 10^{-3} M) was injected. IOP was recorded up to and including 60 min from time zero. The β-adrenoceptor antagonists tested in this way were: timolol, oxprenolol, betaxolol, laevobunolol, DHL, carteolol, metoprolol and metipranolol. Results were compared with control experiments in different

eyes where saline in volumes of 3, 10 and 30μl were injected at time zero, 20 and 40 min respectively.

Effect of Acetazolamide on IOP

Bovine eye preparations were set up as previously; vascular perfusion pressure and IOP was allowed to equilibrate. Acetazolamide, dissolved in saline was injected into the perfusate as bolus doses of 0.3, 1 and 3umol (3, 10 and 30μl 10^{-1} M) at time zero, 20 and 40 min respectively. The effect upon IOP, measured every 5 min, was recorded.

Effect of Dopamine Agonist FPL 65879AA on IOP

The experimental protocol utilised was as that described previously for acetazolamide, except that FPL 65879AA was given in various bolus dosages of: 30, 100 and 300nmol, and 1, 3, 10 and 30μmol.

Single Dose Timolol: IOP-Time Curve

This procedure was carried out in an attempt to determine the absolute change in IOP in response to a single dose of timolol. The experimental protocol was similar to that described above for the β-adrenoceptor antagonists, except that in this case only timolol was utilised, and was injected only once, as a single bolus dose of 10nmol at time zero. IOP was subsequently recorded for a period of 100 min, at 5 min intervals. Results were compared with control experiments in which saline was injected.

Log Dose-Response Curves for IOP Effects of Timolol and Carteolol

In this procedure, single bolus doses of timolol or carteolol were injected and IOP recorded every 5 min for a period of 100 min from time zero, as in the previous procedure described for the single dose of timolol. In this case, however, the absolute effect of a range of different doses upon IOP over time was established for each of the two drugs. This was undertaken with a view to the production of log dose-response curves for the effect of each specific single bolus dose of timolol or carteolol upon IOP. The bolus doses of timolol and carteolol utilised were: 0.03, 0.3, 1, 3, 10 and 30nmol, in volumes of 1 up to 30μl.

Effect of Atriopeptin (AP) on IOP

The experimental protocol was as per that described above for the β-adrenoceptor antagonists, using AP (rat; fragment 1-27) dissolved in Krebs solution. A 10pmol (10μl of 10^{-6} M) bolus injection of AP was given at time zero, followed 40 min later by a 50pmol (50μl of 10^{-6} M) bolus dose. Only 2 doses of AP were thus given. IOP was recorded every 5 min for a period of 80 min. Once again, results were compared with control experiments in which saline was injected at corresponding time intervals and volumes.

Effect of AP on Ciliary Artery Perfusion

Isolated perfused eye preparations were set up and allowed to reach stable IOP and perfusion pressure. Noradrenaline bitartrate was added to the perfusate reservoir at a final concentration of 10^{-5} M, thus causing an increase in vascular tone and hence vascular perfusion pressure, at constant perfusate flow rate. Upon attainment of a stable degree of vascular tone, bolus injections of 10, 30 and 100pmol AP (1, 3 and 10μl 10^{-5} M) were given, allowing full recovery of vascular tone after one dose before proceeding to the next. Results were compared with

experiments in which bolus injections of 0.3 and 3nmol (3μl of 10^{-4} M, and 3μl of 10^{-3} M) sodium azide (a potent vasodilator), were given, and also control experiments in which saline was injected in volumes of 3 and 10μl.

Effects of Other Vasodilator Drugs on Ciliary Artery Perfusion

In addition to AP, the vascular effects of other classes of vasodilator drug were investigated. Perfused eyes were set up, and after equilibration noradrenaline bitartrate was added to the perfusate reservoir at a final concentration of 10^{-5} M, in order to increase uveal vascular tone, as described in the ciliary artery perfusion investigation for AP. After attainment of stable vascular tone, bolus doses of each drug were given in volumes of 1, 3, 10 and 30μl (ranging from 0.01 to 3000nmol, depending upon the particular drug), and the reduction in vascular tone induced by each dose of drug was determined. Results were compared with control experiments in which saline was injected in similar volumes.

IOP Effects of Other Vasodilator Drugs

This procedure was identical to that previously described for the Log Dose-Response curves for IOP effects of timolol and carteolol, except that in this case the ocular effects of the nitrovasodilators sodium azide and SNP, and the potassium channel agonist pinacidil were investigated. Doses utilised were as follows: sodium azide; 0.01 to 30nmol in log increments (all in volumes of 1 or 3μl); SNP: 0.3 to 1000nmol in log increments (all in volumes of 1, 3 or 10μl); pinacidil; 3 to 3000nmol in log increments (all in volumes of 1, 3 or 10μl). After each single dose of vasodilator drug, IOP was recorded every 5 min for 165 min.

Investigation of EDRF Production by Uveal Vascular Endothelium

In order to investigate the possibility that the uveal vascular endothelium produces EDRF, eyes were perfused with Krebs solution containing noradrenaline at 10^{-5} M concentration. Upon attainment of a stable degree of vascular tone, bolus injections of ACh were given, ranging from 30pmol to 0.1μmol (3μl 10^{-5} M to 1μl 10^{-1} M) in log increments. Full recovery of vascular tone was allowed after each dose of ACh before proceeding to the next dose. L-NOARG was then added to the Krebs solution at $3X10^{-5}$ M,

and allowed to perfuse until attainment of stable vascular tone once more, whereupon vascular relaxation responses to each bolus dose of ACh was determined once more. Results were compared with control procedures where saline was injected in volumes of 3 and 10μl.

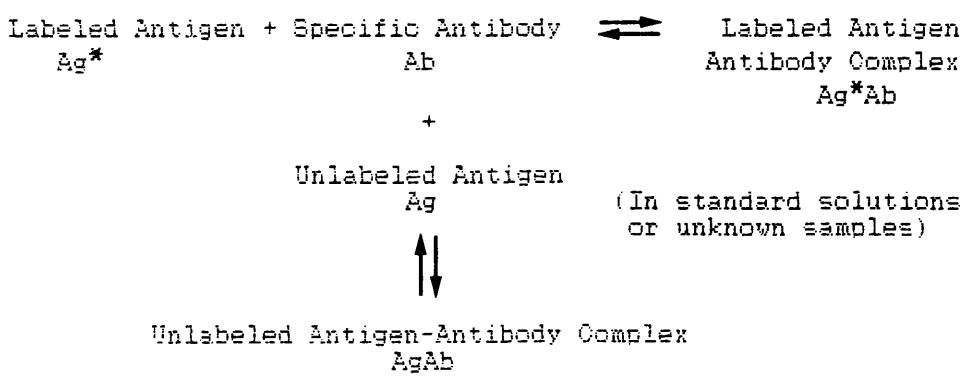
Ciliary Cyclic GMP Determinations

In order to determine the effects of the vasodilators AP and sodium azide upon cyclic GMP in the ciliary epithelium, perfused eye preparations were set up and allowed to achieve stability. Each eye was then challenged with a bolus dose of AP or sodium azide, known from previous experiments to be sufficient to lower IOP, or of saline control. Perfusion was allowed to continue for precisely 2 min more. whereupon perfusion was stopped and the eye was rapidly dissected to remove the: sclera posterior to the ora serrata, the choroid and retina. vitreous, lens and lens capsule. The anterior portion of the sclera, cornea and ciliary body remaining was then inverted over a plasticine sphere which filled the anterior chamber and immersed in liquid nitrogen for 20s, thus effectively halting all chemical or enzymically catalysed reactions. The time between stopping perfusion and immersion in liquid nitrogen was approx. 3 min. After removal from the liquid nitrogen, the anterior eye portions were placed in a freezer at -30°C

for a period of 20 min. They were then removed and the superficial tissue, largely ciliary epithelial cells were carefully scraped from the ciliary body in the region of the pars plicata, taking care to harvest as little as possible of the underlying stroma. The scraped epithelial cells were then placed in Eppendorf tubes containing 1ml 6% TCA, and stored at -30°C until required. The tissue was thoroughly disrupted by subjecting it to a thrice freeze-thaw-sonication procedure. Sonication consisted of 5 min in a Camlab Transsonic T310 sonicator bath. The resulting homogenate was centrifuged at 20000g.min in a DAMON/IEC DPR-6000 centrifuge, and the supernatant layered on top of 1.6ml of a solution of tri-N-octylamine (22.5% v/v) in freon, in a 3ml glass test tube. This was vortex-mixed thoroughly for 20s, then briefly centrifuged to clarify the seperate phases. The supernatant was then transferred to a 0.5x6cm column of Dowex-50W 50X8-100 mesh cation exchange resin (hydrogen form) which had been washed 4 times in distilled H_2O. The eluate from this column, after discarding the dead volume, was collected (approx. 8ml), and evaporated to dryness and subsequently stored at -30°C. The dead volume and eluate volume were previously determined using standard ^3H-cyclic GMP. 0.5 ml of TCA were mixed with 0.1ml ^3H-cyclic GMP, and the mixture was then added to 1.6ml tri-N-octylamine in freon in a 3ml glass test tube. The tube was then vortex mixed for 20s and then centrifuged at 20000g.min. 0.4ml of supernatant was then transferred to a

Dowex-50W 50X8-100 mesh cation exchange resin (hydrogen form), and the resulting eluate collected in a 10ml plastic liquid scintillation counting vial. The 0.2ml of supernatant still remaining in the tube containing the tri-N-octylamine in freon was then transferred to the same column and the eluate collected in another counting vial. The column was then washed with 8x 0.5ml distilled H_2O, and each of the 8 eluates were collected in seperate vials. 10 ml of Ecoscint scintillation cocktail was added to each of the ten vials, and the β radiation counted in a Packard 2000CA Tri-Carb Liquid Scintillation Counter. The liquid scintillation counter included an automatic correction for counting efficiency.

The concentration of cyclic GMP present in each sample was estimated via the technique of cyclic GMP RIA, using a kit. Each dried extract was redissolved in 500μl of 0.05M sodium acetate buffer, pH 6.2. The principle of the assay is the competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. This interaction is represented schematically below:



If increasing amounts of non-radioactive antigen (e.g. standards or unknowns) and a fixed amount of radioactive antigen are allowed to react with a constant amount of antibody, a decreasing amount of radioactive antigen is bound to the antibody. This relationship can be expressed as a standard curve and the amount of unlabeled antigen in a sample determined from this curve. Seperation of bound from free antigen is achieved via centrifugation followed by aspiration of the supernatant, containing the free antigen. The labeled antigen utilised in the kit is ^{125}I-cyclic GMP. Harper and Brooker (1975) and Frandsen and Krishna (1976) demonstrated that acetylating cyclic GMP samples with acetic acid anhydride at the 2'-O-position to yield 2'-O-acetyl cyclic GMP resulted in a better antibody binding reaction, and thus the substituted cyclic GMP displaced the ^{125}I-labeled derivative more efficiently than the unsubstituted cyclic nucleotide. Therefore the acetylated samples method was utilised throughout. The standard curve was prepared according to the acetylated samples protocol supplied with the kit. Duplicate samples were run throughout. Internal standards were utilised to determine recovery efficiency per sample from its respective Dowex column. This was achieved by adding 1500 dpm of ^{3}H-cyclic GMP to each spun-down tissue pellet in 0.5ml TCA, immediately prior to cyclic nucleotide extraction. The added ^{3}H-cyclic GMP standard was subtracted from the final result for the calculation of cyclic GMP per

sample. After extraction, evaporation and redissolving, 100µl of the redissolved extract was placed in a 10ml plastic liquid scintillation vial, and 10ml Ecoscint scintillation cocktail was added. The β radiation was then counted in the same liquid scintillation counter as mentioned above, to determine the dpm. The resultant dpm per vial was multiplied by 5, and the recovery efficiency per sample calculated.

After incubation in a refrigerator at +4°C for 18h, all tubes were decanted and blotted on absorbent paper until dry. All tubes, including standards and blanks, were then counted on a Packard Cobra Auto-Gamma gamma counter. The gamma counter had a computerised calculation of fmol cyclic GMP per sample incorporated into its programming, which included a correction for counting efficiency.

Total protein present in the ciliary epithelial cell tissue residue saved from the initial cyclic nucleotide extraction was estimated by the method of Lowry et al. (1951). To extract the total protein present, the pellet from the centrifugation was transferred to a 10ml glass test tube and dissolved in 5ml N-NaOH, by heating in a water bath at 60°C with occasional mixing for 2h. The protein assay was performed in triplicate (using 20, 30 and 50µl aliquots from each sample) within 3h of extraction. Tissue samples were compared with BSA standards and a blank.

Drug Effects on Vascular Flow Using Radiolabelled Microspheres

In order to determine how various drugs affect the distribution of blood flow within the anterior eye, a radiolabelled microsphere technique was adopted. For infusion of microspheres, a two-way apparatus (Fig.19) was constructed immediately proximal to the arterial cannula whereby a pre-prepared bolus of spheres could be loaded into the system, and subsequently injected into the eye at a suitable point in the procedure without interruption of flow or sudden increase in pressure (after administration of either drug or saline control), via the activation of a two-way valve. After attainment of full flow rate, noradrenaline bitartrate was added to the perfusate reservoir at a final concentration of 10^{-5} M, thus causing an increase in vascular tone. (The concentration of noradrenaline chosen was known to be such as to elicit submaximal uveal vasoconstriction). At this point, a bolus of approximately 4500 carbonized latex ^{141}Ce-labelled 15μm spheres, suspended in 100μl saline containing 0.01% Tween-80, to prevent aggregation of the spheres, and 0.009% benzyl alcohol as a bacteriostat, was agitated on a vortex mixer for a full minute and then loaded into the two-way system behind the arterial cannula. The eye was then treated with either drug, or 30μl saline control injected as a single bolus behind the cannula, and left for up to 1 min until the

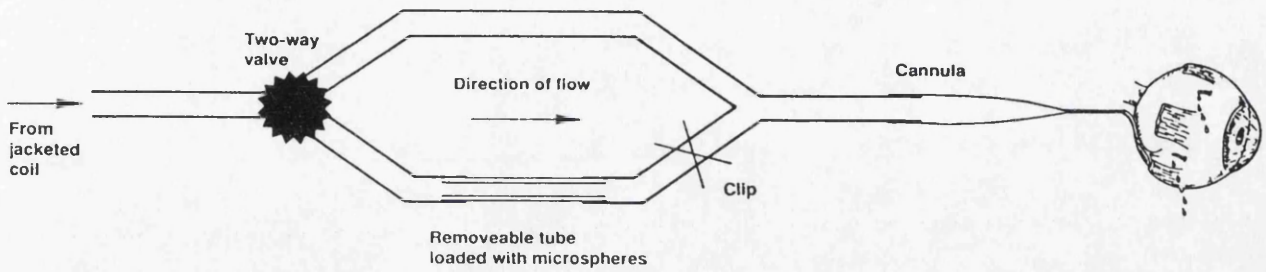


Fig.19. Microsphere Infusion Apparatus.

full extent of the effect of the drug could be seen on the pen recorder indicating vascular perfusion pressure. In the case of timolol or carteolol, bolus doses were chosen (30nmol) which are known to have maximal ocular hypotensive effect on this preparation. In the case of SNP and verapamil, doses which are known to elicit maximal uveal vasodilatation (300nmol) were utilised. After development of the full drug effect, the preloaded bolus of spheres was introduced into the eye and allowed to perfuse for a period of 5 min. The eye was then taken down from the perfusion apparatus and dissected to remove the: cornea, iris, ciliary body, lens, vitreous, aqueous, choroid/sclera (seperation of these two structures was found to be impractical), retina and extra-ocular muscle. Each component was then placed in a preweighed glass scintillation vial, the weight of the vial plus contents determined, and then counted on a Packard 5355 gamma counter in order to determine cpm/g tissue (wet weight). Results were compared with additional experiments in which eyes perfused with Krebs containing no noradrenaline (and thus exhibiting only basal vascular tone) were challenged with bolus doses of timolol or carteolol, or saline control.

RESULTS

Effects of β-Adrenoceptor Antagonists on IOP

All eight drugs tested produced significant decreases in IOP. The graph of % Change in IOP from time zero as ordinate against time as abscissa (Fig.20) indicates that the effects of the β-adrenoceptor antagonists timolol, oxprenolol, betaxolol and laevobunolol could be seen approx. 10 min after bolus injection of 3nmol into the arterial perfusate. The further doses of 10 and 30nmol, administered 20 and 40 min later, were given in an attempt to confirm dose-dependence of the IOP-lowering effect. The drug effects were compared with data from control perfusions in which vehicle was injected. The graph of % Change in IOP from time zero as ordinate against time as abscissa (Fig.21) indicates a similar result obtained with four further β-adrenoceptor antagonists DHL, metoprolol, metipranolol and carteolol. Control eyes showed a slight but statistically significant tendency for IOP to rise: 2.2±1.9% ($P<0.1$, $n=6$, 60 min value vs. time zero value) over the period of perfusion. The falls in IOP mediated by each drug were expressed as percentages in order to obtain a more direct comparison of the effects of each drug, as initial IOP was rather variable. Timolol and oxprenolol yielded a significantly greater drop in IOP than the other β-antagonists ($P<0.025$).

When calculated in absolute terms, control eyes exhibited a statistically insignificant rise in IOP of

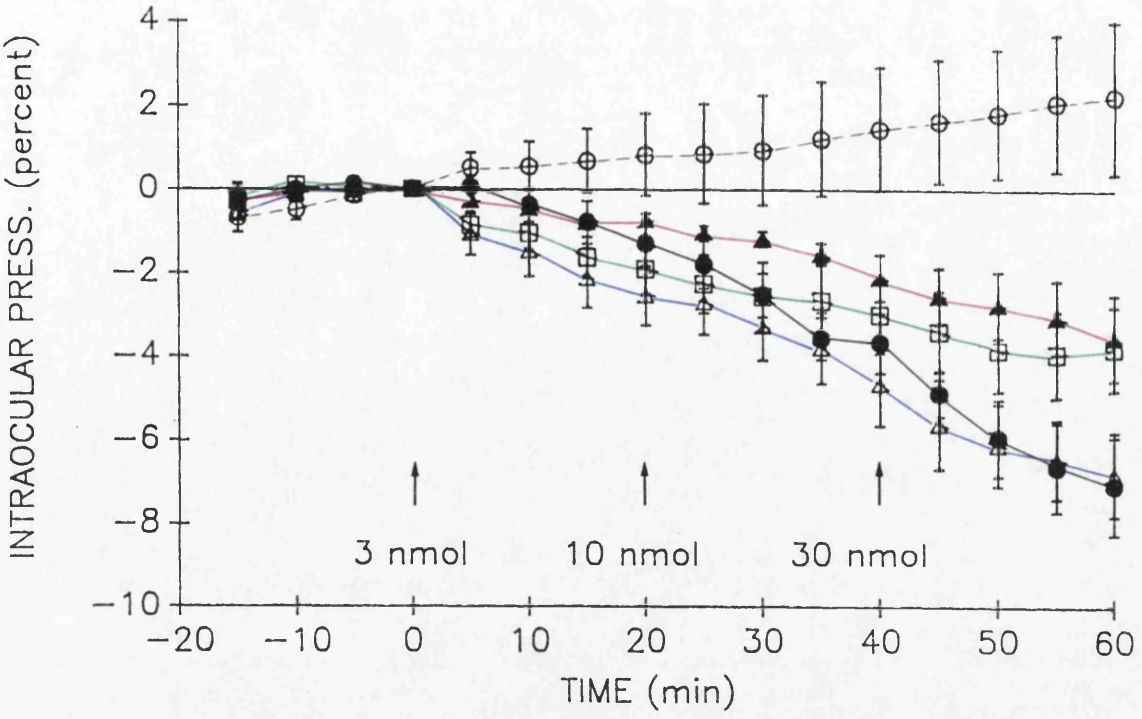


Fig.20. % Change in IOP vs. Time.
P values are identical to corresponding values in Table 2 for absolute fall in IOP. Vertical Bar shows s.e.m.

Saline Control ○- - -○ Timolol ●——● Oxprenolol △——△
Betaxolol ▲——▲ Laevobunolol □——□

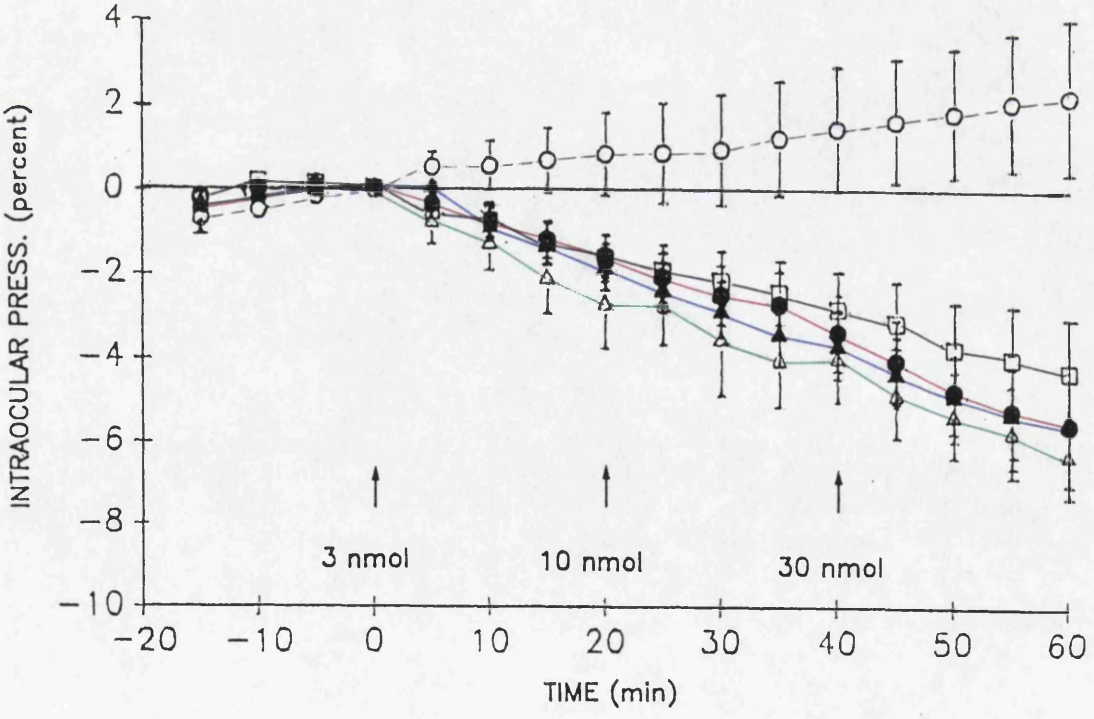


Fig.21. % Change in IOP vs. Time.
P values are identical to corresponding values in Table 2 for absolute fall in IOP. Vertical Bar shows s.e.m.

Saline Control ○- - -○ Dihydrolaevobunolol □——□
Metoprolol ▲——▲ Metipranolol ●——● Carteolol △——△

0.2±0.2mmHg (n=6). The corresponding significant absolute fall in IOP in terms of mmHg pressure in response to 3, 10 and 30nmol bolus injections of the β-adrenoceptor antagonists was as follows in Table 3:

β-Adrenoceptor Antagonist	n	Fall in IOP (mmHg)	P
Betaxolol	9	0.4±0.1	<0.01
Laevobunolol	9	0.4±0.1	<0.005
Oxprenolol	10	0.6±0.1	<0.005
Timolol	11	0.7±0.1	<0.005
DHL	11	0.6±0.1	<0.005
Metoprolol	11	0.6±0.1	<0.005
Metipranolol	10	0.5±0.1	<0.005
Carteolol	9	0.6±0.1	<0.005

Table 3. Fall in IOP in response to β-antagonists, 60 min after bolus injections of 3, 10 and 30nmol of drug. Values given are mean±s.e.m. P represents significance of difference between 60 min and time zero results as indicated by unpaired Student's t-test.

Arterial perfusion pressure of 29.5±0.5mmHg (n=80) was unaltered by any of the β-adrenoceptor antagonists tested under these conditions of low vascular tone.

Effect of Acetazolamide on IOP

A bolus injection of 0.3μmol acetazolamide produced a fall in IOP within 15 min (Fig.22). Again, further doses of 1 and 3μmol were given in an attempt to test dose dependence

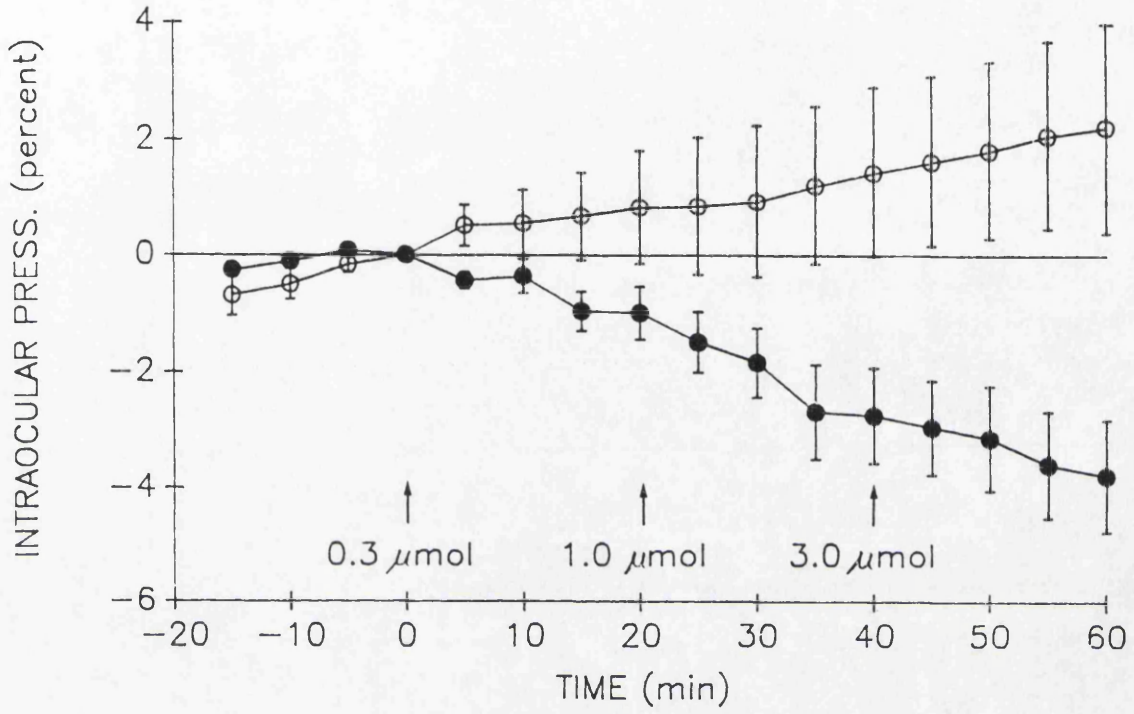


Fig.22. % Change in IOP vs. Time.
Vertical Bar shows s.e.m.

Saline Control o——o Acetazolamide ●——●

of the response. The drug effects were compared with control perfusions in which saline was injected. Control eyes exhibited a slight tendency for IOP to rise over 60 min of perfusion. Acetazolamide (0.3, 1 and 3μmol) produced a small fall in IOP: 3.8±1.0% ($P<0.005$, n=8), corresponding to a significant absolute fall in IOP: 0.4±0.1mmHg ($P<0.005$, n=8).

Effect of Dopamine Agonist FPL 65879AA on IOP

The specific DA_2 agonist FPL 65879AA, in bolus doses from 30nmol to 30μmol, was found to have no significant effect upon IOP in comparison with corresponding injections of saline control.

Single Dose Timolol: IOP-Time Curve

A single bolus injection of 10nmol timolol yielded a sustained drop in IOP, falling to a plateau after a period of 50 min, which persisted over the 100 min of perfusion, as seen in Fig.23. The dose of timolol utilised yielded a significant total drop in IOP of 10.4±1.2% ($P<0.005$, n=6), measured as the difference between the initial IOP at time zero (drug injection) and the new IOP after development of the IOP plateau, 100 min after drug injection. This

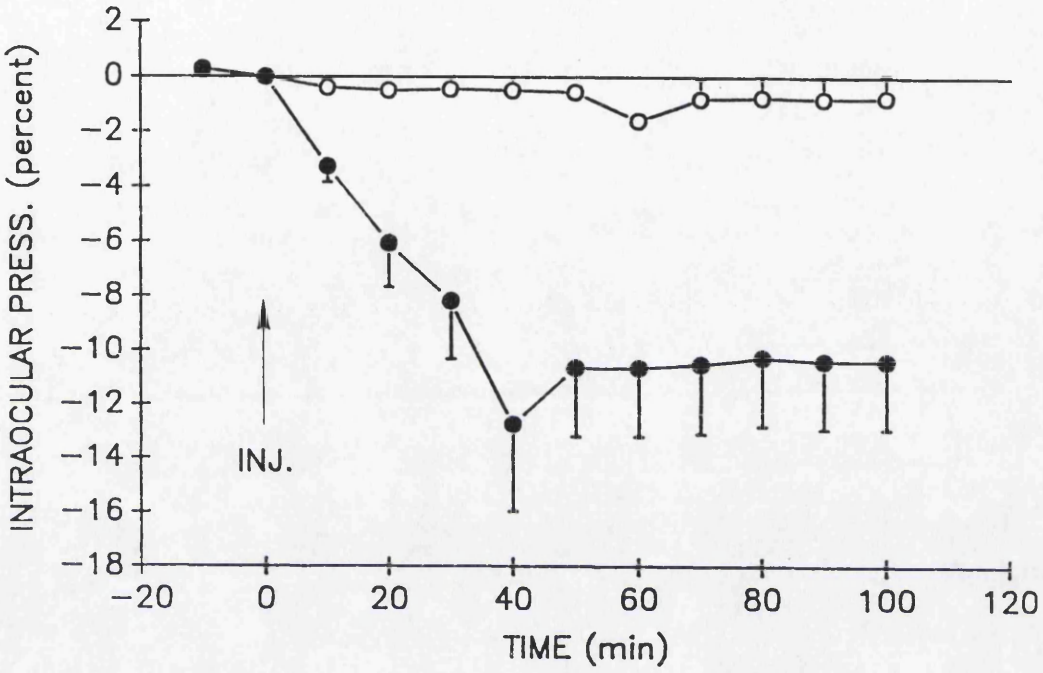


Fig 23 % Change in IOP vs. Time. Single Dose Timolol. Vertical Bar shows s.e.m.

Saline Control o——o Timolol •——•

corresponded to a significant absolute drop in IOP of 1.0±0.3mmHg ($P<0.005$, $n=6$) over the 100 min of perfusion. Control eyes injected with saline in this case exhibited a small but statistically insignificant drop in IOP of 0.1±0.1mmHg ($n=9$).

Log Dose-Response Curves for IOP Effects of Timolol and Carteolol

Experiments were performed as in procedure 4 with a single bolus dose of timolol, however in this case the drop in IOP mediated by a variety of doses of timolol and carteolol were ascertained. The results are shown in table 4.

Drug	Bolus Dose (nmol)	n	Total Drop in IOP (mmHg)	P
Timolol	0.03	6	0.2±0.1	n.s.
	0.3	6	0.3±0.1	<0.05
	1.0	5	0.4±0.1	<0.01
	3.0	6	1.0±0.2	<0.005
	10.0	6	1.0±0.1	<0.005
	30.0	6	1.1±0.2	<0.005
Carteolol	0.03	6	0.2±0.1	n.s.
	0.3	7	0.3±0.2	n.s.
	1.0	6	0.4±0.1	<0.01
	3.0	6	0.5±0.2	<0.025
	10.0	5	0.7±0.1	<0.005
	30.0	6	0.7±0.2	<0.005

Table 4. Drug, bolus dose and total drop in IOP. Values given are means±s.e.m. P represents significant difference between IOP at time zero and IOP at 100 min as indicated by unpaired Student's t-test.

Plotting the data in table 4 as the total drop in IOP as ordinate against $\log_{10}$ (number of moles of drug injected as bolus) as abscissa yields the log dose-response curves for the IOP effects of timolol and carteolol as seen in Fig.24. These log dose-response curves indicate a dose-dependence of the pressure lowering effect of each drug, with a maximum effect seen at 30nmol bolus dose approx. and ED_{50} of 1nmol approx., for both. Carteolol, however, appears to be less efficacious as an ocular hypotensive agent than timolol upon the bovine perfused eye, yielding a maximum drop in IOP of 0.7±0.1mmHg (n=5), as opposed to the drop of 1.1±0.1mmHg (n=6) obtained with timolol.

It appears from these log dose-response curves that the potency ratio of carteolol to timolol as ocular hypotensive

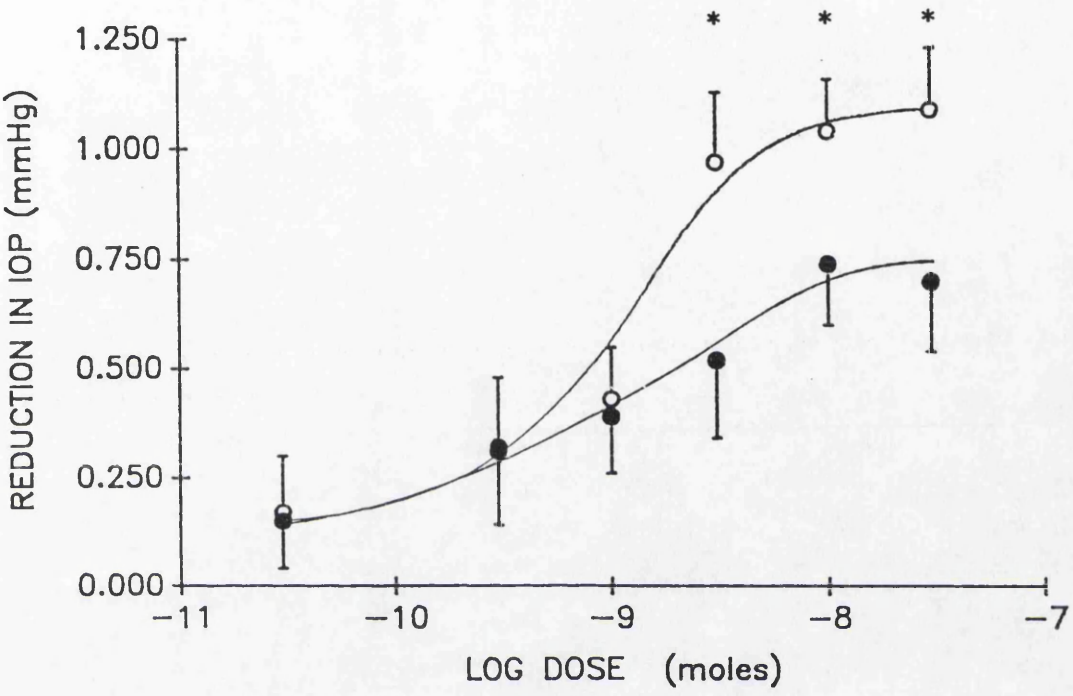


Fig.24. IOP Log Dose-Response.
Vertical Bar shows s.e.m.
*P<0.025. Differences between drug treated groups as indicated by unpaired Student's t-test.

Timolol ○——○ Carteolol ●——●

agents is approx. unity when administered to the bovine perfused eye preparation.

Effect of Atriopeptin (AP) on IOP

The effect of AP (rat: fragment 1-27) upon IOP (Fig.25) could be seen approx. 15 min after bolus injection of 10pmol. A further dose of 50pmol was given 40 min later. Drug effects were compared with data from control perfusions. AP (10 and 50pmol) produced a significant reduction in IOP of 9.2±2.0% ($P<0.005$, n=9), corresponding to a significant absolute drop in IOP of 0.9±0.2mmHg ($P<0.005$, n=9). Arterial perfusion pressure was unaltered by AP under these conditions of low vascular tone.

Effect of AP on Ciliary Artery Perfusion

Vascular tone was significantly increased by 36.5±2.9mmHg ($P<0.005$, n=87) on addition of noradrenaline bitartrate at 10^{-5} M to the perfusate reservoir. Perfusion of noradrenaline resulted in a rapid and continual fluctuation in IOP, which effectively nullified equilibration of IOP, thus rendering it impractical to conduct further investigation of the effects of drugs on IOP. For this reason, investigation of the vascular effects

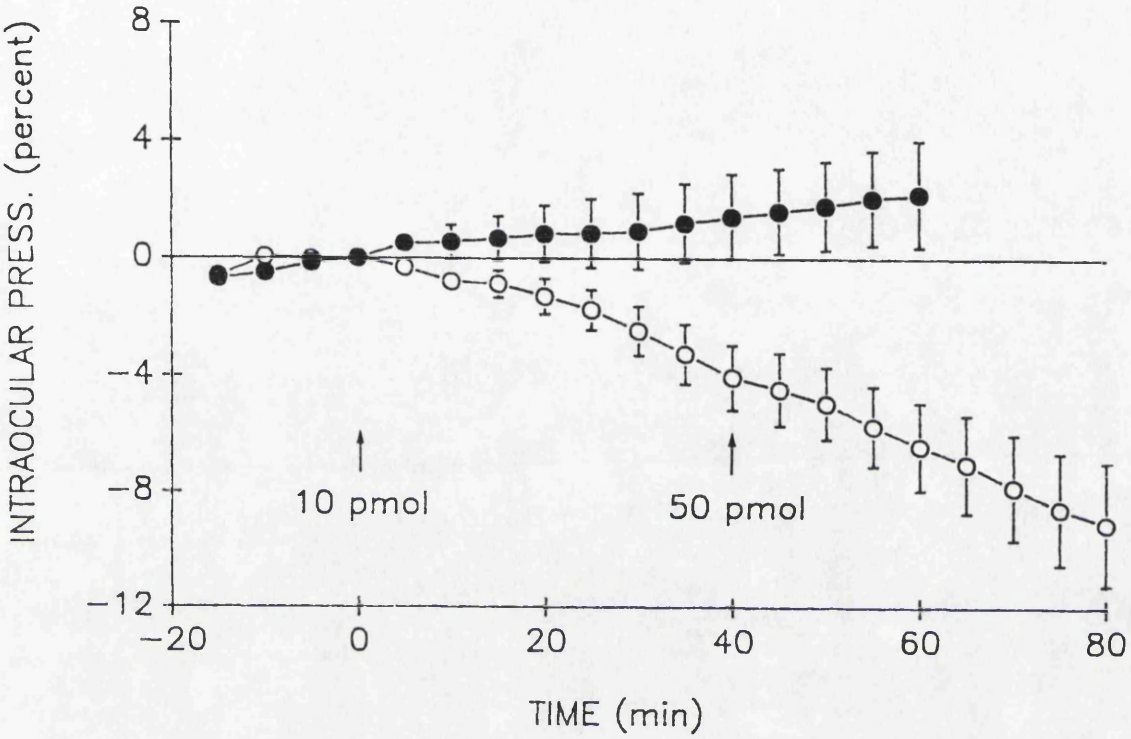


Fig 25. % Change in IOP vs. Time.
Vertical Bar shows s.e.m.

Saline Control ●——● Atriopeptin o——o

of drugs, and their IOP effects, were not undertaken simultaneously. Vascular relaxation to bolus doses of AP (10, 30 and 100pmol) in the presence of noradrenaline was negligible, being similar to that produced by equivalent bolus injections of saline. Out of 10 such experiments, a small reduction in arterial perfusion pressure (approx. 10% of the induced tone) was seen in only 3 eyes. Data from one such experiment are shown in Fig.26. This contrasts with the reproducible and significant dilatation of the ciliary vasculature under tonic constriction produced by bolus injections of 0.3 and 3nmol sodium azide.

Effects of Other Vasodilator Drugs on Ciliary Artery Perfusion

Again, vascular tone was increased by the addition of noradrenaline to the perfusate reservoir at a concentration of 10^{-5} M. Data obtained in one such procedure with sodium azide are shown in Fig.27. The percentage relaxation of the vascular tone induced by noradrenaline was calculated in response to each dose of drug. As can be seen in Fig.27, injections of saline in this preparation produced no effect whatsoever upon vascular tone. Full recovery of tone was allowed before injection of the next dose of drug. The results for sodium nitroprusside, sodium azide, cromakalim,

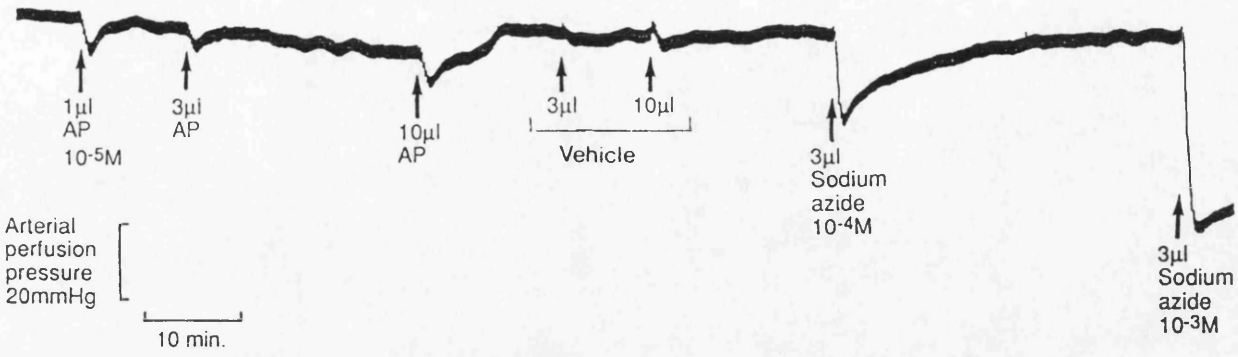


Fig.26. Effect of Atriopeptin upon Ciliary Artery Perfusion Pressure.

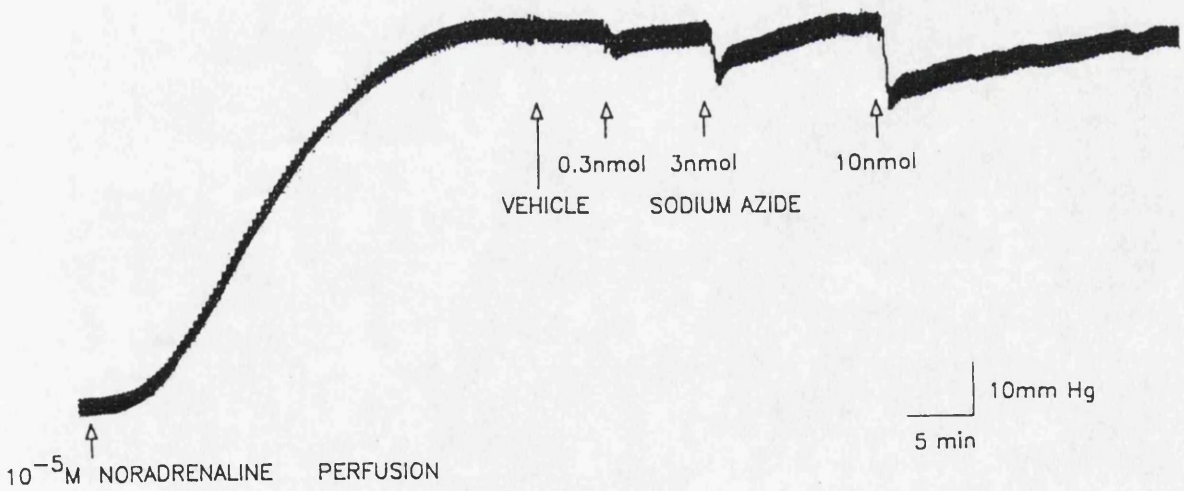


Fig.27. Effect of Sodium Azide upon Ciliary Artery Perfusion Pressure.

pinacidil, verapamil and nifedipine are shown in Fig.28, where all the data has been assembled for ease of comparison of different drugs. For details of the magnitude and statistical significance of these responses, the same data are shown in table 5.

Vasodilator	Bolus Dose (nmol)	n	Relaxation of Total Vascular Tone Induced by Noradrenaline (Percent)	P
SNP	0.01	9	3.6±1.0	<0.005
	0.03	9	1.3±0.7	<0.05
	0.1	18	7.1±0.8	<0.005
	0.3	17	13.0±2.3	<0.005
	1.0	18	33.8±6.5	<0.005
	3.0	13	39.0±4.5	<0.005
	10.0	7	40.3±5.8	<0.005
	30.0	11	61.9±5.2	<0.005
	100.0	7	79.2±12.3	<0.005
	300.0	4	76.3±7.1	<0.005
	1000.0	4	90.9±3.9	<0.005
	3000.0	4	88.3±7.5	<0.005
Sodium Azide	0.01	7	1.9±0.6	<0.01
	0.03	9	9.1±3.2	<0.025
	0.1	12	22.1±6.5	<0.005
	0.3	23	27.3±5.2	<0.005
	1.0	13	39.9±8.4	<0.005
	3.0	23	49.4±5.8	<0.005
	10.0	21	65.6±7.8	<0.005
	30.0	20	62.3±10.1	<0.005
	100.0	5	71.4±7.8	<0.005
	300.0	4	81.2±11.7	<0.005
	1000.0	4	82.5±5.2	<0.005
Cromakalim (BRL 34915)	0.3	6	8.2±1.5	<0.005
	1.0	6	11.3±3.3,	<0.01
	3.0	6	25.1±8.2	<0.025
	10.0	14	56.4±3.6	<0.005
	30.0	11	63.1±7.2	<0.005
Pinacidil	0.1	12	0.6±0.5	n.s.
	0.3	12	1.0±0.4	<0.025
	1.0	12	5.2±3.2	n.s.
	3.0	12	9.1±3.2	<0.01
	10.0	12	35.1±7.5	<0.005
	30.0	10	55.8±8.4	<0.005
	100.0	13	70.1±9.7	<0.005
	300.0	14	79.2±10.4	<0.005
	1000.0	13	98.0±11.0	<0.005
	3000.0	4	98.7±0.9,	<0.005

Table 5 Continued/

Verapamil	0.1	6	12.3±1.0	<0.005
	0.3	7	15.4±2.8	<0.005
	1.0	7	35.4±6.2	<0.005
	3.0	9	36.4±3.8	<0.005
	10.0	8	44.1±8.2	<0.005
	30.0	11	51.8±6.2	<0.005
Nifedipine	0.01	11	4.6±1.8	<0.025
	0.03	12	13.3±3.6	<0.005
	0.1	11	23.6±5.6	<0.005
	0.3	10	29.7±5.7	<0.005
	1.0	7	36.7±4.6	<0.005
	3.0	7	44.1±6.7	<0.005
	10.0	9	51.3±10.1	<0.005
	30.0	9	56.4±9.2	<0.005

Table 5. Effects of vasodilator drugs on ciliary artery perfusion pressure in the presence of noradrenaline (10^{-5} M). Values given are means±s.e.m. P represents significant difference between relaxation in response to drug and control as indicated by unpaired Student's t-test.

The vasodilators tested all produced substantial and reproducible vasodilatation over the dose range 0.1nmol to 30nmol.

Addendum I

A maximum of seven injections of drug per eye were given, as each individual eye remained viable for no more than 2 to 3h.

IOP Effects of Other Vasodilator Drugs

The dose-response relationship was examined in detail for the effect upon IOP of sodium azide, SNP and pinacidil. The data are shown graphically in Fig.29. and the details are given in table 6.

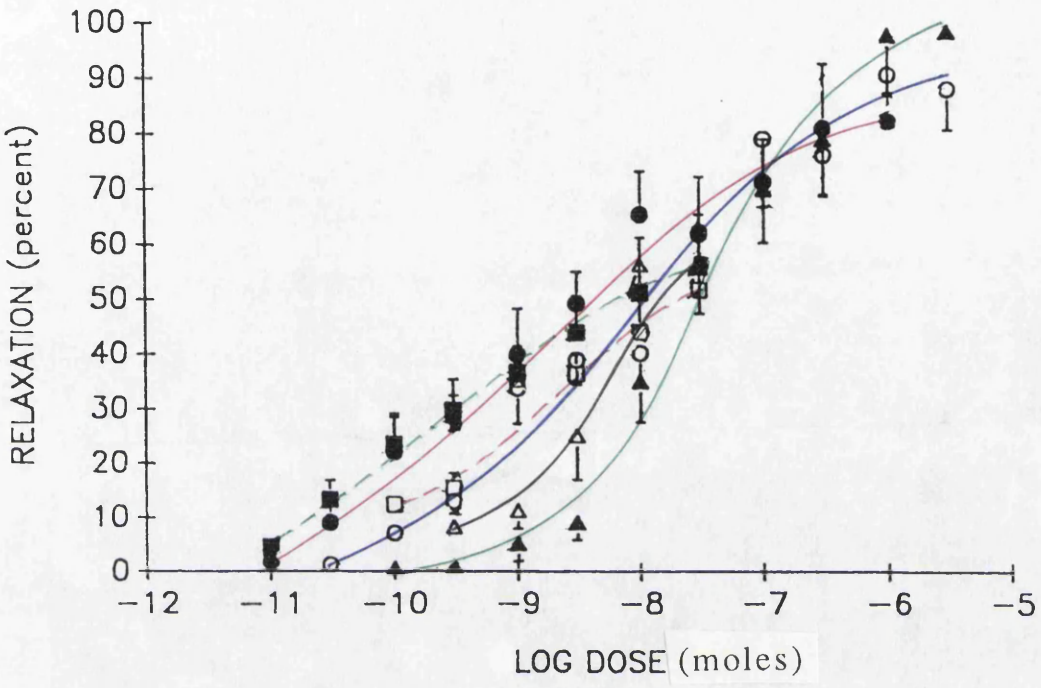


Fig.28. Vascular Log Dose-Response.
Vertical Bar shows s.e.m.

Sodium Nitroprusside
Cromakalim
Verapamil
Sodium Azide
Pinacidil
Nifedipine

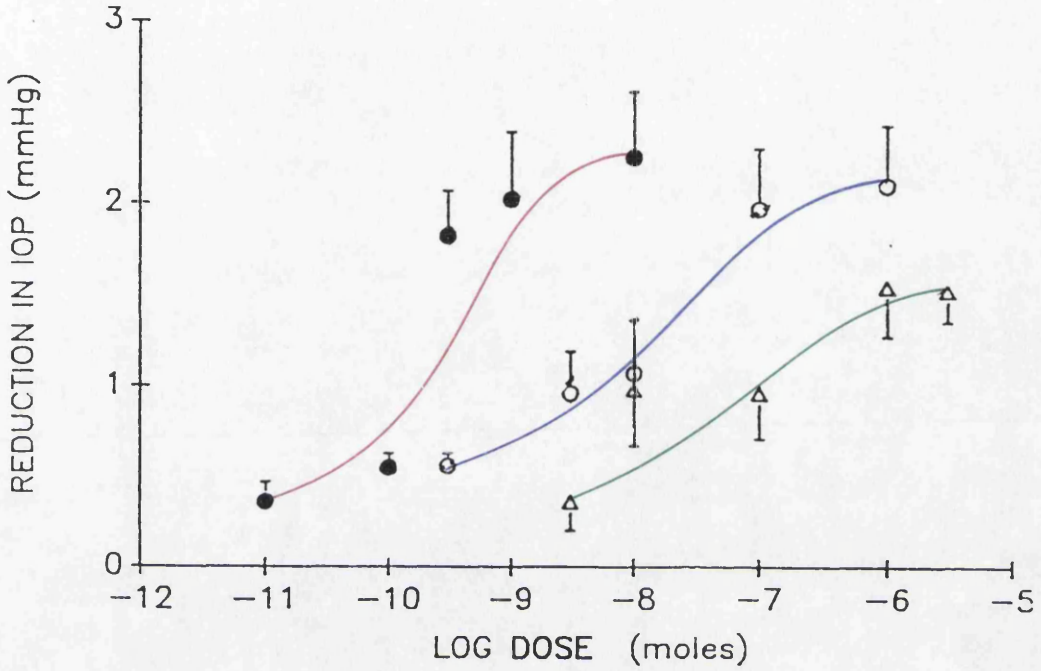


Fig.29. IOP Log Dose-Response.
Vertical Bar shows s.e.m.

Sodium Nitroprusside ○——○ Sodium Azide ●——●
Pinacidil △——△

Vasodilator	Bolus Dose (nmol)	n	Drop in IOP (mmHg)	P
SNP	0.3	6	0.6±0.1	<0.005
	3.0	7	0.9±0.2	<0.005
	10.0	10	1.1±0.3	<0.005
	100.0	8	2.0±0.3	<0.005
	1000.0	7	2.1±0.3	<0.005
Sodium Azide	0.01	7	0.4±0.1	<0.01
	0.1	3	0.6±0.1	<0.005
	0.3	5	1.8±0.2	<0.005
	1.0	5	2.0±0.4	<0.005
	10.0	7	2.2±0.3	<0.005
Pinacidil	3.0	6	0.4±0.2	<0.05
	10.0	6	1.0±0.3	<0.01
	100.0	9	1.0±0.2	<0.005
	1000.0	9	1.5±0.3	<0.005
	3000.0	6	1.5±0.2	<0.005

Table 6. Effects of vasodilator drugs upon IOP. Values given are means±s.e.m. P represents significant difference between IOP at time zero and IOP at 100 min as indicated by unpaired Student's t-test.

The log dose-response curves for the IOP effect of these drugs indicate a dose dependence of the pressure lowering effect of each drug, with maximum doses and ED_{50}'s as listed in table 7.

Vasodilator	Maximum IOP Reducing Dose (nmol)	ED_{50} (nmol)
SNP	1000	10
Sodium Azide	10	0.3
Pinacidil	1000	100

Table 7. Vasodilator, maximum IOP reducing dose and ED_{50}

Examining the maximum response of each drug, pinacidil appears to be significantly less efficacious than either SNP or sodium azide.

Investigation of EDRF Production by Uveal Vascular Endothelium

Table 8 indicates the vascular relaxation response to tone induced by the presence of noradrenaline, mediated by bolus doses of ACh. in the presence and absence of L-NOARG. It can be seen that L-NOARG diminishes significantly the vasorelaxant effect of ACh.

Bolus Dose ACh (nmol)	n	Relaxation of Total Vascular Tone Induced by Noradrenaline (Percent)	Relaxation of Total Vascular Tone Induced by Noradrenaline (Percent - in presence of L-NOARG)	P
0.03	10	3.6±2.5	3.6±2.4	n.s.
0.1	10	10.8±3.0	4.3±4.0	<0.05
0.3	10	11.4±4.8	4.9±1.2	<0.025
1.0	10	16.9±5.9	4.6±3.8	<0.005
3.0	10	21.9±6.7	8.7±5.2	<0.01
10.0	8	23.8±6.4	9.1±5.5	<0.005
30.0	8	22.6±7.1	8.9±5.3	<0.01
100.0	6	28.0±7.5	9.8±5.4	<0.005

Table 8. Effect of ACh on ciliary artery perfusion pressure in presence of noradrenaline (10^{-5} M) and noradrenaline plus L-NOARG ($3X10^{-5}$ M). Values given are means±s.e.m. P represents significant difference between relaxation in response to ACh in eyes perfused with noradrenaline, before and after addition of L-NOARG to perfusate, as indicated by paired Student's t-test.

Ciliary Cyclic GMP Determinations

Table 9. of drug treatment and ciliary cyclic GMP indicates that sodium azide yielded a significant increase in ciliary cyclic GMP compared with saline treated control. AP also yielded a significant increase in ciliary cyclic GMP compared with saline treated control, although the increase was smaller in this case.

Drug Treatment	n	Ciliary Cyclic GMP fmol/mg protein	P
Saline Control	6	126.8±16.8	
Sodium Azide (10nmol)	6	232.9±27.4	<0.005
AP (0.1nmol)	6	209.2±22.1	<0.01

Table 9. Effects of drugs on ciliary cyclic GMP levels. P represents significance of difference between control and drug treated groups as indicated by unpaired Student's t-test.

Drug Effects on Vascular Flow Using Radiolabelled Microspheres

Of all the structures within the eye which were dissected out, significant quantities of spheres were found only within the iris, ciliary body and choroid/sclera. Activity within the choroid/sclera was assumed to be due to the entrapment of spheres in the choroid, as the sclera is relatively avascular. Activity found within the cornea, retina and extra-ocular muscle was negligible, and no activity was detected in the vitreous or aqueous. The introduction of noradrenaline at 10^{-5} M into the perfusate caused a significant increase in arterial perfusion pressure from 34.0±5.1mmHg to 71.0±7.4mmHg ($P<0.005$, n=45), and a significant decrease in the number of spheres entering the ocular circulation, from 1118±120 (24.8%±2.7%), n=20, to 819±138 (18.2%±3.1%), n=21, $P<0.05$. The vast majority of the remaining spheres were found in the perfusate tubing

proximal to the eye, and in the cannula. A very small proportion (0.8±0.2%, n=45) were found in the effluent perfusate leaving the eyes through the venous outflow. The observed activity for each tissue and treatment group is shown in Fig.30. The data indicate that timolol or carteolol at 30nmol bolus dose do not significantly increase the numbers of spheres entrapped in the iris, ciliary body or choroid, as compared with control. Bolus doses of 300nmol SNP or verapamil elicited significant increases in the numbers of spheres entrapped in the iris, ciliary body and choroid in the noradrenaline perfused group ($P<0.01$, Mann-Whitney test), and in separate experiments, significant reduction in arterial perfusion pressure of 29.8±4.3mmHg (mean±s.e.m., $P<0.05$, n=10). The observed activity for each tissue in each treatment group in the absence of noradrenaline is shown in Fig.31. Timolol at 30nmol bolus dose was found to reduce the numbers of spheres entrapped in the choroid ($P<0.01$, Mann-Whitney test). When administered at 300nmol bolus dose, a significant reduction in the numbers of spheres was observed in the iris ($P<0.01$, Mann-Whitney test). Carteolol at 30nmol bolus dose yielded a significant reduction in the numbers of spheres entrapped in the iris, ciliary body and choroid ($P<0.01$, Mann-Whitney test). Carteolol at 30nmol bolus dose, however yielded a small but significant increase in arterial perfusion pressure of 8.22±2.33mmHg (mean±s.e.m., $P<0.025$, n=10, student's t-test) in the non-noradrenaline perfused eyes.

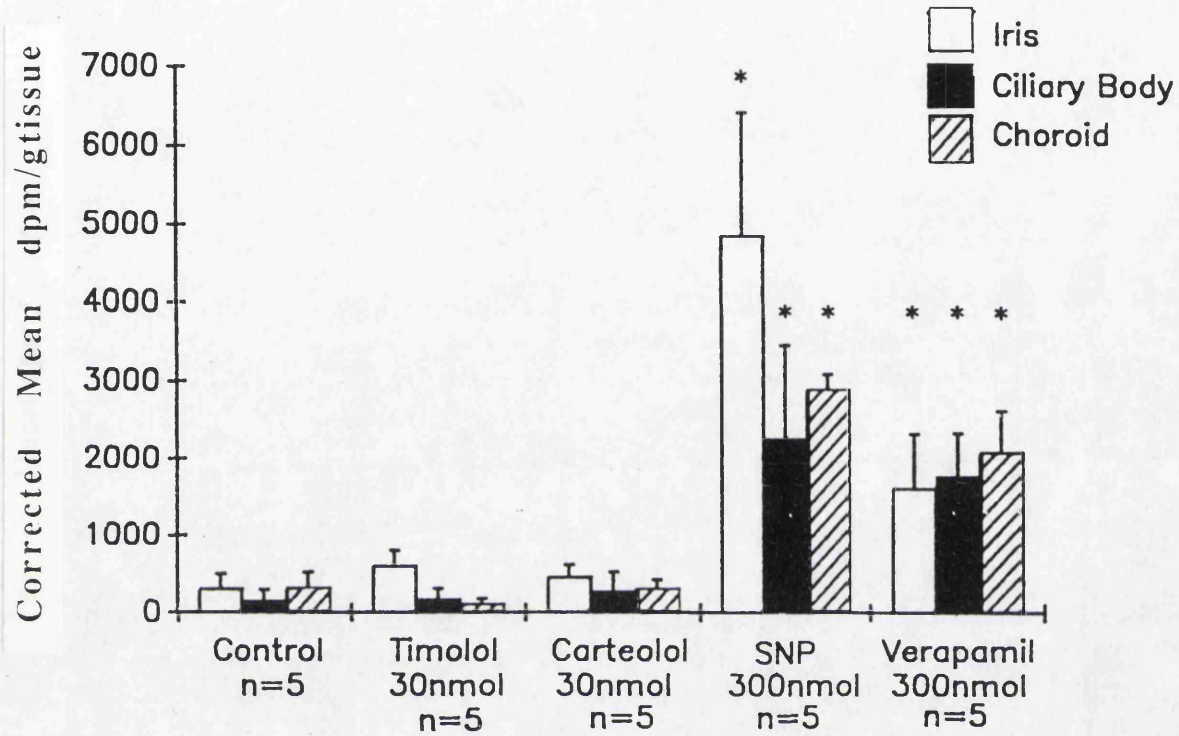


Fig.30. Radioactivity in Various Ocular Tissues following Perfusion with Labelled Microspheres. Results Indicated are Means of 5 experiments. Vertical Bar shows s.e.m. Prior to each experiment, Noradrenaline was incorporated into Krebs solution at 10^{-5} M concentration.
*P<0.01. Differences from control as indicated by Mann-Whitney Test.

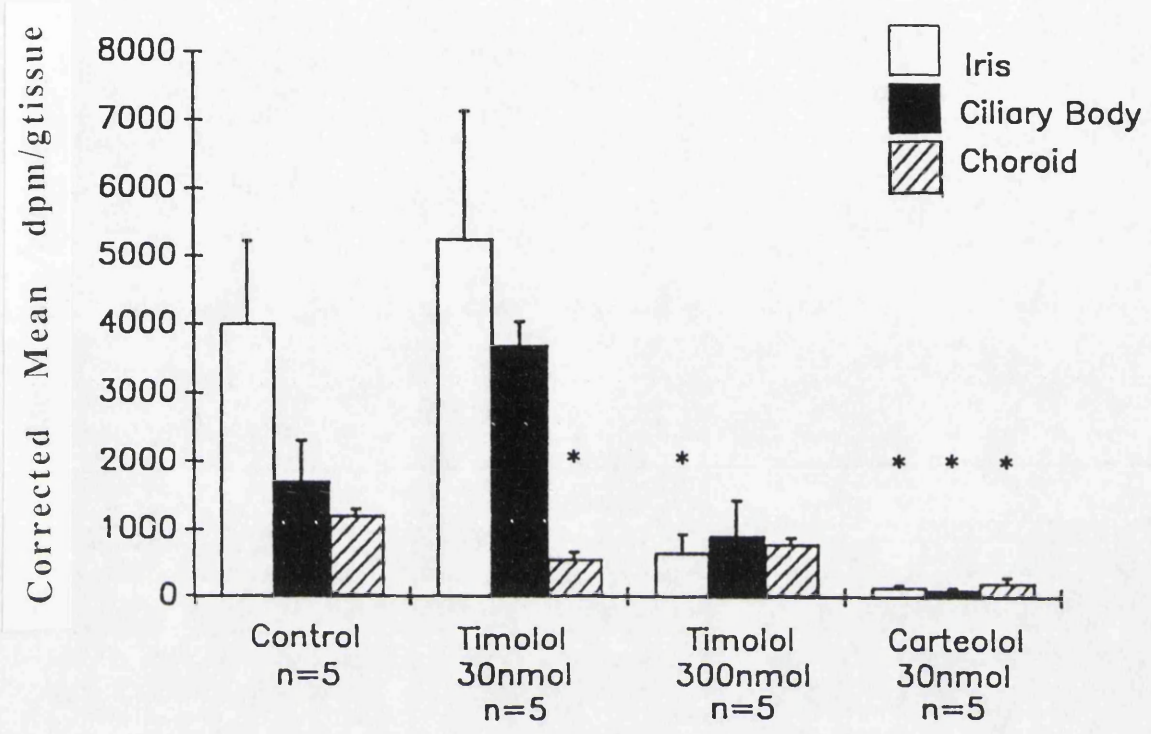


Fig.31 . Radioactivity in Various Ocular Tissues following Perfusion with Labelled Microspheres. Results Indicated are Means of 5 experiments. Vertical Bar shows s.e.m.
*P<0.01. Differences from control as indicated by Mann-Whitney Test.

lasting for approximately 1 min.

DISCUSSION

The Isolated Arterially Perfused Eye

Studies of ocular physiology utilising the isolated perfused eye preparation are scarce in the literature. However, results obtained via experimentation upon perfused eye preparations from several species have been reported. Facility of outflow in the bovine (Erickson-Lamy et al., 1988) and human (Erickson-Lamy et al., 1991; Liang et al., 1992) perfused anterior segment has been measured. Macri and Cevario (1973), using the feline eye, conducted an investigation into the "induction" of aqueous humour formation using the cholinergic drugs acetylcholine and eserine. In the feline perfused eye, some drugs produce the same effects as in the human, although results with timolol are conflicting (Helal et al., 1979; Macri et al., 1980). Arachidonic acid is reported to decrease aqueous humour secretion in this preparation (van Alphen & Macri, 1981). Kodama et al. (1985) employing the rabbit perfused eye, studied the effects of a selection of ocular hypotensive drugs upon aqueous humour formation, which was found to be significantly reduced by timolol, adrenaline and (at high concentration) acetazolamide. The bovine perfused eye has also been described, in a study of glutathione depletion and oxidative stress (Kishida et al., 1985) and in a preliminary communication on drug effects on IOP (Wilson, 1988). Although they recommended the bovine perfused eye for biochemical studies, Kishida et al. judged this preparation

to be unsuitable for the investigation of aqueous humour dynamics. However, these authors commenced perfusion within 1 to 2h post mortem, which may explain their dissatisfaction with their results. The present results indicate that any delay in the commencement of perfusion beyond 1h after slaughter considerably reduces the probability of achieving a stable IOP. However, no correlation was found between success rate and postmortem time when not exceeding 50 min. In the present study, steady state IOP achieved in the isolated perfused bovine eye lies in the range 7 to 11 mmHg. In this preparation, the episcleral venous pressure is probably not much above zero, since the vortex veins are cut. Assuming that all the episcleral veins drain into the vortex veins, these values are equivalent to a range of 15.5 to 21.5 mmHg in vivo, if we are to assume an episcleral venous pressure of 9 mmHg in the living animal. This is in reasonable agreement with the report by Woelfel et al. (1964) that in the calf, IOP in vivo is 16.4±1.6 mmHg. Cannulation of the anterior chamber with a 23 gauge steel needle, and connection to a water manometer was chosen as the method of measurement of IOP in this preparation since this allowed for continual measurement of IOP over the period of experimentation. In addition, the relatively small changes in IOP which occurred could be very accurately and reproducibly determined, since even a small change of IOP in mmHg pressure shows as a considerably greater corresponding change in mmH_2O pressure (there being approx.

13.6 mmH_2O to every 1mmHg). Tonometry does not guarantee as reproducible results as the anterior chamber cannulation method. Electronic measurement and recording might well involve baseline drift in excess of 1mmHg over a 1 to 2h period, and further would probably disturb the equilibration of the preparation at each attempt to take a reading. Additionally, tonometry would be less sensitive to the small changes in IOP being measured.

The constant flow method of perfusion was adopted as the preferred method. Earlier work with the bovine perfused eye by Wilson (1988) included a procedure whereby bovine eye preparations were perfused via a constant pressure method, while flow rate was monitored electronically. However, it was found that this method of perfusion resulted in significantly lower starting IOP, and a rather lower proportion of eyes whose IOP remained stable during the pre-drug part of the experiment.

After arterial cannulation, some eyes were rejected due to a failure to achieve adequate perfusion. Adequate perfusion was judged on the basis of the following criteria: that there should be at least two vortex veins flowing, and that at no point during the initial phase of perfusion should perfusion pressure rise above 100 mmHg. Additionally, in the pre-drug part of the experiment, perfusion pressure and IOP should equilibrate between 20 and 60 mmHg, and 7 and 11 mmHg pressure respectively, within a period of 90 min after commencement of perfusion. The

Addendum II

Control eyes in some experiments showed an acceptable small (but statistically significant) tendency for IOP to rise (Figs. 20, 21, 22 & 25), but not in others (Fig.23). This was probably due to a progressive slight increase in the permeability of the blood-aqueous barrier over the course of perfusion, in some eyes.

overall rejection rate based upon these criteria was 34%, however the rejection rate after drug injection was found to be only 6%.

Wilson (1988), describing the constant pressure perfusion of the bovine eye, inferred that the addition of dextran and albumin to the Krebs physiological saline solution was beneficial towards maintaining the integrity of the blood-aqueous barrier. However, it has been found subsequently that provision of oncotic pressure in the perfusion fluid does not significantly protect the barrier, during the course of the perfusion procedure (Wilson et al., 1993). No provision was made therefore for oncotic pressure in the perfusate: the immediate benefit of this being the absence of albumin in the perfusate and therefore the negation of any problems of drugs injected into the perfusate binding to the albumin, and thus being unavailable for the mediation of a pharmacological effect. The ability of the preparation to yield reproducible results in the absence of oncotic pressure was not found to be significantly compromised. Further, it has been shown that the provision of oncotic pressure in the perfusate via the addition of dextran and albumin does not significantly protect the blood aqueous barrier for the purposes of the study of intraocular pressure. Wilson et al. (1993) report that a correlation exists between rising IOP and leakage of albumin into the chamber.

In an effort to determine that the tissues of the isolated perfused eye are metabolically active, an estimate was made of O_2 consumption and CO_2 production by perfused fresh eyes, and also by perfused dead eyes (Wilson et al., 1993). Fresh bovine perfused eyes showed a significant net consumption of O_2 and production of CO_2 when compared with eyes which had been placed in cold storage for 24h prior to perfusion. By varying the perfusion rate through the ciliary artery it is possible correspondingly to increase the perfusion pressure. By allowing IOP to stabilise at each step, it has been shown that an 80% increase in perfusion pressure results in less than a 9% increase in IOP (Wilson et al., 1993). This clearly provides evidence that the production of aqueous humour in the bovine perfused eye is an active secretory process, not one of passive ultrafiltration or leakage.

The advantages offered by this preparation as a model for the study of aqueous humour dynamics lie in the convenience of an easily obtained and inexpensive tissue, whose use does not require the killing of animals purely for experimental purposes, an issue over which public concern has been mounting (Fox, 1984).

Although drugs are not usually delivered via the arterial route in vivo, in the perfused eye the intra-arterial route of drug administration affords a much more accurate estimate of the speed of drug response than does the topical route which is conventionally used. If a known

concentration of drug is perfused (instead of injecting bolus doses) then the drug concentration in the vicinity of the target cells will be accurately known. This avoids the very large losses of drug which can occur during corneal and conjunctival absorption, and due to diffusion through other ocular tissues and fluids. This preparation lends itself to studies of drug mechanisms on aqueous humour dynamics especially where the drug may have effects on the blood vessels or on the blood-aqueous barrier.

IOP Effects of the β-Adrenoceptor Antagonists

Results obtained with the β-adrenoceptor antagonists confirm that the standard β-adrenoceptor antagonists used in the treatment of COAG are able to lower IOP in this preparation, in a reproducible and dose-dependent manner. However, it was impractical to test numerous drugs at various doses, up to those doses producing maximal responses, due to the great volume of work which this would incur. Hence it is not possible to ascribe accurately any potency differences between them in their pressure lowering effects. It would appear, however, that timolol, oxprenolol and metoprolol are the most potent, whilst betaxolol and laevobunolol are the least. Carteolol appears to be almost as potent as timolol, a little surprising since results to clinical trials indicate that carteolol at 1% is about

equipotent with timolol at 0.25% (Horie et al., 1982). On the other hand, betaxolol in the clinical situation is reportedly less potent than timolol (Allen et al., 1986; Allen & Epstein, 1986).

In order to obtain more useful data concerning the ocular hypotensive effect of the β-adrenoceptor antagonists, a modified experimental approach was initiated. It may be observed from the % Change in IOP - Time curves obtained with the β-adrenoceptor antagonists (Figs.19 & 20) that the total fall in IOP elicited by each drug at the given dosages does not appear to be complete: rather, what is shown is the first part of the fall in IOP. Were the time axis to be extended further, then, based on the experimental results, one would expect to see a further drop in pressure. The pressure drop had commenced approx. 10 min after injection of the initial bolus dose of drug, and showed little sign of abating before injection of the following dose. When the effects of injecting a single bolus dose of timolol were monitored for a longer time (Fig.22), the IOP was seen to stabilise after approx. 50 min. By 100 min the plateau on the IOP-Time curve is completely formed. By repeating the procedure with various bolus doses of timolol, significantly different total drops in IOP can be seen in each case, facilitating the construction of log dose-response curves, as seen for timolol and carteolol (Fig.24).

Clinically, when a dose of β-adrenoceptor antagonist is given topically, IOP starts to fall but eventually forms a

stable lower IOP. This is sustained typically for a period of approx. 12h, whereupon it will start to rise once again, unless a further dose of drug is given to keep it at its lower level. The situation clinically, however, differs from the experimental situation in that the drug, given topically, and at very high concentration (0.006 to 0.06M approx.) sets up a relatively large depot in the inferior fornix, and absorption leads to the establishment of a much smaller depot in the conjunctiva, cornea, iris and ciliary body (Maurice & Mishima, 1984). The drop in IOP is relatively great, of the order of 5 to 10mmHg, as compared with the perfused eye preparation. The fact that only relatively small drops in IOP were seen in the present method was most probably due to the fact that episcleral venous pressure is probably equal to zero in this preparation, as previously discussed, and the drugs were given arterially as a bolus injection, so that within a short space of time the majority of the drug was washed out of the preparation. Further, species differences between the bovine and human eye may play a part in the relatively modest response to the β-adrenoceptor antagonists, as well as the fact that the bovine eyes may have been compromised by trauma, anoxia and lack of normal nutrition.

There is much evidence that the IOP lowering effect of the β-adrenoceptor antagonists is not mediated by binding to a classical β-adrenoceptor, as detailed previously. The ability of the β-adrenoceptor antagonists to reduce IOP in

Addendum III

It is, however, not known whether all neuronal noradrenaline is depleted in nerves associated with the iris/ciliary body of the bovine eye, even after isolation for periods of 2 to 3h.

the perfused eye implies that their effect is not dependent upon intact sympathetic innervation, nor upon the presence of circulating catecholamines. Since an antagonist does not generally produce any effect in the absence of its agonist, this provides further evidence that the ocular hypotensive effect of the β-adrenoceptor antagonists is mediated via some effect other than antagonism of a classical β-adrenoceptor.

It is generally accepted that stimulation of adenylate cyclase via a G protein is the post-receptor mechanism of the β-adrenoceptor. However, Shahidullah and Wilson (1992) reported that bolus injection of the β-adrenoceptor agonist terbutaline did not mediate an increase in ciliary cyclic AMP concentration, but did yield a significant decrease in the rate of secretion of aqueous in the bovine perfused eye preparation. In addition, forskolin was seen to mediate an increase in ciliary cyclic AMP concentration, but had no effect upon aqueous secretion. This, and other evidence (reviewed by Lotti et al., 1984) casts further doubt upon whether conventional β-adrenoceptors are involved in the ocular hypotensive effect of timolol. For example, Vareilles et al. (1977) noted that in rabbits a 500 times greater concentration of timolol was required in order to reduce water load induced elevation of IOP, than that required to antagonise the inhibitory effects of isoprenaline in the same assay. Further, the concentrations of timolol in the aqueous humour following instillation of

0.5% solution in rabbits were approx. 1000 times that required to antagonise β-adrenoceptors in other tissues. Bonomi et al. (1979) observed that the effectiveness of various β-adrenoceptor antagonists in lowering IOP in ocular hypertensive rabbits did not correlate with their peripheral β blocking potencies judged by inhibition of isoprenaline induced tachycardia. Similarly, Sears (1979) reported that preliminary studies indicate that the β-adrenergic binding and blocking potencies of propranolol, timolol and pindolol do not appear to parallel their ocular hypotensive effects. More recently, Schmitt et al. (1981b) found little relationship between the ability of timolol, propranolol, oxprenolol and practolol to antagonise the ocular hypotensive effect of isoprenaline in water-loaded rabbits. Additionally, the ability of these β-adrenoceptor antagonists administered topically to antagonise isoprenaline-induced elevation of cyclic AMP in the aqueous humour, was not correlated with their ocular hypotensive activity.

IOP Effects of Inhibitors of Carbonic Anhydrase

As a means of validating the bovine perfused eye as a preparation for studying drugs which lower IOP, the carbonic anhydrase inhibitor acetazolamide was tested. This compound is well documented as an ocular hypotensive agent in the

human eye and that of several animal species (Becker, 1954, 1955; Friedland & Maren, 1984). However, in the present preparation, the results with acetazolamide indicate that a very high concentration is required to produce only a relatively modest reduction in pressure. On the face of it this would suggest that the dependence of aqueous humour production on the active secretion of bicarbonate ion is probably of minor importance in the bovine eye. This result, however, contrasts with the findings of Wilson et al. (1993), that the new carbonic anhydrase inhibitor MK-927 will reduce both aqueous secretion rate in the bovine perfused eye, over the range 1 to 100nmol bolus dose. MK-927, however, has considerably higher lipid solubility than acetazolamide. Therefore it may be that MK-927 gains access more readily than acetazolamide to bovine ciliary carbonic anhydrase.

Involvement of Dopamine Receptors upon IOP

The DA_2 agonists bromocriptine, lergotrile and pergolide are known to lower IOP in the rabbit and monkey eye (Potter & Burke, 1983), and human eye (Geyer et al., 1987). The Fisons DA_2 agonist, FPL 65879AA, however, was found to be without effect over a relatively large dose range, up to and including some high doses. This is perhaps not entirely surprising, however since the site of action of

dopamine DA_2 agonists when acting as ocular hypotensive agents may well be central (Potter & Burke, 1982). The perfused eye preparation has no capacity for responding to a substance which elicits its effects in this way. No DA_2 receptors have been found in the ciliary tissue or elsewhere in the ocular structures of the rabbit (Mallorga & Sugrue, 1987).

Effect of AP on IOP

AP is observed to decrease IOP in rabbits (Sugrue & Viader, 1986; Steardo & Nathanson, 1987; Nathanson, 1987). Bianchi et al. (1986) postulated an inhibition of adenylate cyclase as the mechanism by which AP achieves this effect. Nathanson (1987), Mittag et al. (1987), Korenfeld and Becker (1989) and Fawcett and Wilson (1989) suggested that activation of guanylate cyclase is responsible. Nathanson demonstrated that AP receptors, coupled to the activation of guanylate cyclase are present in the rabbit ciliary processes. Further, it was shown that an intravitreal injection of AP into rabbits resulted in a marked decrease in IOP in the ipsilateral eye persisting for more than 48h, with a smaller reduction in IOP in the contralateral eye. Mittag et al. investigated the effect of a synthetic AP upon both ciliary guanylate cyclase and ciliary adenylate cyclase activity, in the albino rabbit eye. This group concluded

that ciliary guanylate cyclase is partially stimulated by intravitreal injection of AP (2 to 4μg per eye), and the concomitant rise in ciliary cyclic GMP leads to a stimulation of ciliary adenylate cyclase. Further, a significant reduction in IOP was observed, lasting for a period of 40h. Korenfeld and Becker reported a significant decrease in IOP following intravitreal injection of AP into rabbits. with a concomitant increase in cyclic GMP in iris-ciliary body preparations. This group also presented evidence that after intravitreal injection of AP there is a decrease in aqueous secretion. associated with the decrease in IOP. Fawcett and Wilson reported that cyclic GMP in isolated bovine ciliary processes increased 5-fold in response to incubation with AP. The rise occurred within 90s of addition of AP and reportedly persisted beyond 12 min. This was confirmed in the present work by the observation of a significant increase in cyclic GMP in ciliary processes taken from perfused eyes following treatment with AP. In all cases, however, the in vitro work cited involved ciliary tissue which was composed of vascular as well as secretory cells. Since AP is known to increase cyclic GMP in vascular tissue (Winquist. 1985). these observations may just have been a reflection of the ciliary blood vessels' response to AP. The present results show that AP lowers IOP in the perfused eye preparation. The time course reported by Fawcett and Wilson (1989) for the rise in ciliary cyclic GMP is compatible with the observed

decrease in IOP occurring approx. 15 min after the first bolus injection of AP (Fig.25).

Vascular Effects of AP

It is clear from several observations reported in the literature (Nilsson & Bill. 1984; Nilsson et al., 1985; Chiou & Yan. 1986) that a uveal vasodilatation is often accompanied by a change in IOP. Receptors specific for AP have been reported in rabbit ciliary body (Bianchi et al., 1986). It was important to determine the effect of AP upon the ciliary vasculature since AP is reported as relaxing resistance arteries in various vascular beds. for example, in kidney, pituitary gland and intestine (Napier et al., 1984), brain (Contin & Genest. 1985) and adrenal gland (Gibson et al., 1986). This effect occurs via the activation of guanylate cyclase and hence a cyclic GMP dependent protein kinase. which leads to inhibition of calcium ion release from the sarcoplasmic reticulum of vascular smooth muscle (Murad. 1978. 1986). It has however been demonstrated in the present study that this is not in fact the case. The bolus dose of AP required to lower IOP lacks detectable vasodilator effects in the perfused eye preparation. By the same reasoning, the rise recorded in ciliary cyclic GMP is probably related to a decrease in

aqueous humour formation, rather than to a relaxation of the arterioles present in the ciliary tissue assayed.

Effects of Other Vasodilator Drugs

The results obtained with AP in this preparation were fundamentally significant, and led to the examination and comparison of the ocular and vascular effects of a number of other vasodilators on a similar basis. Since AP is known to increase ciliary cyclic GMP (Nathanson, 1987), other vasodilators which are also known to activate guanylate cyclase were studied, specifically, the nitrovasodilators sodium azide and SNP (drugs which reportedly also have ocular effects in vivo). Since sodium azide is also known to stimulate the release of EDRF from the endothelium of various blood vessels (Katsuki et al., 1977), it was further decided to test the bovine uveal blood vessel endothelium for the production of EDRF. L-NOARG acts as an inhibitor of nitric oxide synthase, and thus reduces the synthesis and release of EDRF by the vascular endothelium in response to ACh, thereby reducing the effectiveness of ACh as a vasodilator. This was seen in the bovine perfused eye preparation (Table 8). On the basis of these experimental results, therefore, it appears that EDRF is indeed released by the endothelium of the uveal vasculature. Since EDRF mediates activation of a soluble guanylate cyclase, it may

directly affect secretion from the ciliary epithelium.

Among the other vasodilators chosen for study was the ATP-sensitive potassium channel agonist pinacidil. Numerous reports in the literature indicate that this drug opens plasma-membrane K^+ channels which respond to ATP, and in so doing mediates a decrease in intracellular free K^+. This in turn leads to an inhibition of release of Ca^{2+} from intracellular stores. Such reports include examples of vascular smooth muscle from several sources, for instance rabbit aorta (Gelband et al., 1988; Bray et al., 1991), rabbit superior mesenteric artery (Meisheri et al., 1991) and rat portal vein (Hamilton et al., 1986; Hamilton & Weston, 1989; Hu et al., 1990).

From the results obtained it is clear that sodium azide, SNP and pinacidil all possess the ability to lower IOP in a reproducible and dose-dependent manner in this preparation. The nitrovasodilators are more efficacious in this respect than the potassium channel agonist (Fig.29), a possible consequence of the different mechanism of action of this drug. The potency of sodium azide is approximately 100x greater than that obtained with SNP, perhaps reflecting the dual mode of action of azide upon both guanylate cyclase and EDRF. Also, against a noradrenaline-induced vascular tone, the three vasodilators all yielded significant and dose-dependent relaxation (Fig.28). In this respect all three drugs appear equally efficacious. The order of potency for all three drugs is the same for both parameters,

sodium azide being more potent than SNP, which in turn is more potent than pinacidil. Again, the greater potency of sodium azide than SNP may be a reflection of its dual mode of action upon both guanylate cyclase and release of EDRF (Murad, 1986). Although the range of potency is spread over three orders of magnitude for the ocular effect, however, it is spread over only one order of magnitude for the vascular effect.

If the log dose-response curves for the IOP effect (Fig.29) and the log dose-response curves for the vascular effect (Fig.28) are drawn together on a common set of axes, an important result is seen (Fig.32). Comparing the effects of pinacidil and SNP upon IOP and upon the vasculature, it can be seen that each drug exhibits an ED_{50} which does not differ significantly for each parameter. Therefore it may be suggested that the ocular effect of pinacidil and SNP could be related to their vascular effects in this preparation. However, when the effect of sodium azide upon IOP is compared with its effect on the vasculature, some 30-fold difference is seen in the respective ED_{50} values. The data indicate that for sodium azide, as for AP, IOP reduction can be measured at doses below the threshold for vasodilation of the ciliary vascular bed. The direct assays of cyclic GMP show that in the ciliary tissue collected (which must consist mainly of ciliary epithelium), an increase in cyclic GMP occurs in response to the same dose of sodium azide or AP which lowered IOP. This provides good

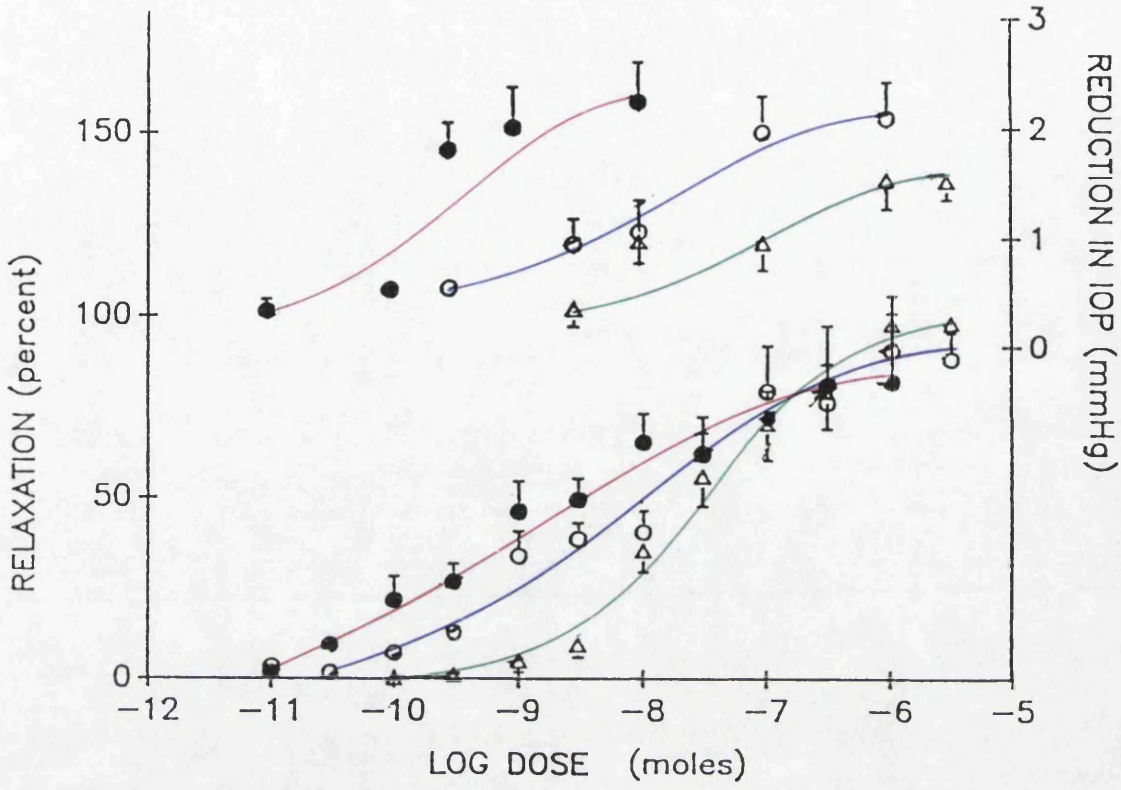


Fig.32. Combined Ocular and Vascular Log Dose-Response. Vertical Bar shows s.e.m.

Sodium Nitroprusside ○——○ Sodium Azide ●——●
Pinacidil △——△

evidence that cyclic GMP is involved in this response, particularly since sodium azide and AP are known to affect synthesis of this nucleotide by different mechanisms: sodium azide by stimulation of a soluble guanylate cyclase (Murad et al., 1978), and AP by stimulation of a particulate guanylate cyclase (Nathanson, 1988). The present results allow for the correlation, in one preparation, of data for sodium azide and AP-induced changes in all three parameters: IOP, vascular resistance and ciliary cyclic GMP content.

In summary, the data show that the four vasodilators investigated (including AP) all lower IOP in the perfused eye preparation. The data further suggest that, at least in the cases of AP and sodium azide, the ocular effects cannot be the result of a vascular action, and that cyclic GMP may operate as a second messenger in the ciliary epithelium. The finding that sodium azide produces corresponding results to AP (unlike the other drugs tested) suggests that they are particularly efficacious with respect to their ability to lower IOP. There is no reason to expect that vasodilation per se should lower IOP when no change in perfusion pressure is evident. This has been emphasized by the finding in the bovine perfused eye that a large (80%) increase in perfusion pressure produces a very small (9%) increase in IOP (Wilson et al., 1993). Hence it is quite likely that the other vasodilators tested (SNP and pinacidil) also lower IOP independently of their vascular relaxant effects. Since SNP is believed to stimulate a soluble guanylate cyclase

(Katsuki et al., 1977), it may well act by this mechanism in the ciliary epithelium also. The present results, however, suggest that it is less efficacious than sodium azide in this regard.

Vascular Flow Determinations Utilising Radiolabelled Microspheres

There are several practical difficulties which require to be overcome when utilising labelled microspheres. Firstly, it is essential to introduce sufficient spheres into the system under study in order that statistically significant changes in flow in response to a drug may be measured. Warren and Ledingham (1974) suggest introducing a minimum of 1000 to 5000 spheres, when dealing with a whole animal. It is important that the number of spheres infused in any experiment is sufficient to yield good counting conditions, even in the smallest organ counted, usually the spleen. Hales (1974) states that a minimum of 400 spheres is required to be trapped in a tissue in order to come within 95% confidence limits in calculation of mean activity. Utilising ^{141}Ce-labelled spheres, which emit highly energetic gamma rays, it was of course desirable to keep the numbers of spheres to a minimum. When dealing with the isolated perfused eye, however, it was felt that a

minimum number of 400 spheres per structural component was necessary, in order to yield reliable data.

Another problem associated with the use of microspheres is the question of how to introduce the spheres into the system under study. The spheres are prepared as a suspension in saline, which must be agitated directly before use. Simple injection of this suspension, however, into the animal will result in the majority of the spheres being trapped in either the lumen of the syringe needle, or the needle connection (Hales, 1974). Thus very few spheres will successfully enter the system under study. It is necessary therefore to design an injection chamber whereby a suspension of spheres can be directly introduced into the flowing system. Several such designs have been utilised for in vivo studies, for example, the special glass vial (Rudolph & Heymann, 1967), the catheter (Sasaki & Wagner, 1971), and the disposable plastic syringe connected to a length of catheter rather than a needle (Kaihara et al., 1968). In the present study, however, it was considered most appropriate to utilise a two-way flow system, as previously related (Fig.19). This was because this design enabled spheres to be introduced into the perfusate without interruption of flow, an important consideration when dealing with an isolated organ perfused in vitro, when there is an inherent need for equilibration of the system. Any interruption to perfusate flow during sphere infusion could adversely affect the final results. Utilising this system

it was found that sufficient spheres entered each eye, in a reproducible manner.

Selection of suitable sphere diameter is also crucial. If the spheres are too small then the majority will simply pass completely through the circulatory system under study; too few would actually be trapped in the tissue capillaries for attainment of reproducible results. If the spheres are too large, then few will enter the tissues under study. Henkind et al. (1979) quotes a range of diameters of the uveal capillaries from 8 to 40μm. Several workers, studying uveal circulation or total circulation in the eyes of cats, monkeys and rabbits (Alm & Bill, 1973; Oksala, 1988; Nilsson et al., 1985) successfully utilised spheres of 15μm diameter. 15μm diameter spheres were therefore chosen in the present study of uveal perfusion in the isolated bovine eye. Such a diameter of microsphere is, however, probably too large to enable measurements of retinal flow to be made, as the retinal capillaries reportedly vary from 8 to 15μm in diameter (Henkind et al., 1979). But in this study uveal flow was of major interest, and the retinal artery was not cannulated. The fact that only negligible counts were found in the retina may reflect the lack of any branches leading from the long posterior ciliary arteries to the retinal circulation, distal to the point of cannulation, and negligible retinal supply from the choroid. As very few spheres were found in the effluent perfusate arising from the venous outflow through the vortex

veins. it appears that a good choice of sphere diameter was made. This was further confirmed by the significant and reproducible sphere entrapment found in the uvea.

Investigation of Drug Effects on Vascular Flow

From the data obtained. when comparing control eyes perfused with Krebs containing noradrenaline with control eyes not perfused with noradrenaline. it is clear that the perfusion of noradrenaline alone significantly reduces entrapment of spheres in the iris. ciliary body and choroid. This. together with the observation that more spheres were found proximal to the eye in the noradrenaline perfused group. but no more in the perfusate effluent. indicates that noradrenaline mediates a constriction of the uveal blood vessels throughout the structures of the eye examined.

From data obtained with drugs where noradrenaline was perfused. it is clear that the β-adrenoceptor antagonists timolol and carteolol do not mediate an increase in the entrapment of spheres in the iris. ciliary body or choroid. at a dose of 30nmol. This is a maximal IOP reducing dose in this preparation for each of these drugs (Fig.23). Since timolol and carteolol exert no effect upon vascular perfusion pressure. in the eyes with vascular tone induced. it seems likely that neither drug had any effect upon flow to the separate parts of the vascular bed. Indeed, it

appears that the known effects of these drugs upon aqueous formation at the doses given in this study are independent of any effect upon the state of constriction of the uveal blood vessels in this preparation. This is particularly so as the tone induced by noradrenaline was submaximal and hence likely to be reversible. if timolol or carteolol at the given concentration possess any uveal vasodilator action.

In the non-noradrenaline perfused group, a more complex situation is seen. The reduction in sphere entrapment which timolol was found to elicit in the choroid at 30nmol bolus dose suggests that a maximal IOP reducing dose of timolol reduces flow to the choroid in the eye with a relaxed vasculature. However, the total number of entrapped spheres in the non-noradrenaline treated eyes challenged with 30nmol timolol did not differ significantly from the numbers in the control group, therefore timolol at this dosage had no effect upon overall vascular resistance. Timolol (300nmol) results in a significant reduction in the numbers of spheres entrapped in the iris, and also a statistically insignificant reduction in the ciliary body. There is also a significant reduction in the overall total numbers of spheres entrapped in the ocular vasculature. This suggests either that the higher dose of timolol does mediate an increase in vascular perfusion pressure, thus reducing flow, or alternatively, this dose of timolol mediates a dilatation of previously constricted arterio-venous (AV) shunts.

leading to an increase in the numbers of spheres simply passing through the ocular vascular beds and draining from the preparation through the venous outflow. However, analysis of the small numbers of counts per minute present in the effluent and its container indicates that no such increase in the numbers of spheres passing directly through the eye could be detected. In the case of the group of eyes treated with timolol at 300nmol, the significant decrease in the total number of spheres entering the ocular circulation corresponded with a significant increase in the numbers of spheres which failed to enter the eye. This then indicates that at a dose which was supramaximal for IOP reduction, timolol does mediate vasoconstriction in the iris and ciliary body. However, as the perfusate flow rate was constant, this dose of timolol should have mediated an increase in perfusion pressure. No such increase was seen. It may be that the transducer - pen recorder system was simply not sensitive enough to register the possibly small increase in perfusion pressure in response to timolol at this dose.

The results obtained in this study using timolol are broadly in agreement with those of Watanabe and Chiou (1983), who noted a reduction in flow to the choroid in the rabbit eye in response to a 1h pretreatment with 0.25% timolol. However, although these workers also observed a reduction in flow to the iris root-ciliary body in response to timolol, they reported no change in flow to the iris

itself in response to this drug. This contrasts with the observation in the present study that at supramaximal dosage, a reduction in sphere entrapment and hence vasoconstriction occurred in the iris. However, at no point was a reduction in sphere entrapment in the ciliary body observed in the bovine eye in response to timolol. Why there should be a vasoconstriction in the choroid but not the iris at a dose which maximally reduced IOP, and the iris but not the choroid at supramaximal dose, is unclear. One would expect to see such an effect in the choroid at the higher dose as well as the lower dose. The failure of timolol to mediate a vasoconstriction in the iris at the lower dose may be due to the presence of ß-adrenoceptors in the vessels of the iris with a lower binding affinity for timolol than in the choroid.

Carteolol, 30nmol dose (which yielded maximal ocular hypotensive effect) was found to significantly reduce sphere entrapment within the iris, ciliary body and choroid in the non-noradrenaline perfused group, as compared with saline control. This corresponds to a small but significant rise in overall perfusion pressure as measured via the transducer - pen recorder system. Sphere entrapment proximal to the eye in this group was also significantly increased; no significant change in the numbers of spheres present in the perfusate effluent was found. At constant flow rate, therefore, these results are indicative of a significant

uveal vasoconstriction in response to carteolol, rather than to any dilatation of ocular AV shunts.

In comparison with the 300nmol dose of timolol, the 30nmol dose of carteolol gave rise to a greater reduction in the overall number of spheres present in the ocular vasculature, and a small but measureable pressure increase as recorded via the transducer. This suggests that a smaller vasoconstriction in response to timolol may indeed have been simply too small for the pressure transducer to measure, given its limit of resolution of approx. 0.5 mmHg pressure. The results suggest that in the perfused eye model, in addition to a reduction in aqueous humour formation, both carteolol and timolol may differentially affect flow to the various ocular regions. The more pronounced uveal pressor responses to carteolol suggest that this drug may cause ocular vasoconstriction at doses similar to those which lower IOP. This represents a possible adverse effect of carteolol, particularly if it promotes vasoconstriction in the retinal vessels. These data are in conflict with the findings of Baxter et al. (1992), using a technique of Colour Doppler Ultrasound, reported that the unilateral instillation of timolol (0.5%) mediated a significant fall in the resistive index of the central retinal artery and posterior ciliary arteries of both eyes, thus probably increasing the blood flow through these vessels.

It has been suggested that the binding of β-adrenoceptors in the ocular vasculature by timolol may lead to a reduction in flow via a vasoconstriction as the drug may be blocking adrenergically stimulated vasodilation of blood vessels (Neufeld. 1979; Neufeld et al., 1983). This hypothesis is somewhat weakened by the observation that timolol will reduce sphere entrapment in the choroid and iris in the isolated perfused eye, which has no intact sympathetic innervation. It may be possible, however, that even in the isolated eye there may be some release of endogenous noradrenaline from intracellular stores, promoting a β-adrenoceptor mediated vasodilation against which timolol can act. However, noradrenaline is relatively selective for α-adrenoceptors, and so even if it is released endogenously in this preparation, it would promote vasoconstriction (indeed as seen in experimental results when noradrenaline is added to the perfusate), rather than dilation. The observation that the β-adrenoceptor antagonist carteolol elicits a more pronounced vasoconstriction to a greater proportion of the uveal circulation than timolol, further weakens this hypothesis, as carteolol also possesses intrinsic sympathomimetic activity (Koch, 1983). Intrinsic activity at the α-adrenoceptor may account for the vasoconstrictor effect of carteolol, however Wilson (1989) found that the vasoconstrictor effect of carteolol was not blocked by addition of the selective α-adrenoceptor antagonist

phentolamine, thus suggesting that carteolol possesses intrinsic activity at the β-adrenoceptor.

The question of whether a reduction in flow to various structures within the eye is responsible for the ocular hypotensive action of a drug is further complicated by the observations that in the noradrenaline perfused eyes, the nitrovasodilator SNP, and the calcium channel antagonist verapamil, both elicit an increase in sphere entrapment in the iris, ciliary body and choroid. These drugs, in addition to mediating a significant increase in ocular entrapment, and a reduction in the proximal entrapment, caused a large reduction in perfusion pressure. This underlines the vasodilator action of these drugs upon the uveal vasculature. Both SNP and verapamil have been found to lower IOP, at the same dosages as those which mediate vasodilation. The picture in the literature is somewhat confused. For example, nitrovasodilators have been reported to increase IOP in the human eye (Krupin et al., 1977), although Karnezis and Murphy (1988) report that i.v. infusion of SNP reduces IOP in human volunteers, and Elliott et al. (1991) report that topical application of SNP to hypertensive human volunteers results in a reduction in blood pressure, but no change in IOP. In the case of calcium channel blockers, Monica et al. (1983) and Abelson et al. (1988) reported a decrease in IOP in human volunteers in response to topical verapamil. However, Beatty et al.

(1984) and Kelley & Walley (1988) reported an increase in IOP following topical nifedipine in the normal human eye.

Despite the claim of Watanabe and Chiou (1983), a reduction in flow to the ciliary body probably does not play an important role in the effect of β-adrenoceptor antagonists upon IOP in the perfused eye preparation. Dilation or constriction of AV shunts within the uveal vasculature in response to the drugs studied in the isolated eye preparation probably does not occur, therefore this mechanism probably plays no part in the ocular hypotensive action of these drugs. Reduction in flow to the choroid or iris mediated by these drugs may be involved in their effect upon IOP. However the increase in flow to the choroid, ciliary body and iris elicited by SNP and verapamil (drugs which also reduce IOP in the perfused eye preparation), suggests that the drugs studied which lower IOP have direct effects upon the secretory ciliary epithelial cells, and their varying ability to modify ocular blood flow is probably incidental to their ocular hypotensive action. Such a conclusion is compatible with the work of Bill (1975).

Involvement of Ca^{2+} in Control of IOP

The experimental observations obtained may in part be due to the modification of intracellular Ca^{2+} concentration,

probably in the non-pigmented ciliary epithelium. Intracellular free Ca^{2+} is known to be important in a great many changes in cellular function. Some ion channels are known to be Ca^{2+} dependent (Adams et al., 1982; Yoshida et al.. 1991; Masao et al.. 1992) and cytosolic free Ca^{2+} may be important in modulating secretion of aqueous. This would possibly explain the reported effect of the L-type Ca^{2+} channel antagonists verapamil and nifedipine upon IOP in the bovine perfused eye; in each case they reduce intracellular free Ca^{2+} via the blockade of L-type Ca^{2+} channels. although as cited previously there is conflict in the literature concerning the ocular effects of these drugs.

The ATP-sensitive K^+ channel agonists cromakalim and pinacidil. in addition to reportedly mediating a decrease in cytosolic free K^+. are also reported as having an effect upon cytosolic free Ca^{2+} (Hamilton & Weston. 1989: Quast & Cook. 1989). These workers. investigating various blood vessel preparations. reported that cromakalim and pinacidil give rise to a hyperpolarization of the cell membrane and subsequent closure of voltage-operated Ca^{2+} channels, leading to a decrease in cytosolic free Ca^{2+} concentration and smooth muscle relaxation. Further. it has been noted that by the addition of a K^+ channel agonist (BRL 38227) together with SNP in the guinea-pig internal anal sphincter, an enhancement of the relaxation effect produced is seen. as compared with the effect of either drug alone (Rae & Muir. 1992). A similar effect is seen when SNP is added together

with the Ca^{2+} channel antagonist diltiazem. A possible explanation for this effect is that the presence of the K^+ channel agonist, or the Ca^{2+} channel antagonist, promotes hyperpolarization of the cell and thus enhances the relaxatory effect of SNP. Thus there is the suggestion of a further link between cyclic GMP, Ca^{2+} and K^+ in various tissues: there may also exist a relationship between these intracellular second messengers in the secretory epithelium of the ciliary processes.

Agents which promote an increase in intracellular cyclic GMP (AP, sodium azide and SNP) may be eliciting a concomitant decrease in release of Ca^{2+} from intracellular stores within the ciliary epithelium, as is reported in vascular smooth muscle, thus leading once again to a reduction in aqueous secretion and hence a reduction in IOP. This, however, still begs the question of how a β-adrenoceptor selective antagonist would promote a reduction in aqueous secretion, particularly in an isolated eye preparation in which there is no intrinsic sympathetic tone and hence there should be no agonist effect with which the antagonist could compete.

Many ion channels are directly regulated by G proteins (Sternweiss & Pang, 1990). α and/or β subunits of G_i or G_o proteins coupled to the muscarinic acetylcholine receptor open an inwardly rectifying K^+ channel in cardiac muscle by a membrane-delimited pathway (Kurachi et al., 1992). In addition to G protein opening ligand-gated channels, G

proteins also affect voltage-gated channels. For example, G_o protein inhibits Ca^{2+} current in neurones (Scott et al., 1991). Recent evidence is consistent with direct opening of dihydropyridine-sensitive (L-type) Ca^{2+} channels by a G_s protein, without the involvement of a cyclic AMP-dependent channel phosphorylation (Yatani et al., 1987, 1988). The same G_s protein that opens L-type Ca^{2+} channels can also stimulate adenyl cyclase, however (Yatani et al., 1988; Brown & Birnbaumer, 1988). Furthermore, the discovery of β_2 adrenoceptors in feline cardiac right-ventricular papillary muscle (Lemoine & Kaumann, 1991) which may be positively coupled to an L-type Ca^{2+} channel, indicates a possible mechanism for the action of β-adrenoceptor selective antagonists upon aqueous secretion, should such receptors also exist in bovine ciliary processes. However, Yatani et al. (1988) and Brown and Birnbaumer (1988) suggest that the concomitant production of cyclic AMP as a result of the binding of the receptor by agonist itself promotes activation of the cardiac L-type Ca^{2+} channel, via phosphorylation of cytosolic protein kinase A (Fig.33), in addition to direct activation of the Ca^{2+} channel by G_s protein. However, in the isolated perfused eye model, agents which directly increase cytosolic cyclic AMP levels such as forskolin have no effect upon aqueous secretion rate (Shahidullah & Wilson, 1992). Perhaps such receptors may exist in the ciliary epithelium coupled to the

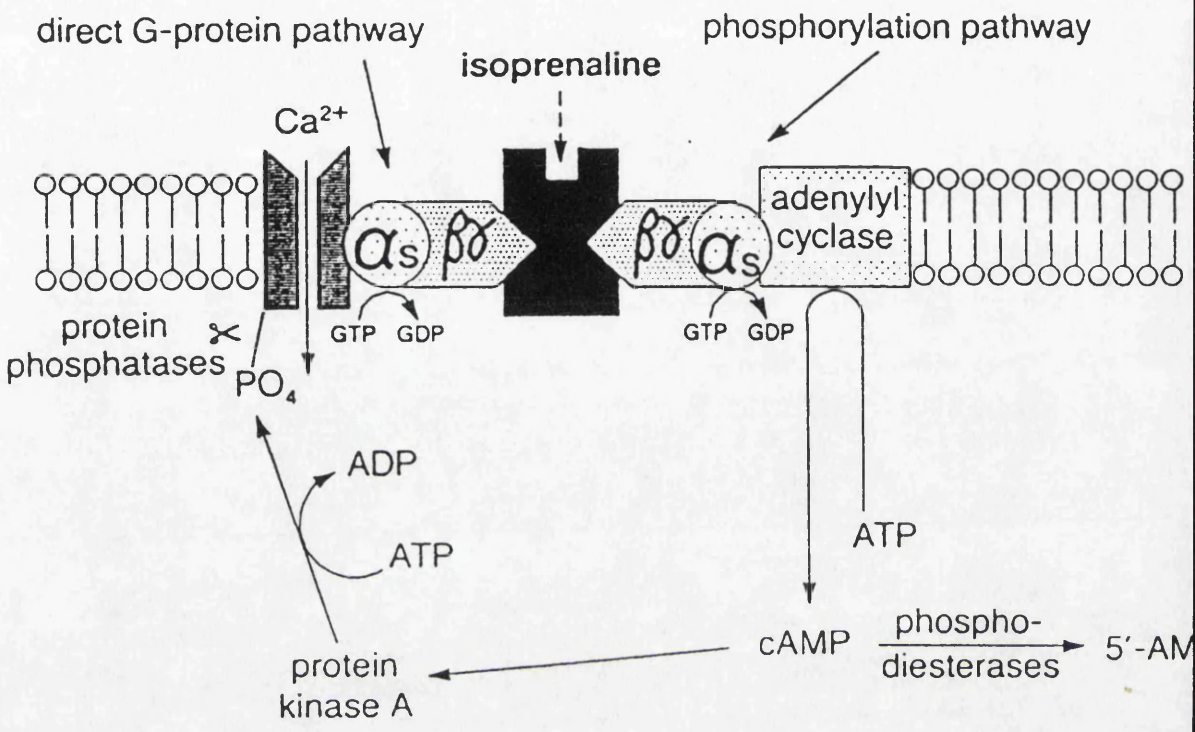


Fig.33. Proposed mechanisms of regulation of the voltage-gated L-type Ca^{2+} channel in cardiac muscle. When isoprenaline binds to the ß-adrenoceptor (dark rectangle), nucleotide exchange on the α_s subunit of G_s ($\alpha_s\beta\gamma$) is accelerated, resulting in activated $G_s\alpha$. $G_s\alpha$ can then activate the Ca^{2+} channel directly by binding to (direct G protein pathway) or indirectly (phosphorylation pathway) by activating adenyl cyclase which synthesises cyclic AMP, which activates cyclic AMP-dependent protein kinase (protein kinase A), which presumably phosphorylates the Ca^{2+} channel. Cyclic AMP may be degraded by a variety of phosphodiesterases and the Ca^{2+} channel may be dephosphorylated by one or several protein phosphatases (Hartzell & Fischmeister, 1992).

dihydropyridine-sensitive Ca^{2+} channel directly by G_s protein only, without being associated with adenyl cyclase.

The regulation of Ca^{2+} channels by a G_s protein has been refuted (Hartzell et al., 1991; Hartzell & Fischmeister, 1992), however Hartzell et al. base this upon data obtained from experiments performed upon frog, rat and guinea-pig cardiac myocytes - in each case a different species and tissue to the feline cardiac muscle as investigated by Lemoine and Kaumann (1991). Hartzell & Fischmeister conclude that although G proteins affect cardiac Ca^{2+} channels in lipid bilayers and excised membrane patches, there is little evidence that this pathway is physiologically significant. For example, these workers found that less than 10% of purified L-type Ca^{2+} channels would bind to added endogenous G-protein.

There is a possibility that ciliary β_2-adrenoceptors are conformationally different from extraocular β_2-adrenoceptors, and will bind both to (-)-timolol and (+)-timolol, in both cases yielding antagonism of the receptor, despite the observations that the (+)-enantiomer of timolol possesses very limited ability to block β-adrenoceptors present at extraocular sites (Share et al., 1984; Richards & Tattersfield, 1985, 1987). However, the theory that there may be β_2-adrenoceptors positively coupled via a G_s protein to transmembrane Ca^{2+} channels in the non-pigmented ciliary epithelium does not adequately explain all of the experimental observations reported to date concerning the

ocular effects of β-blocking drugs. If such receptors were involved in the control of aqueous secretion then intact sympathetic innervation would be required. It would be expected that secretion would be reduced in one eye of patients with unilateral Horner's syndrome, and that timolol would have an effect only upon the unaffected eye. This is not in fact the case, however (Wentworth & Brubaker, 1981). Additionally, there is still no explanation as to why betaxolol possesses similar efficacy to timolol as an ocular hypotensive agent, or why the β-adrenoceptor agonists terbutaline (Shahidullah & Wilson, 1992) and isoprenaline (Colasanti & Trotter, 1981) reportedly reduce the rate of aqueous secretion in certain species. Once again, one may speculate that perhaps the ciliary β_2-adrenoceptor, although not selective for most β_1 agonists and antagonists, is conformationally different and has different binding characteristics from extraocular β_2-adrenoceptors, and will bind betaxolol. Indeed the topical administration of betaxolol to rabbits is associated with levels of the drug in the aqueous which are capable of blocking β_2-adrenoceptors and this has been suggested as a possible mechanism of the ocular hypotensive action of the drug (Polansky & Alvarado, 1985).

CONCLUSIONS

The bovine perfused eye preparation allows for the rapid and inexpensive comparative screening of drugs for possible ocular hypotensive activity. Additionally, comparisons of the effect of a drug upon both IOP and the vasculature may be undertaken. Such comparison allows for study of the fundamental mechanisms of actions of such drugs. The preparation has also proved useful in the more sophisticated investigation of the effects of drugs which lower IOP upon the vasculature. using a radiolabelled microsphere technique. This may be the first time such an attempt to assess vascular flow in an isolated perfused organ has been made.

The bovine perfused eye offers the opportunity to study the effect of ocular hypotensive drugs upon intracellular mechanisms. specifically, upon intracellular second messenger pathways. The increase in cyclic GMP concentration as measured in the ciliary processes in response to sodium azide and AP. and measurements of ciliary cyclic AMP concentration (Shahidullah & Wilson. 1992) in response to terbutaline and forskolin. in association with aqueous flow rate determinations, demonstrates the usefulness of this preparation for analysis of biochemical changes and their relation to aqueous humour dynamics.

Intact adrenergic innervation is not necessary for aqueous secretion. nor the reduction in secretion and IOP seen in response to ocular hypotensive drugs.

The ocular hypotensive effect of a number of different classes of compound has been successfully demonstrated. The ability of the β-adrenoceptor antagonists to lower IOP in this preparation is well established. However, this provides evidence opposing the hypothesis that the effect is mediated via a conventional β-adrenoceptor, since this occurs in the absence of sympathetic tone. The evidence obtained with AP and sodium azide that cyclic GMP may act as an important post-receptor mechanism in the ciliary epithelium supports the growing body of literature which suggests that many cells utilise several of the post-receptor mechanisms, specifically, cyclic nucleotides, inositol phosphates and ion channels. Results obtained with sodium azide and AP indicate that their effect on IOP cannot be mediated by vasodilation. Data obtained with SNP and pinacidil do not exclude the possibility of a causal relationship between vasodilation and a reduction in IOP, however they by no means prove this. Data obtained with timolol and carteolol during the microsphere perfusion experiments suggest a vasoconstrictive effect of these drugs, but only at supramaximal IOP reducing dose in the case of timolol. There are also reports in the literature, albeit conflicting, of the ocular effects of selected Ca^{2+} channel antagonists: indeed, pilot studies with the present preparation indicate a small IOP lowering effect of bolus injections of verapamil or nifedipine. Ciliary intracellular Ca^{2+} may play a role in the control of IOP.

FUTURE WORK

It has been shown that in the bovine perfused eye, standard drugs which lower IOP do so by reducing the rate of aqueous humour formation, as measured by a fluorescein dilution method (Shahidullah & Wilson, 1992). Further work with this method should confirm that AP acts by depressing aqueous humour formation, as reported by Korenfeld and Becker (1989). Similar experiments with the other vasodilators used in the present work may indicate that they also suppress aqueous secretion. Extending the study of cyclic GMP in the ciliary epithelium to examine the effects of a range of doses of SNP as well as AP and sodium azide, may confirm the hypothesis that all these agents lower IOP through their actions on guanylate cyclase.

More precise information concerning the concentration of drug actually present at the site of action (probably the ciliary epithelium) would be obtained by administering ocular hypotensive drugs such as timolol at a fixed concentration in the perfusate, rather than as bolus doses.

Investigation of the effects of some other compounds upon IOP, such as Ca^{2+} ionophores, 8-bromo cyclic AMP, 8-bromo cyclic GMP and L-NOARG would perhaps provide evidence of the mechanisms by which IOP is controlled. Additionally, in order to confirm that the ocular hypotensive effect of pinacidil is mediated by an ATP-sensitive K^+ channel, the effect of pinacidil upon IOP could be investigated in the presence of perfused glibenclamide, an ATP-sensitive K^+ channel antagonist. Although aqueous secretion rate has

been estimated in this model, investigation of drug effects upon facility of outflow has yet to be attempted.

Further evidence of the mechanism of action of β blockers in the bovine eye might emerge if eyes were perfused with Krebs containing a β_2 selective agonist, such as salbutamol or butoxamine.

A study of the effects upon IOP and vascular relaxation of the addition of SNP together with a K^+ channel agonist, or together with a Ca^{2+} channel antagonist would perhaps be informative.

An attempt to culture bovine ciliary epithelial cells would represent a logical extension of work performed to date with this preparation. There is of course, the reservation that during the establishment of a viable cell culture, the structure of cellular components, including plasma membrane receptors, often changes. However, there would be several opportunities offered by this approach. For example, changes in ciliary intracellular free Ca^{2+}, cyclic AMP, cyclic GMP and IP_3 could be determined in response to incubation with selected drugs. This would avoid the potential complication of the presence of ciliary stroma and small blood vessels, which may have contaminated to some degree the scraped ciliary epithelial cell preparations assayed for cyclic GMP in the present work. Additionally, since changes in intracellular free Ca^{2+} are involved in the activation of very many cell types, micro-scale measurements of intracellular free Ca^{2+}, using the

fura-2 technique (Morgan & Morgan, 1982), could be performed upon cell cultures.

By using cell cultures, it is possible to apply membrane patching techniques in association with G-protein/Ca^{2+} excision and reinsertion into lipid bilayers. Measurements of Ca^{2+} currents induced by the addition of GTPγS and GTPβS (activator and inhibitor of G-proteins, respectively), as reported by Yatani et al. (1987) might then provide further information as to the possible presence of G-protein gated Ca^{2+} channels in the ciliary epithelium.

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APPENDIX

Appendix I

Statistics

All data is given as: mean±s.e.m..

With the exception of the data obtained from the EDRF investigation and the radioactive microsphere perfusion experiments, all data was analysed using the 2-tailed unpaired Student's t-test (Gosset, 1908) of difference between population means. Population variances were not assumed to be equal.

The test statistic (t) is given by the relation:

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where: $\bar{x}$ = sample mean

μ = population mean

s = sample standard deviation

n = sample size

Degrees of Freedom (df) is given by the relation of Dixon and Massey (1969):

$$df = \frac{\left[\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right]^2}{\frac{(s_1^2/n_1)^2}{n_1} + \frac{(s_2^2/n_2)^2}{n_2}}$$

Computed values of t were compared with the calculated percentiles of the t-distribution.

Data obtained from the EDRF investigation was analysed using the 2-tailed paired Student's t-test (Gosset, 1908) of difference between population means of nonindependent samples.

The test statistic (t) is given by the relation:

$$t = \frac{\bar{d} - \mu_d}{s_{\bar{d}}}$$

where: $\bar{d}$ = mean difference between pretreatment and post-treatment values of sample

μ_d = mean difference between pretreatment and post-treatment values of population

$s_{\bar{d}}$ = sample standard error of difference between pretreatment and post-treatment values of sample

Degrees of freedom (df) is given by the relation:

$$df = n-1$$

where: n = sample size

Computed values of t were compared with the calculated percentiles of the t-distribution.

Data obtained from the radioactive microsphere perfusion experiments was analysed using the 2-tailed Mann-Whitney test (Mann & Whitney, 1947) of difference between population medians.

The test statistic (T) is given by the relation:

$$T = S - \frac{n(n+1)}{2}$$

Where: S = Sum of Ranks assigned to the sample observations from one population

Computed values of T were compared with the calculated quantiles of the Mann-Whitney test statistic.

Appendix II

Materials

Timolol Maleate	Sigma Chemical Company
Carteolol Hydrochloride	Sigma Chemical Company
Betaxolol Hydrochloride	Sigma Chemical Company
Metipranolol	Sigma Chemical Company
Metoprolol	Sigma Chemical Company
Oxprenolol	Sigma Chemical Company
Laevobunolol	Strathclyde University
DHL	Strathclyde University
Verapamil Hydrochloride	Sigma Chemical Company
Nifedipine	Sigma Chemical Company
Cromakalim (BRL 34915)	Smith Kline & Beecham
Pinacidil	Sigma Chemical Company
SNP	Sigma Chemical Company
Sodium Azide	Sigma Chemical Company
Acetazolamide	Sigma Chemical Company
FPL 65879AA	Fisons plc

141Ce-Labelled 15μm Carbonized Latex Microspheres	DuPont New England Nuclear (NEN)
Cyclic GMP Radioimmunoassay Kit	DuPont New England Nuclear (NEN)

Appendix III

List of Abbreviations

The following abbreviations have been used throughout this thesis:

IOP:	Intraocular Pressure
OAG:	Open-Angle Glaucoma
COAG:	Chronic Open-Angle Glaucoma
ICB:	Iris-Ciliary Body
SNP:	Sodium Nitroprusside
DHL:	Dihydrolaevobunolol
Cyclic AMP:	3'5' Cyclic Adenosine Monophosphate
Cyclic GMP:	3'5' Cyclic Guanosine Monophosphate
IP_3:	Inositol Trisphosphate
GTPβS:	Guanosine Triphosphate Beta-S
GTPγS:	Guanosine Triphosphate Gamma-S
AP:	Atriopeptin (Atrial Natriuretic Factor)
ACh:	Acetylcholine

L-NOARG:	L-Nitroarginine
TCA:	Trichloroacetic Acid
BSA:	Bovine Serum Albumin
RIA:	Radioimmunoassay
N-YAG Laser:	Nitrogen Yttrium-Argon-Garnet Laser
s.e.m:	Standard Error of Mean
mm:	Millimetres
mmH_2O:	Millimetres of Water
mmHg:	Millimetres of Mercury
i.v:	Intravenous
°C:	Degrees Celcius
M:	Molar
h:	Hours
min:	Minutes
s:	Seconds
g:	Grammes
mg:	Milligrammes
µg:	Microgrammes
ml:	Millilitres
µl:	Microlitres
mol:	Moles
µmol:	Micromoles
nmol:	Nanomoles
pmol:	Picomoles

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