MECHANISMS OF ACTION OF DRUGS WHICH ALTER AQUEOUS HUMOUR FORMATION

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Declaration

I hereby declare that this thesis has been composed by myself and that it embodies the results of my own special study and research, that was carried out in the Ocular Pharmacology Laboratory within the Department of Pharmacology, The University of Glasgow, between October 1990 and September 1993.

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

DEDICATED TO THE MEMORY
OF
MY FATHER

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SUMMARY

There is a great need for an in vitro model for studying aqueous humour dynamics. A large number of antiglaucoma drugs is currently in clinical use or are in various stages of development, and in some cases their underlying biochemical mechanisms of action are unclear. The present work was undertaken to find out whether the easily obtainable inexpensive bovine eye could be established as such a model to study the physiology and pharmacology of ocular hypotensive drugs. The specific objective was to try to explore the mechanism of action of the extensively used β-blocker, timolol, to determine whether it acts through the conventional β-adrenoceptor-adenylate cyclase mechanism, as is often assumed.

The isolated arterially perfused bovine eye has been proved to be a valid model for studying aqueous humour dynamics and the pharmacology of various antiglaucoma drugs. Its usefulness has been shown by significant effects of standard and well-established ocular hypotensive agents, such as timolol and MK-927, a carbonic anhydrase inhibitor, on both intraocular pressure and aqueous humour formation. MK-927 produced a dose-dependent decrease in intraocular pressure and aqueous humour formation with moderate doses of 1, 10 or 100 nmol.

In addition to the responses to the standard drugs, the viability and metabolic activity of the isolated perfused eye has been shown by significant consumption of O_2 and elaboration of CO_2 during the course of perfusion. This was achieved by comparing the two parameters between the perfused living and dead eyes.

The bovine perfused eye has been shown to offer the opportunity to study the effect of ocular hypotensive drugs upon intracellular mechanisms, especially upon second messenger pathways. It allows rapid and convenient sampling of substantial amounts of target tissue for biochemical analysis after drug challenge.

The usefulness of the perfused eye has also been shown for isolating and culturing ciliary epithelial cells. Two new methods of isolating and culturing ciliary epithelial cells have been developed. One by aseptic and consecutive perfusion of the isolated eye with DMEM, calcium-free buffer and collagenase and the other by incubating the excised ciliary processes with collagenase. The cells were successfully propagated up to 10 passages without any obvious sign of change of growth characteristics. In first passage cells cultured in this way, there occurred highly significant and concentration-dependent increases in cyclic AMP and cyclic GMP in response to terbutaline and atriopeptin, respectively, indicating that these cells retain functional receptors. This will open up broad opportunities for physiological, pharmacological and biochemical studies on specific target cells responsible for aqueous humour production.

To explore the mechanism of action of timolol, we studied four different drugs which are known either to alter aqueous humour formation or intracellular cyclic AMP content. The drugs used were timolol, a β-adrenoceptor antagonist; terbutaline, a β-adrenoceptor agonist; forskolin, a potent direct stimulator of adenylate cyclase and 8-bromo cyclic AMP, a membrane permeable analogue of cyclic AMP.

Both timolol (10 nmol) and terbutaline (30 nmol) were found to cause a significant reduction of intraocular pressure and aqueous humour formation by bolus injection into the perfusate, but neither of them had any effect on levels of

ciliary epithelial cyclic AMP. Even in the case of terbutaline, a three times higher dose also failed to alter ciliary cyclic AMP content.

However, when excised ciliary processes from fresh bovine eyes were incubated with terbutaline, concentrations of 10^{-4} to 10^{-6} M produced a graded increase in cyclic AMP . The same concentrations of terbutaline also produced a more pronounced and concentration-dependent increase in cyclic AMP in cultured ciliary epithelial cells. 10^{-4} M terbutaline produced about 800% increase in ciliary epithelial cyclic AMP content.

Forskolin (30 nmol) on the other hand, produced a highly significant increase in ciliary cyclic AMP in the perfused eye, but had no effect on aqueous humour production. A 100 nmol dose of 8-bromo cyclic AMP also failed to produce any effect on aqueous humour formation. Finding no relevance of ciliary cyclic AMP levels to aqueous humour formation in all these experiments, we turned to specific inhibitors of cyclic nucleotide-dependent protein kinases. A 10 nmol bolus dose of KT-5720 (protein kinase A inhibitor) completely blocked the effect of terbutaline (30 nmol) on intraocular pressure and aqueous humour formation. In contrast, KT-5823 (protein kinase G inhibitor) was unable to block the intraocular pressure-lowering effect of terbutaline.

Thus experiments with timolol, terbutaline, forskolin and 8-bromo cyclic AMP showed that there is no correlation between the aqueous humour formation rate and the ciliary epithelial content of cyclic AMP. On the other hand, experiments with protein kinase A and protein kinase G inhibitors indicate that cyclic AMP may be an important modulator of aqueous humour formation. From the convincing irrelevance in the former case and an apparent relevance in the latter case we were led to suggest the following possibilities: that cyclic

AMP may be an important modulator of aqueous humour formation but at changes in concentration which are too low to be measured by radioimmunoassay; that functional compartmentalization of cyclic AMP may be involved in ciliary epithelial cell function; that the increase in cyclic AMP in some critical foci of ciliary epithelial cells may be important to modulate cell function, yet this change is not sufficient to affect the total cyclic AMP content of the cells.

The vasodilator cardiac peptide, atriopeptin and the nitrovasodilator, sodium azide, were found to be highly effective in reducing aqueous humour formation and intraocular pressure in the perfused bovine eye. Atriopeptin at concentrations of 10^{-6} to 10^{-8} also produced a concentration-dependent increase in cyclic GMP in cultured ciliary epithelial cells.

The intraocular pressure-lowering effect of a 10 nmol bolus dose of sodium azide was found to be completely abolished by the specific inhibitor of protein kinase G (KT-5823). In contrast, protein kinase A inhibitor (KT-5720) was unable to block this effect of sodium azide. It was concluded that both atriopeptin and sodium azide lower intraocular pressure by reducing aqueous humour formation and that cyclic GMP may be an important 2nd messenger involved in aqueous humour formation.

The calcium-channel blocker verapamil in bolus doses of 1, 10 or 100 nmol produced a dose-dependent decrease in intraocular pressure in the bovine perfused eye. On this basis future work on drugs which can modulate intracellular Ca^{2+} is suggested.

INTRODUCTION

INTRODUCTION

General Consideration

The supply of nutrients to the avascular structures of the eye, such as the cornea, the lens and the trabecular meshwork is accomplished by aqueous humour (AH). The existence of two systems of blood vessels - the retinal and the uveal - together with the blood-retinal and blood-aqueous barrier and the complex mechanism of AH formation and drainage make it possible to supply nutrition to the intraocular tissues without blood interference in transmission of light through the eye. This complex arrangement for ocular nutrition contributes to good optical properties of the eye.

AH is a transparent fluid contained in the anterior and posterior chambers of the eye and is formed by the ciliary epithelium (CE) of the ciliary processes projecting from the ciliary body. As the aqueous is formed it enters the posterior chamber from which it flows in three different directions: (1) most of the aqueous flows forward between the lens and iris and through the pupil into the anterior chamber, (2) a small portion flows into the vitreous to be absorbed in the posterior part of the eye and (3) some of the aqueous apparently is reabsorbed at the ciliary body. Apart from supplying nutrition to the ocular tissues AH has other functions including draining of metabolic wastes into the venous system and maintaining optimum pressure in the eye, which is important to give the rigidity necessary for optical alignment of the cornea, lens and the retina. The intraocular pressure (IOP) is also responsible for the bulk flow of aqueous out of the anterior chamber through the two major outflow pathways - the trabecular and the uveoscleral. Such pressure is inherent in a system where fluid is to flow through passive routes. IOP is determined by the relationship

between the rates of AH formation and drainage. In the normal eye the rate of production is equal to the rate of outflow of AH. Any deviation from the normal relationship will affect the IOP. If, for example, the rate of production of AH is more than the rate of its drainage, IOP will increase. Increased IOP can also occur in eyes with normal AH production but defective drainage, such as complete or partial blockage to the outflow pathways. A high IOP is the important cause of chronic open-angle glaucoma (COAG), a condition in which IOP rises to the point of damaging the retina and its nervous outflow. Reduction of IOP by various means to a level at which optic nerve damage ceases, is currently the only available and clinically acceptable treatment of COAG which is the most common cause of blindness throughout the world (Leopold and Duzman, 1986; Everitt and Avorn, 1990).

The IOP can be reduced by two basic mechanisms: (1) decreasing the production of AH from the ciliary processes, (2) increasing the outflow of AH through the trabecular (conventional) and/or uveoscleral (unconventional) pathways (Hurvitz. et al., 1991)

One of the important contemporary issues in glaucoma research is to determine which of the two basic mechanisms of reducing IOP (decreasing the inflow and increasing the outflow) is the preferable therapy. There is widespread belief that COAG is invariably associated with an increased resistance to the outflow of AH. Also there is apprehension that the secretory suppressant may have long term side effects on some of the avascular structures in the eye which depend upon adequate nutrition from the AH. Thus it follows that rational therapy should be directed at decreasing the resistance to outflow. The paradox is that drugs most commonly used in treating glaucoma in fact suppress secretion. The following may throw light on this paradox:

1. Decreasing outflow resistance is often resistant to drugs.

2. The pathological changes in the outflow channel, whether in the superficial trabecular meshwork nearer to the anterior chamber angle or deep in the juxtacanalicular tissue, have always been poorly understood. Ocular hypertension is due to an increased resistance to outflow but what happens to the trabecular meshwork to cause this is totally unknown (Epstein, 1987). Treatment to increase outflow is not aimed at the actual pathological changes in the outflow channel. Most of the available treatments, e.g. pilocarpine, are aimed at activating the ciliary muscle. But there is no evidence to suggest that in COAG, cholinergic tone is defective or the cholinergic transmitter or its receptors is in any way lost or altered (Sears, 1991).

3. Several classes of secretory suppressants are in extensive use in the treatment of glaucoma, such as carbonic anhydrase inhibitors and beta blockers. Very few ocular side effects have been reported to result from these drugs despite their substantial reduction of the rate of AH formation (Gilmartin et al., 1984; Bron et al., 1989; Higginbotham et al., 1989).

4. In the context of secondary glaucoma, the goal is to eliminate the cause. Medication aimed at improving outflow may not work at first and may have effects that aggravate the resolution of the primary inflammatory process. In such cases IOP must be kept at a reduced level by reducing AH flow, until the cause is eliminated (Sears, 1991).

5. Increased resistance to outflow as a result of compression of the trabecular meshwork or the apposition of the inner wall of the Schlemm's canal to the outer wall, has a deleterious effect on the outflow channels. Under such

conditions the effectiveness of the outflow medication is lessened (Sears, 1966).

6. In acute angle-closure glaucoma where there is ischaemia to the iris sphincter, the reduction of IOP can usually be achieved either by a drug which suppresses secretion or, temporarily, by an osmotic agent. Ischaemia renders the tissue unresponsive to pharmacologic agents (Sears, 1991).

For all these reasons, reducing IOP by reducing AH inflow is as logical as increasing the outflow of AH.

The present study is concerned with the mechanisms of action of drugs which decrease the production of AH from the ciliary process. Thus a brief review of the structures responsible for AH production, AH physiology (its composition, production, drainage etc.) as well as a discussion on the pharmacology of the agents which may influence AH dynamics deserve worth attention.

Gross Structures of the Eye

In shape the eyeball is a sphere, with the segment of a smaller sphere, the cornea, in front. It is composed of three coats or tunics. The outermost protective coat is made up of the sclera posteriorly and the cornea anteriorly. The middle coat is mainly vascular, consisting of the choroid, ciliary body and iris. The innermost coat is the retina, containing the essential sensory elements responsible for vision - the rods and cones. Within the three coats are the refractory media - namely, the AH, the crystalline lens and a clear jelly, the vitreous humour (Fig. 1).

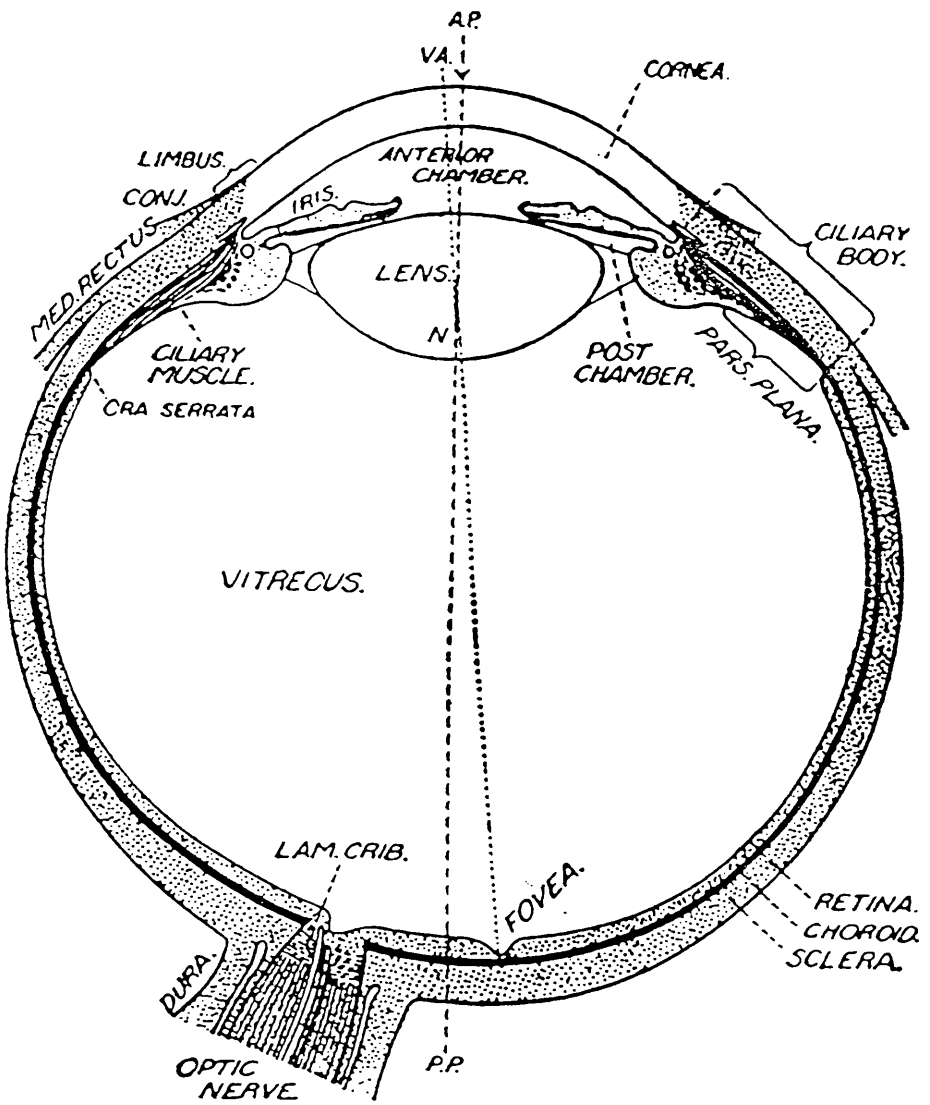

Fig. 1. Horizontal section of the eye. P.P., posterior pole; A.P., anterior pole; V.A., visual axix (Wolff, Anatomy of the Eye and Orbit, 1968).

The cornea, the first and the most powerful refracting surface of the optical system of the eye, occupies the anterior sixth of the outermost coat. The crystalline lens is a double convex transparent body positioned between the iris and AH in front and the vitreous humour behind and is supported by an elastic capsule and the ciliary zonule (the suspensory ligaments) which is itself attached to the ciliary body. The iris, a pigmented membrane, is the most anterior portion of the vascular tunic of the eye made up of a flat bar of circular muscle fibres surrounding the pupil and a thin layer of smooth muscle fibres by which the pupil is dilated, thus regulating the amount of light entering the eye. It is covered by two layers of epithelia which are continuous forward extensions of the pigmented and nonpigmented layers of the ciliary body. The ciliary body is the middle thickened portion of the vascular tunic anterior to the ora serrata (the terminating point of the retina at the ciliary body), connecting the choroid with the iris. It is composed of corona ciliaris (the ciliary processes and folds), ciliary ring, ciliary muscle and a basal lamina. The ciliary processes are finger like projections, approximately 70 in number in man and 90 to 110 in bovine (Prince et al., 1960), projecting from the ciliary body. Contained in them is a mass of capillaries which are covered by a double layer of epithelium, which has an extraordinary apex to apex arrangement. The ciliary epithelia are thought to be the key structures responsible for AH production. The choroid is the thin portion of the vascular coat extending from the ora serrata to the optic nerve. It furnishes blood supply to the retina and conducts arteries and nerves to the anterior structures of the eye. The retina is the innermost layer of the three tunics of the eyeball surrounding the vitreous body and continuous posteriorly with the optic nerve. It is divided into pars optica, which rests upon the choroid, pars ciliaris which rests upon the ciliary body, and pars iridica which rests upon the posterior surface of the iris. Grossly, the retina is composed of an outer pigmented layer (pars pigmentosa), and an inner transparent layer (pars nervosa)

which make up the pars optica. The rods and cones layer in the latter forms the percipient element of the retina (i.e., the element that responds to visual stimuli by a photochemical reaction) and is connected with the nerve fibre layer by nerve fibres which join to form the optic nerve. The optic nerve in turn carry the impulse to the brain.

The AH and vitreous body are contained in the two spaces within the eyeball. The larger space is situated between the lens and retina and contains the vitreous humour. The smaller space, termed the aqueous chamber, is the space between the internal surfaces of the cornea and lens. The aqueous chamber is itself divided into the anterior and posterior chambers. The anterior chamber contains most of the aqueous and is the space between the anterior surface of the iris and the internal surface of the cornea. The posterior chamber is the name given to the smaller space between the lens and the iris.

The vitreous is a clear mass and physiologically a hydrogel bounded by a hyaloid membrane. Forward extensions of the hyaloid membrane form the suspensory ligaments supporting the lens, known as the zonule. The vitreous is probably not a tissue in proper sense, but rather a product of the surrounding tissue, the neuroretina, the water content being extremely high, between 98% (Redslob, 1932) and 99.7% (Sullmann, 1951). It allows light to reach the retina and nutrients to diffuse from the ciliary body to the retina. The cornea and lens are nourished by a process of diffusion from the vitreous and AH of dissolved oxygen and nutrients, derived from the various capillaries. The cornea also absorbs oxygen by diffusion directly from the atmosphere.

The Ciliary Epithelium

AH is formed by the CE of the ciliary processes projecting from the ciliary body (Fig. 2). The bulk of the ciliary body consists of unstriated muscle arranged in three bundles: longitudinal, radial and circular. The inner surface of the body can be divided into two parts: the anterior third consists of ciliary processes and is termed the pars plicata, while the smooth posterior two thirds is termed the pars plana (fig. 2). Projecting inwards from the pars plicata region into the posterior chamber are approximately 70 radial ridges - the ciliary processes. Each process is 1 mm high, 2 mm long antero-posteriorly and 0.5 mm wide. In the bovine these processes are particularly well developed. The ciliary processes have a rich blood supply and are probably the most heavily vascularised part of the eye. Each process contains a mass of capillaries, so arranged that each comes into close relationship, at some point in its course, with the surface of the CE covering the ciliary processes.

The CE consists of two layers of cells, the columnar non-pigmented (NPE) and the cuboidal pigmented epithelial (PE) cells. The inner, NPE layer is next to the AH or vitreous. The outer layer is heavily pigmented and represents the forward continuation of the retinal pigment epithelium whilst the NPE layer is the forward continuation of the neuroepithelium from which the retinal cells are derived. The cells of this epithelium are considered to be responsible for AH secretion and show characteristic features of secretory epithelia under the electron microscope, i.e. interdigitations of the lateral surfaces of adjacent cells and basal infoldings (Pappas and Smelser, 1958, 1961). The relations of the epithelial cell layers are of importance, since the secreted aqueous must be derived from the blood in the capillaries of the ciliary stroma and thus secretion must occur across both layers. The bases of the NPE cells line the posterior

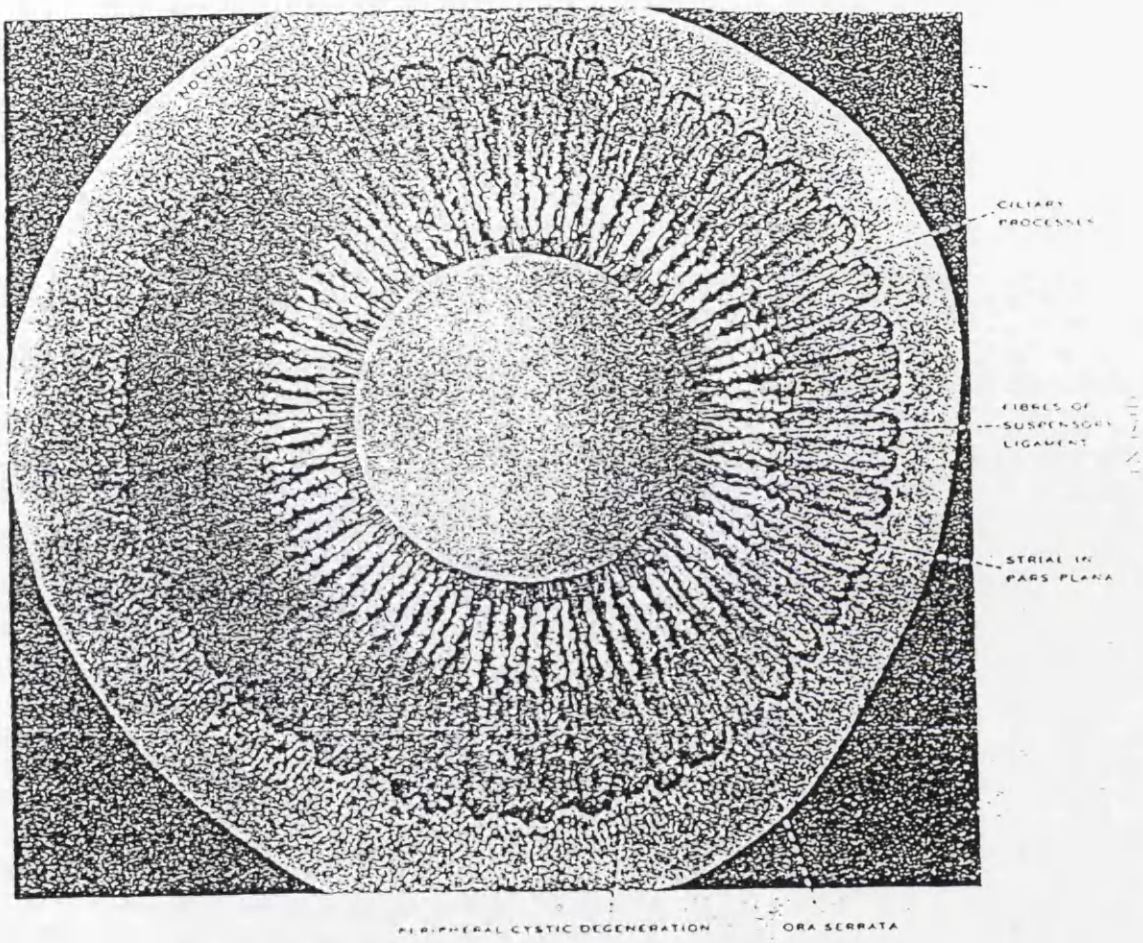

Fig. 2. Posterior view of the ciliary body and lens, showing ciliary processes (Wolff, Anatomy of the Eye and Orbit , 1968).

chamber whereas the bases of the PE cells rest on the ciliary body stroma (Fig. 3). The apices of the PE and NPE cells are thus in contact with one another. This unusual arrangement is the result of the invagination of the neuroepithelial layer during embryogenesis. Despite this arrangement, the secretory process is directed from apex to the base of the non-pigmented cells along the lateral intercellular canals which are 'closed' at the apical ends by dense junctional complexes (Cole, 1977) (Fig. 3).

Tight junctions are always present at the apices of the NPE cells. Many gap junctions usually are found between the lateral surfaces of the PE cells and less frequently between the lateral surfaces of the NPE cells. Gap junctions and puncta adherentia are located between the PE and NPE cells (Fig. 4). This highly integrated epithelium is responsible for secretion of AH and affords attachment to the ciliary zonules (Fig. 5). All ancillary functions to maintain proper transport activity, diffusional characteristics, mechanical stability of the epithelium etc. depend upon the properties of these specialized intercellular junctions. (Raviola and Raviola, 1978). AH production is only possible if the transport activity of the multitude of epithelial cells is precisely coordinated and is not dissipated by free diffusion of water and solutes along the intercellular clefts of the epithelium. On the other hand, mechanical stability is an essential prerequisite for the epithelium to withstand the tensile force of the elastic zonular fibres. The tight junctions between the NPE cells and nonfenestrated iris capillaries exclude large molecules from the AH (Green, 1984; Novack and Leopold, 1988). Thus these specialized junctions constitute the most important part of the blood-aqueous barrier.

Tight junctions between NPE cells represents the permeability barrier which prevents diffusion of blood-borne macromolecules into the AH (Shiose, 1970;

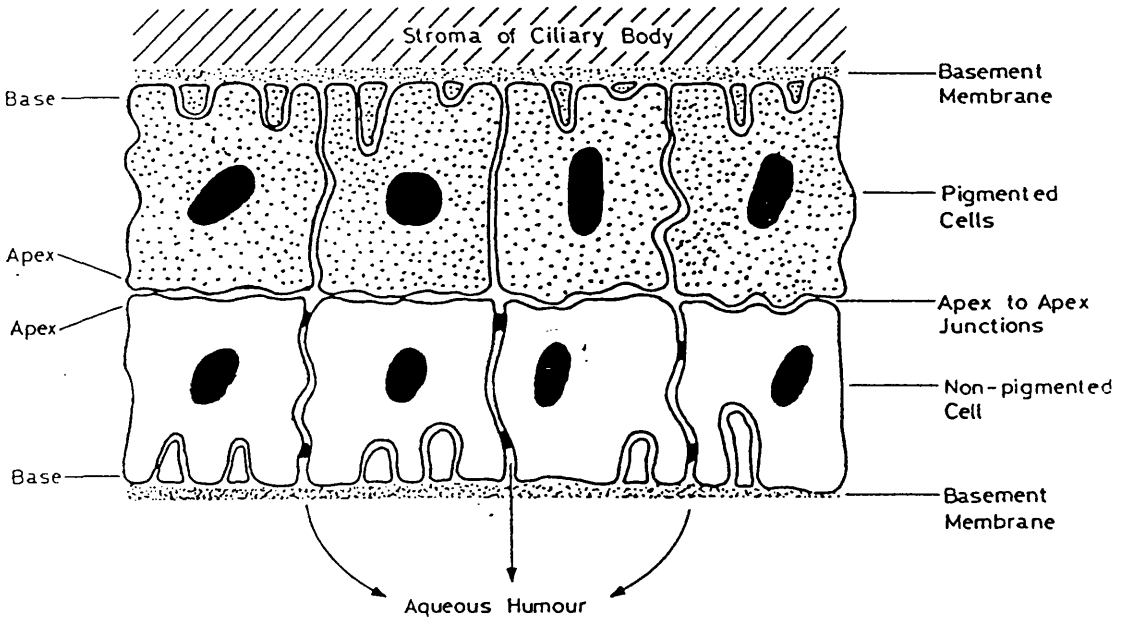

Fig. 3. The apex-to-apex relation of the two layers of cells of the ciliary epithelium (Davson, Physiology of the Eye , 1990).

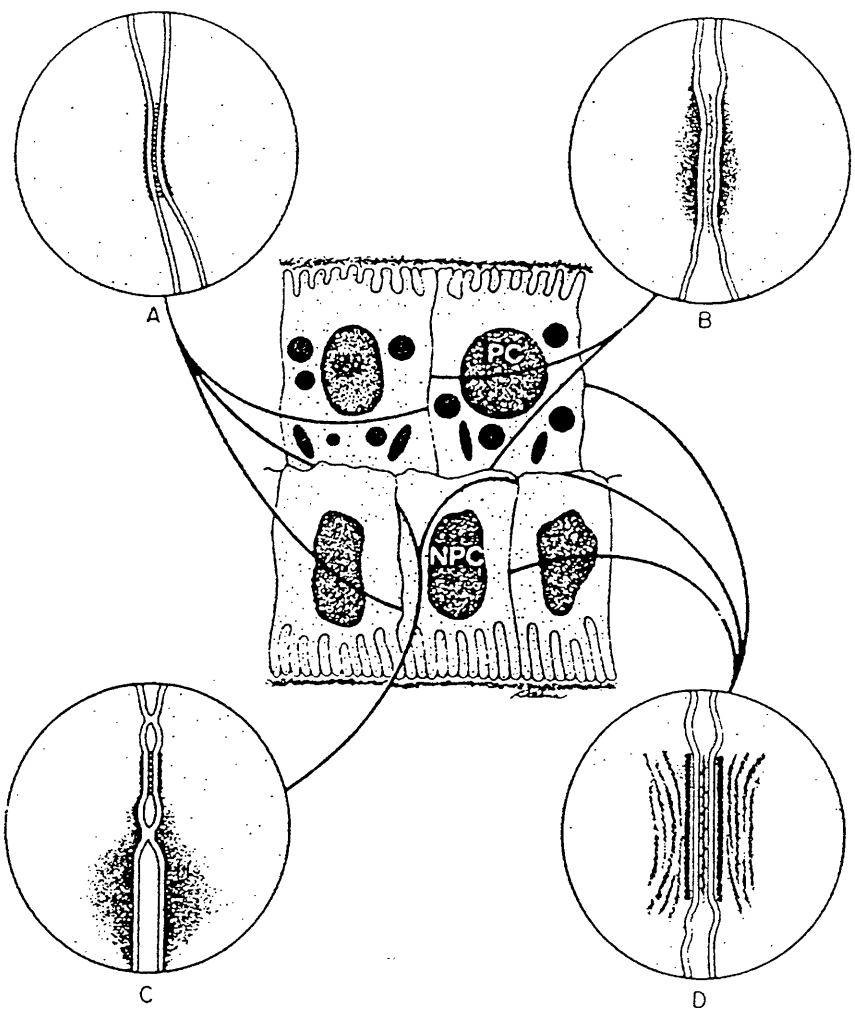

Fig. 4. Diagram of the intercellular junctions in the ciliary epithelium (Raviola, 1977).

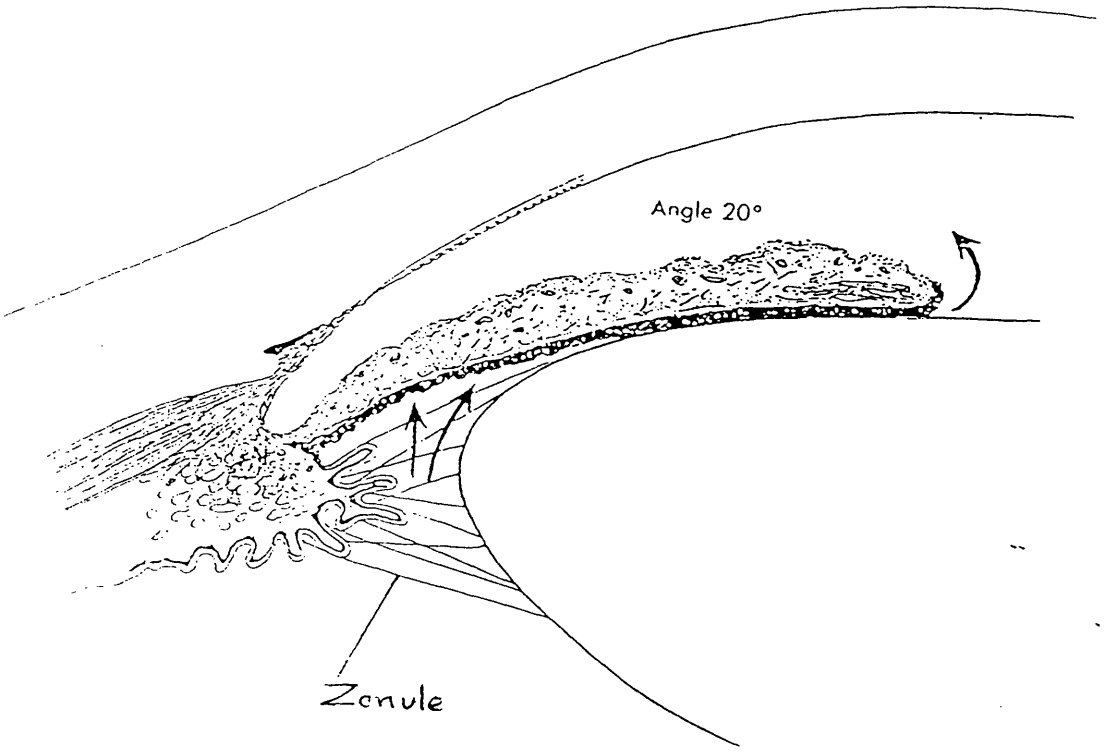

Fig. 5. Diagram showing ciliary zonules and direction of flow of AH from the posterior to the anterior chamber (from Kolker, A. E. and Hetherington, J., Jr.: Becker-Shaffer's diagnosis and therapy of the glaucomas, ed. 4, St. Louis, 1976, The C. V. Mosby Co.).

Vegge, 1971; Smith and Rudt, 1973; Raviola, 1974, 1977; Raviola and Raviola, 1978). Zonula occludens (tight junction) are impermeable to macromolecules and restrict the paracellular movement of ions and small molecules without preventing it altogether.

A large number of gap junctions between both the NPE and PE cells with greater concentration at the interface between the two epithelial layers constitute a striking morphological feature of the CE. These junctions, less commonly referred to as nexuses or macula communicans, are membrane specializations containing channels that mediate movement of ions and small molecules (Saez et al., 1993). In electron micrographs of thin sections, they appear as regions of intercellular contact where apposed plasma membranes of adjacent cells are separated by a gap junction of 2 - 3 nm (Saez et al., 1993). Gap junctions mediate both electrical and metabolic coupling between cells (Revel and Karnovsky, 1967; Pappas et al., 1971; Gilula et al., 1972)

Desmosomes and their organization in the CE indicates that they are typically similar to other epithelia (Staehelin, 1974; McNutt and Weinstein, 1973). Desmosomes occur in small numbers throughout the CE of monkey but are absent between NPE of rabbit (Raviola and Raviola, 1978).

Puncta adherentia are large and numerous between pigmented cells and at the interface between the two epithelial layers (Raviola and Raviola, 1978). They are associated with cytoplasmic microfilaments and may anchor the contractile apparatus of the CE cells to the plasmalemma.

The Blood-Ocular Barriers

The striking differences between the composition of plasma and the AH means that substances encounter difficulty in passing from one fluid to the other. And also there is no barrier between the posterior chamber and the vitreous body and between the vitreous humour and the retina. Horseradish peroxidase and even Thorotrast particles can pass from the vitreous into the intracellular spaces of the retina (Smelser et al., 1965). The movement of horseradish peroxidase is only stopped at the tight junctions of the PE of retina (Peyman and Apple, 1972; Peyman and Bok, 1972). Thus to maintain the normal composition of AH, which is distinct from the plasma, some kind of barrier must be imposed in all the associated structures separating the AH, vitreous and the retina from the plasma. All such structures have either endothelial and/or epithelial barriers. In the mammalian eye, endothelial barriers are localized in the vessels of the retina, optic nerve, ciliary muscle and the iris. Epithelial barriers are present in the PE of retina, the NPE of the ciliary body and the posterior layer of the iridial epithelium (Raviola, 1977). All these constitute the two important barrier systems, namely the blood-vitreous or the blood-retinal barrier and the blood-aqueous barrier. Both these barriers thus have endothelial and epithelial parts and prevent almost all protein movement and they are effective even with respect to low molecular weight solutes, such as sucrose and fluorescein (Bill, 1975).

Blood-Vitreous or Blood-Retinal Barrier

The endothelial cells of the retinal capillaries and the PE of the retina represent respectively the endothelial and epithelial parts of the blood-retinal barrier. The endothelial cells of the retinal capillaries are relatively thick, without fenestrations and attached to each other by tight junctions. They prevent both

outward and inward movement of horseradish peroxidase as examined by injecting intravenously and into the vitreous. However, after intravenous injection, horseradish peroxidase was observed in pinocytic vesicles both in the retinal and optic nerve capillaries but absence of the material outside the vessels even after 2 hours indicated that in these vessels pinocytosis did not cause any appreciable outward transport (Peyman and Apple, 1972; Peyman and Bok, 1972). The epithelial part of the barrier, i. e. the PE separates the choroidal fluid from the retinal tissue fluid and is very important because choroidal tissue fluid is likely to be very similar to plasma.

The Blood-Aqueous Barrier

The ciliary and the iridial epithelia constitute the epithelial part of the barrier and protect the posterior chamber from circulating macromolecules. The other is the endothelial part and is constituted by the non-fenestrated iris capillaries. This prevents movement of macromolecules from the lumen of the iris vessels into the iridial stroma. Horseradish peroxidase does not pass through the walls of iris capillaries (Vegge, 1971).

The double layer CE is in fact a forward continuation of the retina. It is the NPE which constitutes the barrier. These cells are attached to each other by tight junctions and several studies have shown that penetration of horseradish peroxidase from the stroma of the ciliary processes into the posterior chamber is prevented by these junctions (Shabo and Maxwell, 1972; Vegge, 1977)

Since the blood-ocular barriers are largely impermeable even to small water soluble substances, such as glucose and amino acids, important metabolic substrates have to be transported through these barriers by means of carrier-

mediated transport systems. The blood-aqueous barrier contains transport systems for glucose and several amino acids (Reddy, 1979) and transport system for glucose, amino acids, lactate and choline have been identified in the blood retinal barrier (Dollery et al., 1971; Millar and Steinberg, 1976; Tornquist, 1979; Alm et al., 1981; Tornquist and Alm, 1982; Karlsson et al., 1984; Alm and Tornquist, 1985)

Blood Supply to the Eye

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 and the anterior ciliary arteries. These 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 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. The venous blood from the retina drains into the retinal veins and then into the ophthalmic veins.

Special Features of Ocular Blood Supply in Bovine

The bovine eye has a unique vascularization in which the ciliary artery provides most of the blood flow to the eye (Prince et al., 1960). Also in respect of anterior uvea, the greater arterial circle, formed by the anastomosis of the two

long posterior ciliary arteries and the anterior ciliary arteries, is very well developed in this animal (the greater arterial circle in the human has been named the major circle of the iris, inappropriately, because the circle is actually located in the ciliary body and also formed by the ciliary system of arteries). Thus cannulating one of the long posterior ciliary artery in bovine eye allows circulation to the anterior uvea; and catheterizing the ciliary artery before its division into medial and lateral ciliary arteries, the whole eye can be kept viable for a considerably longer period of time and indeed a very recent attempt by de Coo et al. (1993) has proven to be successful in keeping the eye metabolically active for up to 9 days.

In bovine, orbital blood supply is provided largely by the external ophthalmic artery. This is a branch of the internal maxillary artery, which itself is a continuation of the external carotid artery. A smaller artery, the internal ophthalmic artery, arising from the internal rete, also contributes to the orbital supply by entering the orbit through the optic foramen and then joining with the medial long posterior ciliary artery.

The ciliary artery originates from the external rete (an arterial network formed by the arborization of the external ophthalmic artery) (Fig. 6), and it passes superior to the optic nerve. It then divides usually into the larger medial and smaller lateral ciliary arteries which travel parallel to each other, both loosely attached to the optic nerve. Both arteries bifurcate before reaching the globe and each further breaks up into several short posterior ciliary arteries at the globe. Two branches of the large medial ciliary artery each form a long posterior ciliary artery, penetrate into the globe, one along its medial side and the other along its lateral side, well before the equator is reached. These two long posterior ciliary arteries form the chief supply to the major arterial circle and hence to the ciliary

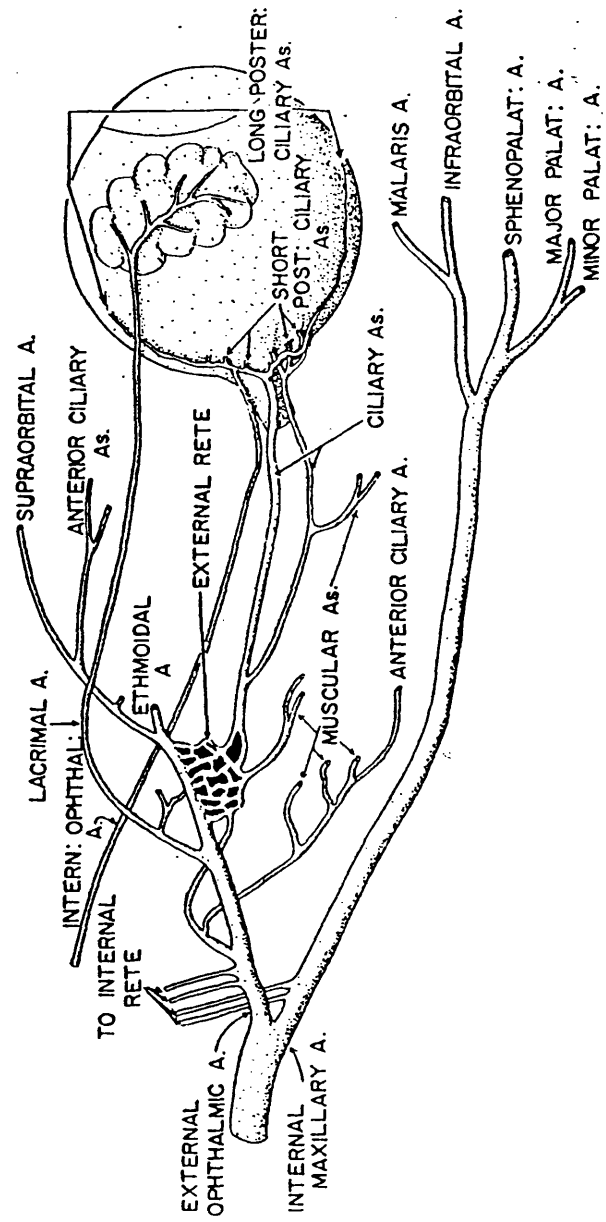

Fig. 6. A schematic diagram of the orbital arterial system of ox (Anatomy and Histology of the Eye and Orbit in Domestic Animals. Prince et al., 1960).

processes of the ciliary body. The short posterior ciliary artery penetrates the sclera at a variable distance from the optic nerve entrance. The retinal arteries in bovine are branches of some of the short posterior ciliary arteries and they enter the globe near where the optic nerve penetrates the globe. A schematic diagram of the orbital arterial system in ox is shown in fig. 6.

The four vortex veins, as a rule one vein in each quadrant of the posterior pole of the eye, drain all the blood from the ciliary body and the iris. Most of the blood is drained by the vortex veins although there are minor anastomotic communications with anterior episcleral vessels. AH is drained into these episcleral vessels from the collector channels leaving the canal of Schlemm. Prince et al. (1960) noted that the ox has a true canal of Schlemm. The vortex veins, after leaving the globe, join to the supraorbital vein, inferior orbital vein and to the external rete which, after multiple anastomotic communications among them, are finally drained by the orbital vein.

Ocular Nerve Supply

The eye has a very rich nerve supply. Apart from the optic nerve, there are sympathetic nerves which are branches of the cervical sympathetic chain, and parasympathetic nerves from the oculomotor and facial nerves. Additionally, there are sensory nerves which are branches of the trigeminal nerve. These nerves are distributed only within the uvea and the extraocular parts of the retinal blood vessels (Bill and Sperber, 1990)

Sympathetic nerves originate in the superior cervical ganglion, and parasympathetic nerves in the pterygopalatine and ciliary ganglia. With the exception of the visual apparatus, sensory impulses are conveyed through the long and short ciliary nerves. 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 gangion 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 vessels of the globe and the dilator pupillae 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.

Composition of Aqueous Humour

AH has a unique composition that differs from plasma in many respects. Several laboratory studies suggest that AH is not simply a filtrate. Rather it is an intraocular fluid whose production is homeostatically controlled and some of whose individual components are in a state of rapid turnover. The complex composition of AH is modulated by many factors, and changes and/or

imbalances in its carefully tuned chemical composition are thought to be both the cause and consequences of pathological condition in the anterior segment of the eye. For example, biochemical analysis of AH has identified high molecular weight lens protein as a cause of elevated IOP in phacolytic glaucoma (Epstein et al., 1978) and ascorbic acid concentration in AH differs in normal subjects and in COAG patients (Lam and Lee, 1975). Several studies have shown that AH can act in tissue culture to promote proliferation (Albrink and Wallace, 1951; Benezra and Sachs, 1974; Herschler et al., 1980; Herschler and Tucson, 1983) or to inhibit cellular growth (Kornblueth and Tenenbaum, 1956). While these opposing findings can be explained by the different experimental conditions and procedures, the possibility of AH being a growth modulator should be considered. Thus AH may be a growth medium in which stimulatory agents and cytotoxic factors act in a concerted manner to affect changes in cell number, morphology and function. Understanding the nature of these modulators and their functions may provide a clue to solving the problems of glaucoma. It is possible that derangement of such a modulator system in the AH may lead to changes in the proliferative capacity, biosynthetic properties and ultimately the survival of the cells of the trabecular meshwork resulting in an increased resistance to outflow .

The AH has a wide range of chemicals in its composition including ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^- etc.), crystalloid or low molecular weight substances (glucose, ascorbate, lactate, pyruvate, urea, H_2O_2, amino acids etc), colloidal or high molecular weight substances (proteins, lipids), biologically active substances (catecholamines, eicosanoids, hormones etc.) as well as some miscellaneous substances such as hyaluronic acid, hyaluronidase etc.). These chemical components derive from a number of sources, the principal ones being the plasma (by passive diffusion) and the CE (by active secretion). Specific

substances also enter the AH by diffusion or secretion from surrounding tissues: corneal endothelium, the crystalline lens, trabecular meshwork, iris and the vitreous. These tissues also utilize a number of nutrients present in the AH, e.g. the main source of glucose for the cornea and the lens is the AH. Thus the composition of AH depends on the nature of the freshly secreted fluid from the CE plus the subsequent passive and active exchange across adjacent tissues. The rate of turnover of AH also contributes to modulation of the composition by way of accumulating waste products of the surrounding tissues. The metabolic requirements of tissues such as cornea, lens and trabecular meshwork are met by the continuous flow of AH through the posterior and anterior chambers of the eye. Diffusional and metabolic alterations of the AH occur continually. For example the lens alters the AH by using glucose, amino acids and other solutes, releasing metabolic wastes, such as lactic acid and may act as homeostatic reservoir for amino acids. Normal composition of AH reflects the normality of all the associated structures contributing in AH production and drainage. Tables 1, 2 and 3 show normal concentration of important components of AH in comparison to the plasma.

Table 1. Biologically active substances in AH and plasma.

Components	Aqueous Humour ($ng.ml^{-1}$)	Plasma ($ng.ml^{-1}$)
Prostaglandins	2	—
Cyclic AMP	8	—
Catecholamines		
Noradrenaline	[a]0.8 - 1.14	0.311
Adrenaline	0 - 0.13	0.097
Dopamine	0.12	0.037

Adapted from Cooper et al. (1984); [a]Trope and Rumley (1985).

Table 2. Protein composition of AH in comparison to plasma.

Components	AH ($\mu g.ml^{-1}$)	Plasma ($\mu g.ml^{-1}$)
Protein (total)	[a]12.4 ± 2.0	7000
Albumin	[b]5.5 - 6.5	3400
Transferrin	[b]1.3 - 1.7	—
Prealbumin	[b]0.3 - 0.4	—
Fibronectin	[c]0.25	29
Immunoglobulins		
IgG	[c]3.0	1270
IgE ($Iu.ml^{-1}$)	[c]<0.75	16 - 218

Sources: [a]Tripathi et al. (1989); [b] Inada et al. (1984); [c]Berman (1991).

Table 3. Concentration of electrolytes and low molecular weight solutes in human AH and plasma.

Components	Aqueous Humour	Plasma
Electrolytes (mM)		
Na^+	142	130 - 145
K^+	4	3.5 - 5.0
Ca^{2+}	1.2	2.0 - 2.6
Mg^{2+}	1	0.7 - 1.1
Cl^-	131	92 - 125
HCO^{-3}	20	24 - 30
Organic solutes(mM)		
Ascorbate	1.1	0.04 - 0.06
Lactate	4.5	0.5 - 0.8
Citrate	0.1	0.1
Glucose	2.7 - 3.9	5.6 - 6.4
Urea	4.1	3.3 - 6.5
Glutathione	0.001 - 0.01	—
[a]H_2O_2	0.024 - 0.069	—
[b]Amino acids (total)	0.17	0.12

Adapted from Riley (1983); [a]Spector and Garner (1981); [b]Davson (1969).

Aqueous Humour Formation

There is now general agreement that AH is produced by the double-layered CE and enters the posterior chamber in the first instance. There are three basic mechanisms by which materials may cross an epithelial barrier, namely diffusion, ultrafiltration and active transport.

All three basic processes contribute to the formation of AH at one step or other. Two major steps are implicated in the formation of AH. The first step is the development of plasma filtrate within the stroma of the ciliary processes by means of ultrafiltration. Due to the fenestrated nature of the ciliary capillaries this ultrafiltrate contains a higher percentage of plasma proteins. Still the capillary wall is a considerable barrier for the plasma proteins. Studies on the dynamics of extravascular plasma proteins in the ciliary processes indicate that the net filtrate from the capillaries contain about 4% of the albumin and 3% of the γ-globulin in the plasma (Bill, 1968). Protein content in the ciliary stroma may be extremely high in some species, e.g. in rabbit it is about 75% of that in the plasma (Bill, 1968). The high protein concentration in the ciliary stroma reduces transcapillary difference in the oncotic pressure, which in turn is very important for filtration from the capillaries. The second step is the extraction of materials (electrolytes and other substances) by the CE into the AH, against a concentration gradient, by means of active secretion of solutes followed by osmotic flow of water to form the AH (Cole, 1977)

The presence of tight junctions between the NPE cells and the non-fenestrated iris capillaries is considered to be morphological basis of the blood-aqueous barrier. Blood-borne large molecules, such as proteins cannot pass this barrier (Green, 1984b; Novack and Leopold, 1988) The ocular barriers are effective

even with respect to low molecular weight solutes, such as sucrose and fluorescein (Bill, 1975)

AH formation was once considered a simple process of diffusion or ultrafiltration of fluid from the plasma across the CE. However, the concentration of certain solutes in the plasma was inconsistent with the view (Davson, 1955; Reddy, 1957) and it is now well accepted that active transport is the most important factor in AH formation and that transepithelial ultrafiltration by hydrostatic pressure may not occur (Sears, 1991). The rate of AH formation depends on the rate of active transport by the CE (Cole, 1977). The arguments against transepithelial ultrafiltration are: First, Bill's (1975) calculation indicates that the value for the transciliary epithelial difference in hydrostatic pressure is less than the oncotic pressure of the stromal tissue fluid. The stroma of the ciliary processes has oncotic pressure (approximately 14 mmHg) as a result of protein leakage through the fenestrated capillaries. Assuming an IOP of 15 mmHg, Bill showed that a capillary hydrostatic pressure greater than 29 mmHg would be necessary for the formation of any ultrafiltrate. The value of capillary hydrostatic pressure has been estimated to be 27 to 28 mmHg (Bill, 1973) and 25 to 33 mmHg (Cole, 1977). Green and Pederson (1972) calculated that a capillary pressure greater than 50 mmHg would be necessary to promote ultrafiltration through the CE. Thus, the estimated values of capillary hydrostatic pressure in the CE and a consideration of the hydrostatic and oncotic pressures involved do not favour ultrafiltration at all. Second, a good deal of formation of AH in subhuman primates continues at a very low arterial blood pressure. Third, although AH formation is oxygen dependent, there is an independence of AH flow from increments of ciliary blood flow (Chiou et al., 1990; Chiou and Chen, 1993). In a recent study Hong and Chiou (1993) showed that the extensively used β-adrenergic antagonists, timolol, levobunolol

and betaxolol, which reduce IOP by reducing AH formation, all increased ocular blood flow considerably (up to 55%). A systemic carbonic anhydrase inhibitor, ethoxzolamide, was also shown to cause an increase of blood flow up to 56%. Increased blood pressure should increase the ultrafiltration. Hence an increase in AH formation would be expected with these drugs if ultrafiltration were a contributing factor in AH formation. Finally, Brodwall and Fischbarg (1982) showed that the hydraulic conductivity of the rabbit CE was too low to be compatible with the production of AH by transepithelial ultrafiltration.

Active secretion by the CE is accomplished by the transport of one or more ions, such as Na^+ (Kinsey, 1971; Cole, 1977; Pesin and Candia, 1983; Krupin et al., 1984; Helbig, et al., 1989a, 1989b; Sears et al., 1991b), Cl^- (Cole, 1969; Holland and Gipson, 1970; Kishida et al., 1983; Pesin and Candia, 1983; Helbig et al., 1989a) and HCO_3^- (Cole, 1977; Maren, 1977; Helbig et al., 1989b; Wolosin et al., 1991)).

The membrane bound enzyme complex Na^+,K^+-ATPase is an energy-dependent active transport system present predominantly in the basolateral membranes of the NPE but also on the PE (Cole, 1964; Cole, 1984; Riley and Kishida, 1986; Coca-Prados and Lopez-Briones, 1987; Flugel and Lutjen-Drecoll, 1988; Usukura et al., 1988). Na^+,K^+-ATPase is an enzyme that catalyses the hydrolysis of ATP (ATP $\rightarrow$ ADP + P) whilst energizing the Na^+,K^+-pump. A marked decrease in AH formation occurs after poisoning of Na^+,K^+-ATPase with ouabain (Cole, 1977; Bonting and Becker, 1964). Vanadate also blocks Na^+,K^+-ATPase activity and its topical administration reduces AH formation in rabbit (Becker, 1980; Krupin et al., 1980a) and in monkeys (Podos et al., 1984). It is likely that Na^+ is the actively transported ion, with Cl^- or HCO_3^- following to maintain electroneutrality. Measurement of electric potential across the CE

indicates that the aqueous is positive with respect to the stroma and ouabain reduces this potential (Cole, 1961; Pesin and Candia, 1983; Krupin et al., 1984). It is clear from these studies that sodium is the transported ion and its pump generates the transepithelial potential difference. In a recent study (Sears et al., 1991) in intact isolated ciliary processes, free of vascular and connective tissue components but in which tight junctions and gap junctions of the epithelial bilayer were preserved, ouabain has been shown to reduce the transepithelial potential difference virtually to zero, confirming the singular importance of the sodium pump to generate the potential difference required for transport.

Active transport of Cl^- may also occur, although the magnitude of this transport is probably small compared to that of Na^+ (Cole, 1969). Although there is no strong evidence to suggest Cl^- as a primary secreted ion coupled to cell metabolism, there appears to be a role for chloride transport in the CE. Some of the short circuit current and transepithelial potential difference across isolated iris-ciliary body has been shown to be chloride-dependent (Cole, 1969; Holland and Gipson, 1970; Kishida et al., 1983). Net chloride fluxes towards the aqueous by active transport have been reported (Saito and Watanabe, 1979; Saito et al., 1980). Presence of chloride channels has been demonstrated in shark CE and intracellular chloride activity in these cells has been shown to be greater than that predicted from considerations of electrochemical equilibrium (Wiederholt and Zadunaisky, 1986). Single chloride channel activity have been demonstrated in rabbits (Yantorno et al., 1987).

Formation of HCO_3^- by the carbonic anhydrase, which catalyzes the reaction $H_2O + CO_2 \rightarrow HCO_3^- + H^+$ is important in the formation of AH. There exists substantial evidence that acetazolamide (a carbonic anhydrase inhibitor) directly affects the transport mechanisms in the CE (Cole, 1977). The exact mechanism,

however, is not known. The existing hypotheses are (i) inhibition of carbonic anhydrase causes a decrease in HCO_3^- available for diffusion into the intercellular channels to maintain electroneutrality, (ii) a change in intercellular pH may inhibit Na^+,K^+-ATPase, (iii) decreased availability of H^+, produced by the carbonic anhydrase-catalysed reaction, decrease the exchange of H^+ for Na^+ and reduces the availability of intracellular Na^+ to be transported into the intercellular channel, (iv) HCO_3^- dependency of sodium pump (Sears et al., 1991). Acetazolamide decreases the rate of Na^+ and HCO_3^- transport into the posterior chamber by equimolar amounts, suggesting a linkage of the accession of these two solutes (Maren, 1976; 1977).

Among other mechanisms recently uncovered for transport of HCO_3^- across the CE, in cultured bovine PE, is a chloride-dependent uptake (Helbig et al., 1988; 1989a). Uptake is not the same as vectoral transport; nonetheless, it has been demonstrated that a sodium-bicarbonate cotransporter and a chloride-bicarbonate exchanger are present in bovine PE.

Other ions and low molecular weight solutes, such as amino acids, glucose, inositol, ascorbic acid etc. are also reported to be secreted by the CE, but the exact mechanism of their transport into the posterior chamber is not clearly known (Berman, 1991). There appears to exist an independent transport system for many of them. A statistical study of the covariation of the concentrations of amino acids and related compounds in the human AH suggested existence of six transport systems in the CE: three independent mechanisms for neutral amino acids and independent mechanisms for basic amino acids, acidic amino acids and urea (Ehlers, 1978). The concentration of ascorbic acid in the aqueous is about 20 times that in the plasma and there is evidence that it is transported actively to the AH (Becker, 1967; Chu and Candia, 1988; Helbig et al., 1989).

In view of the greater concentration of certain enzymes including Na^+,K^+-ATPase, adenylate cyclase and carbonic anhydrase etc. (Tsukahara and Maezawa, 1978; Mishima et al., 1982a) and greater development of intracellular organelles and greater metabolic activities of NPE compared to PE (Shantaveerappa and Bourne, 1964; Caprioli and Sears, 1983), it has been suggested that the NPE plays the dominant role in AH formation.

The mode of action of fluid formation by the transporting epithelium (CE) has been best described by the standing-gradient osmotic flow model (involving active transport of solutes) proposed by Diamond and Bossert (1967). A steady state, standing osmotic gradient is maintained in the lateral intercellular channel, with the greater concentration of solute occurring proximally in the channel adjacent to the tight junction (Fig. 7). This hypertonic fluid in the

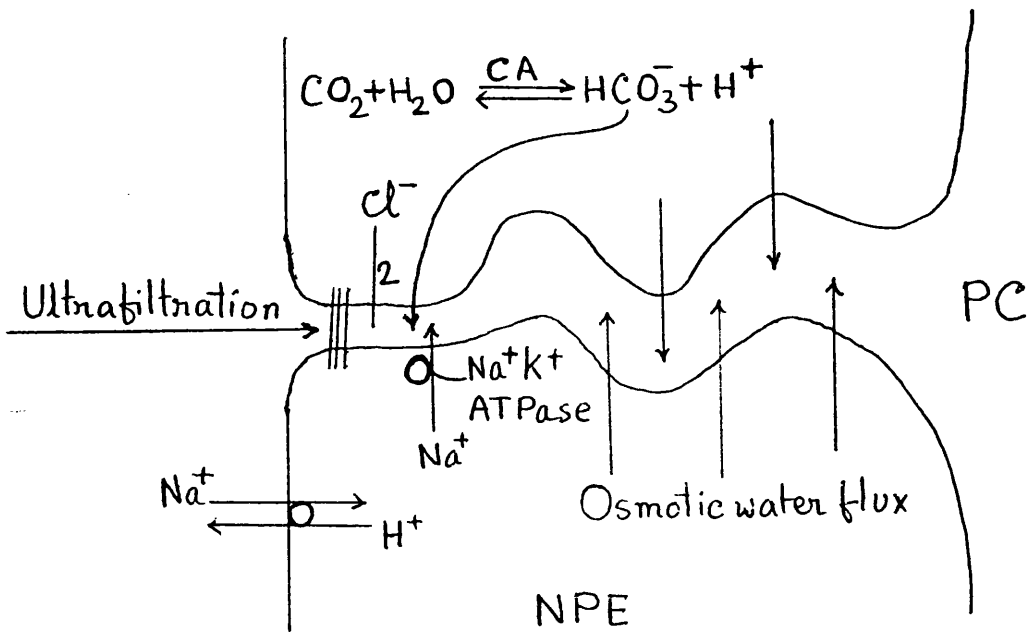

Fig. 7. A diagram of the hypothetical standing-gradient osmotic flow model (Diamond and Bossert, 1967). Na^+,K^+-ATPase is located in higher concentration along lateral cellular interdigitations. PC = posterior chamber.

proximal region of the channel forces an osmotic flow of water into the channel. The solute concentration decreases from the proximal to the distal end of the channel as water enters; slightly hypertonic fluid flows into the posterior chamber as nascent AH. The success of this model in explaining AH production requires the restriction of fluid and solute entry into the intercellular channel by the tight junction, i. e. there should be no ultrafiltration and in fact this may be the case.

Drainage of Aqueous Humour

AH enters the posterior chamber from the ciliary process flows around the lens and through the pupil into the anterior chamber (Fig. 5). From the anterior chamber it leaves the eye and enters the general venous circulation, by bulk flow via two pathways, both commencing at the anterior chamber angle. One pathway runs through the trabecular meshwork across the inner wall of Schlemm's canal and then into collector channels, aqueous veins and the general venous syetem. This is called the anterior, trabecular or conventional route. There are three layers of meshwork between the anterior chamber and the inner wall of Schlemm's canal. The fisrt layer is the uveal meshwork, which is the forward extension of the ciliary muscle inserting in the cornea (Bill and Svedberg, 1972). The second layer is constituted by several sheets of connective tissue extending between the scleral spur and the cornea. The third layer, called the juxtacanalicular tissue, is an endothelial meshwork between the corneoscleral meshwork and the inner wall of Schlemm's canal. It contains collagen and elastic fibres, a ground substance and several layers of endothelium enmeshed in a matrix of glycosaminoglycans, proteoglycans and other macromolecules. The major resistance to outflow resides at the juxtacanalicular

tissue (also called cribriform plexus) and the inner wall of Schlemm's canal. The second pathway is the posterior, uveoscleral or unconventional route. Aqueous flows from the chamber angle across the iris root and the anterior face of the ciliary muscle, through the connective tissue spaces between the muscle bundles of the ciliary body. These spaces in turn open into the suprachoroid from which fluid can pass through the scleral substance or through the perivascular spaces into the episcleral tissue and then into the venous circulation. The pressure in the suprachoroid is lower than in the anterior chamber, the difference being at least a few mmHg under normal conditions, thus favouring the uveoscleral outflow.

In primate most of the drainage occurs through the trabecular route and a small portion is drained via the uveoscleral route. A small part of the AH flows into the vitreous to be absorbed into the posterior part of the eye; some of the aqueous apparently is absorbed at the ciliary body (Moses, 1990). Lower animals have no well developed canal of Schlemm, but a sinus structure having the same function (Bill, 1975). In human eyes, the uveoscleral outflow accounts for about 5 to 20 percent of total outflow (Bill and Phillips, 1971; Bill, 1975), while in monkey outflow is fairly equally distributed between the two routes (Bill, 1966a, 1971). In rabbit and cat, there is comparatively little uveoscleral drainage (Bill, 1966b; Cole and Monro, 1976).

Intraocular Pressure

The contents of the eye are under a pressure, which is larger than in most other tissues, due to continuous formation of AH. Mean normal IOP in man is about 15 mmHg with the highest and lowest accepted values of 21 mmHg and 10.5 mmHg respectively (Davson, 1990; Hurvitz et al., 1991). IOP can vary between

species, individual and even between eyes of the same individual. This pressure is governed by several factors, such as the rate of secretion of AH, resistance to outflow (by at least 2 pathways) and episcleral venous pressure.

Dynamics of Aqueous Humour

The flow of aqueous through the different compartments and outflow pathways of the eye is one of typical hydraulic flow where the source of energy is the difference of pressure at the ends of the passage of flow; the pressure at the upstream end is of course greater. The difference of upstream and downstream pressure is called pressure head (ΔP). The rate of hydraulic flow is related to Poiseuille's law. It has been incorporated into the Goldman equation:

$$F = C (P_i - P_e) \text{ or } F = \Delta P.C$$

This equation relates aqueous flow (F) to facility of outflow (C) and ΔP. The pressure head in case of AH drainage is the difference between IOP (P_i) and the episcleral venous pressure (P_e), i.e. the pressure in the vessels into which AH drains. The mean normal value for outflow facility in human is estimated as 0.25 $\mu l.min^{-1}$. $mmHg^{-1}$ applied pressure (Davson, 1990)

Glaucoma

The term glaucoma encompasses a group of eye diseases characterised by progressive defects in the visual field and ultimately loss of vision. It is usually, though not necessarily, associated with an increase in IOP. The loss of visual field is associated with damage to the optic nerve which is manifested clinically as cupping of the optic disc. The boundaries between glaucoma, low tension glaucoma and ocular hypertension are often indistinct. However, glaucoma is often associated with an IOP of greater than 21 mmHg. Low

tension glaucoma is 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 21 mmHg, but there are no pathological changes present associated with glaucoma, although this condition frequently heralds the development of glaucoma and should be treated accordingly (Jay, 1992). This is because IOP is the most important risk factor of glaucoma; the greater the IOP the greater the risk. A patient with an IOP >24 mmHg is 10.5 times more likely to develop subsequent field loss than a patient with an IOP <16 mmHg (Sommer, 1989).

The disease may be caused by a number of factors, such as: (i) the bowing of the iris over the drainage angle, thus partially occluding the passage of aqueous through the trabecular meshwork into the canal of Schlemm (narrow angle glaucoma, NAG). This leads to an elevated IOP which stretches the scleral layer and further leads to cupping of the optic disc. IOP may rise as high as diastolic pressure in the retinal vessels, thus giving rise to progressive retinal ischemia and detachment, with concomitant increasing loss of visual field (scotoma). (ii) Complete occlusion of the trabecular meshwork by bulging of the peripheral iris (closed angle glaucoma, CAG). (iii) Partial occlusion of the trabecular meshwork by build-up of cellular debris or glycosaminoglycans in the juxtacanalicular tissue (chronic open-angle glaucoma, COAG), thus increasing the resistance to outflow of AH and consequently IOP. This is the most common form of glaucoma all over the world. Again all these types of blockade can be caused either from intrinsic ocular problems (primary glaucomas) or in relation to other ocular or systemic problems (secondary glaucomas). Further, the conditions may be acute, presenting with a sudden increase in IOP and a rapid development of visual loss secondary to optic neuropathy and corneal oedema; or chronic, developing insidiously over a long period of time, with a

comparatively lower IOP, sometimes even in the normal range, presumably caused by the optic nerve being particularly susceptible to the development of glaucomatous optic neuropathy. While much has been written about the predisposing factors, such as family history of damage from glaucoma (Heilman and Richardson, 1978; Schottensten, 1989), diabetes mellitus, high myopia, systemic vascular diseases, age etc., the immediately recognisable causative factor is the insufficient drainage of AH leading to an elevated IOP in most types of glaucomas. Reduction of IOP by various means, to a level at which optic nerve damage ceases, is still the only available and clinically acceptable treatment of the most commonly occurring COAG.

GENERAL OUTLINE OF SIGNAL TRANSDUCTION MECHANISMS AND CELLULAR FUNCTIONS

The unique conceptual framework and the central event for the regulation of most, if not all, cellular functions is the reversible phosphorylation of specific substrate proteins in specific tissues, mediated by hundreds of extracellular and intracellular messenger molecules through their association or interaction with various protein phosphorylation systems (Cohen, 1988, 1989; Hardie, 1990; Walaas and Greengard, 1991). All protein phosphorylation systems have at least three components. The central ones are the phosphoprotein themselves, whose biological properties change during phosphorylation-dephosphorylation. The other components are the two classes of enzymes which are needed for phosphorylation and dephosphorylation reactions. One class includes protein kinases which biochemically are phosphoryltransferases. These enzymes catalyze the transfer of the γ-phosphate group of ATP to the hydroxyl group of a serine, threonine or tyrosine residues of the acceptor substrate proteins (Krebs and Beavo, 1979; Walaas and Greengard, 1991). Another class includes the

phosphoprotein phophatases, which dephosphorylate the phosphoproteins, thereby returning the specific phosphorylation system to its basal state (Cohen, 1989; Hardie, 1990; Shenolikar and Nairn, 1991; Garg, 1992). Additional components are the associated modulators (Nairn et al., 1985b). Thus a substrate protein is converted from dephosphorylated to phosphorylated form by a protein kinase, and the phosphorylated form is converted back to the dephophorylated form by a protein phosphatase. Physiological activity depends on the state of phosphorylation of the substrate protein. So the relative activities of protein kinase and protein phosphatase determine the state of phosphorylation and physiological activity of the specific substrate protein concerned.

Protein Phosphatases

There are hundreds of protein (serine/threonine) kinases in mammalian cytosol and nucleus (Hunter,1987), and also there are certainly hundreds of target phosphoproteins. In contrast dephosphorylation appears to be catalysed by a relatively smaller number of protein phosphatases. A protein phosphatase is a phosphoesterase which catalyses the removal, by hydrolysis, of a phosphate group from a hydroxylated amino acid residue of a protein. The protein phosphatases involved in the dephosphorylation of serine and/or threonine residues appear to be only four in number (Cohen, 1989; Ingebritsen and Cohen, 1983a,b; Shenolikar and Nairn, 1991).

Mechanism of Phosphorylation of Substrate Proteins

Activation of protein kinases represents the most common and direct causal activation mechanism of substrate proteins, although increases in the state of phosphorylation can be brought about indirectly either by inhibition of protein

phosphatases (Walaas and Greengard, 1984; Nairn et al., 1988; Hemmings et al., 1984a, 1990) or by changes in the properties of the protein itself as a substrate for distinct protein kinases or phosphatases. Changes in the state of phosphorylation of substrate proteins mediate, either directly or through additional steps, the generation of biological responses.

Protein Kinases and Their Activation

As mentioned earlier, protein kinases are phosphoryltransferases activation of which causes the phosphorylation of specific substrate proteins. There are numerous protein kinases as there are hundreds of substrate proteins in various tissues (Hunter, 1987; Cohen, 1989). Two important ways have so far been identified for the activation of protein kinases:

One is the activation by autophosphorylation which is the intramolecular event within the protein kinase itself. A large number of protein kinases undergo autophosphorylation and the majority of such kinases have autophosphorylation sites, the phosphorylation of which may profoundly change the activity of the enzyme involved (Blackshear et al., 1988). Important examples are cyclic AMP-dependent protein kinase II (Erlichman et al., 1974; Rubin and Rosen, 1975), cyclic GMP-dependent protein kinase (Gill, 1977; Lincoln and Corbin, 1983), Ca^{2+}-calmodulin-dependent protein kinase II (Lou et al., 1986; Lai et al., 1986,1987).

The second and most important way of activation involves a large number of extracellular and intracellular messengers and their specific receptors on the cell surface or within the cytoplasm. This is extremely complicated and involves four basic mechanisms, known as signal transduction systems. These are:

(1) Cyclic nucleotide systems, mediated by the intracellular second messengers cyclic AMP, and in certain cases, cyclic GMP. Very recently some evidence in support of cyclic CMP [cytidine 3′,5′-monophosphate] as a third intracellular nucleotide messenger has been put forward (Newton, 1992).

(2) The phosphoinositide system, mediated by the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), the ultimate effect of which is mediated by Ca^{2+}.

(3) Ion channel systems (e.g. ligand-gated ion channels) whose effects are usually ultimately mediated by Ca^{2+}.

(4) The tyrosine kinase system, mediated directly by the receptor-kinase complex without generation of any second messenger (Robert, Kirk and Venis, 1990; Nestler and Greengard, 1984; Walaas and Greengard, 1991).

Thus the large number of messenger molecules (hormones, neurotransmitters, local mediators, growth factors etc.) which act at cell surface receptors on mammalian cells, produce their effects on target cells through the above mentioned basic mechanisms.

It is customary to name a protein kinase after the name of the first or the second messenger which is responsible for its activation. Thus the protein kinase which is activated by the second messenger cyclic AMP is named as cyclic AMP-dependent protein kinase. Similarly cyclic GMP-dependent protein kinase,

Ca^{2+}-calmodulin-dependent protein kinase, insulin-dependent protein kinase, growth factor-dependent protein kinase and so on.

In addition to the above mentioned four basic mechanisms, there appear to be other means of regulation of protein kinases. Some first messengers appear to produce biological responses by regulating the total amount of specific protein kinases in target tissues, e.g. interferon which produce its effect by increasing the double stranded RNA-dependent protein kinase (Krust et al., 1982, Sen, 1982) and some oncogenic viruses increase the tyrosine kinases (Erikson et al., 1980; Hunter and Sefton, 1982) in target tissues. Haeme appears to regulate substrate protein phosphorylation by inhibiting rather than activating haeme-regulated protein kinase, although the mechanism by which this protein kinase is inhibited in the presence of haeme and activated in its absence remain unknown (Hunt, 1980). Still several other cellular messenger, including polyamines (Kuehn and Atmar, 1982; Sekar et al., 1982) and the steroid hormones have been shown to regulate protein phosphorylation in target tissues. Each of the three classes of steroid hormones, namely glucocorticoids, mineralocorticoids and gonadal steroids regulate the state of phosphorylation of type II cyclic AMP-dependent protein kinase in target tissues (Liu and Greengard, 1974, 1976; Liu et al., 1981). Thus with a few exceptions, protein phosphorylation appears to be a common pathway through which a large number of regulatory agents produce metabolic and physiological responses in diverse tissues.

Thus, the original protein kinase hypothesis (Kuo and Greengard, 1969) which states that all of the diverse effects on cell function in nervous and non-nervous tissues are mediated through the activation of only one enzyme - the cyclic AMP-dependent protein kinase - and the later extension of this hypothesis which states that a wide variety of biologically active molecules produce many of their

metabolic and physiological responses through the regulation of specific protein kinases, of which cyclic AMP-dependent protein kinase is just one subclass (Greengard, 1978a) are still invaluable assets in understanding cellular function. Existence of numerous pathways for the activation of protein kinases supports the view that protein phosphorylation is a final common pathway of paramount importance in biological regulation.

According to the extended protein kinase hypothesis extracellular 1st messenger (hormones, neurotransmitters, local mediators etc.) through their association with specific receptors, in most cases also involving a membrane-associated guanine nucleotide binding protein (G-protein), stimulate some intermediary enzymes (such as adenylate cyclase, guanylate cyclase, phospholipases etc.) to produce a generation of second messengers (cyclic AMP, cyclic GMP, Ca^{2+}). The newly formed second messengers activate their respective protein kinases, which in turn catalyse the phosphorylation of specific substrate proteins. This phosphorylation, then, through one or more steps, results in the metabolic or physiological response characteristic of the 1st messenger and the specific tissue involved. A simplified illustration of the overall process is shown in fig. 8.

Receptors and Signal Transduction Mechanisms

The receptor can be defined as a molecular structure on the surface of a cell or within the cell, which binds with a specific substance, called the agonist or ligand, thereby producing a specific physiological response characteristic of the agonist and the cell/tissue involved. The basic requirement for a receptor is the ability to discriminate signal from 'noise'. To receive the signal, the receptor must have an affinity for the messenger (agonist). At the same time the receptor must have specificity, in other words, an appropriately low affinity for less

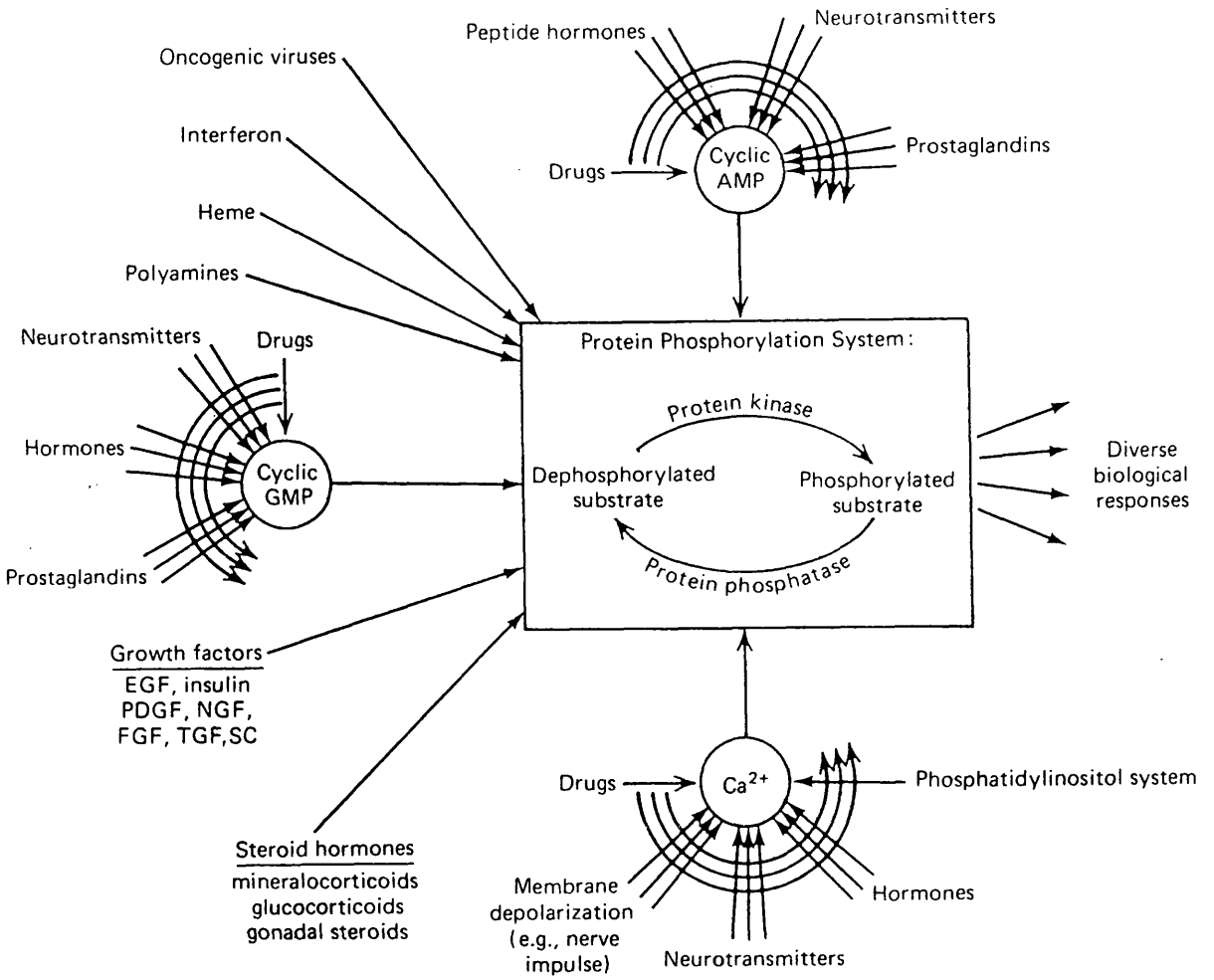

Fig. 8. Schematic diagram of postulated role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents. Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; TGF, transforming growth factor; SC, somatostatin C (Nestler and Greengard, 1984)

active agents. Examples of cell surface receptors are receptors for peptide hormones, neurotransmitters, local mediators (eicosanoids, histamine, bradykinin) etc. and the steroid hormone receptors constitute the examples of cytoplasmic receptors. Obviously there are numerous receptors (known or unknown) as there are numerous messenger molecules both endogenous (associated with and produced by the living body) and exogenous (synthetic chemicals such as drugs, allergens etc.)

Types of Receptor-Effector Linkages

The overall process illustrated in fig. 8, i.e. starting from the extracellular messenger through its occupation of specific receptors (cell surface or cytoplasmic) to the ensuing physiological responses constitute the different steps and components of the signal transduction mechanism. Many different types of linkage between receptor occupation and the resulting physiological response are involved. Some of them involve a G-protein and other intermediates. According to the nature of signal transduction mechanism or receptor-effector linkage, four types of receptors have so far been recognised (Rang and Dale, 1991):

(1) Direct ligand-gated channel type. These are the receptors for fast neurotransmitters where coupling between the receptor and the ionic channel is a direct one not involving any biochemical intermediaries in the cell or within the membrane. Examples are the nicotinic acetylcholine receptor, the $GABA_A$, glycine and glutamate receptors. The action of these transmitters reaches a peak in a fraction of a second, and usually decays within a few seconds.

(2) G-protein-coupled type. Signal transduction by G-protein is a widespread mechanism used by a wide variety of hormones, neurotransmitters, and autocrine and paracrine factors, to regulate cellular functions. G-proteins modulate not only cyclic AMP formation, but also intracellular Ca^{2+} mobilization, arachidonic acid release and, very importantly, membrane potential. Membrane potential is not only trigger for neurotransmitter release and conduction of nerve impulses. In tissues, such as in some secretory cells, it is the main regulator of Ca^{2+} entry. The G-protein-coupled receptor family comprises most of the receptors that are familiar to pharmacologists, e.g. muscarinic acetylcholine receptor, adrenergic receptors, dopaminergic receptors, serotonin receptors, histamine receptors, opiate receptors, receptors for many peptides and many others. Most of these have a variety of subtypes on pharmacological grounds. A large number of G-proteins and their receptors have so far been identified. As many as 86 molecularly and/or pharmacologically defined G-protein-coupled receptors have been reported. These are coupled to a large variety of effectors by a family of up to 15 G-proteins (9 already cloned) in mammalian cells (Birnbaumer, 1990).

(3) Tyrosine kinase-linked type. These are quite different in structure and function fron either the channel-linked or the G-protein-coupled receptors. Examples are receptors for insulin and various other growth factors (Yarden and Ulrich, 1988; Carpenter and Cohen, 1990). In these cases, binding of the extracellular messenger to its receptor appear to directly activate the protein kinase, which often is an integral part of the receptor itself, without any intervening second messenger generation. In certain cases, of course, such tyrosine-specific protein kinases are distinct from the receptors

themselves, although they are often associated with the membranes and functionally linked to membrane receptors through unknown mechanisms.

(4) Intracellular steroid/thyroid-type receptors. These are quite different from the other three types. They are soluble cytosolic or nuclear proteins, so the ligands must first enter the cells to produce their effects. The receptor-mediated regulation of DNA transcription is characteristic of steroid and thyroid hormones and these act by stimulating transcription of selected genes, leading to the synthesis of particular proteins and the production of cellular effects. Receptors of the first three categories are all membrane proteins whereas steroid receptors are soluble cytosolic or intranuclear proteins. Only the mechanisms which are involved or are reported to be involved in aqueous humour dynamics will de discussed further.

SIGNAL TRANSDUCTION SYSTEMS INVOLVED IN AQUEOUS HUMOUR DYNAMICS

Several signal transduction systems appear to be involved in the tissues responsible for AH production (the CE) and drainage (the trabecular meshwork cells). These include (i) the adenylate cyclase/cyclic AMP system which is reported to be involved in decreasing AH formation (Caprioli and Sears, 1984; Sears, 1985; Bartels et al., 1987; Mittag et al., 1993), increasing AH formation (Mittag et al., 1987; Nilsson et al., 1990) and increasing AH outflow (Kaufman, 1987; Erickson-Lamy and Nathanson, 1992); (ii) guanylate cyclase/cyclic GMP system which is reported to cause a decrease in AH formation and IOP (Nathanson, 1987; Korenfeld and Becker, 1989; Becker, 1990; Busch et al, 1992); (iii) Phospholipase/inositol system which is recently reported to be

present in the ciliary epithelium of different species, though its physiological role is yet to be elucidated, e.g. in rabbit (Yoshimura et al., 1989; Ohuchi et al., 1992) and in man (Wax and Coca-Prados, 1987; Lee et al., 1989); (iv) Ion channel system, e.g. Ca^{2+} channel (Jacob, 1991).

All of the above systems are operated through G-protein-coupled receptors. The first three involve their respective second messengers such as cyclic AMP, cyclic GMP or inositol trisphosphate (IP_3) and diacylglycerol (DAG). The ion channel functions do not appear to involve any second messenger; instead, the G-protein interacts directly with the channel macro-molecule to produce the effect.

G-proteins and Their Functions

G-proteins, so called because of their interaction with the guanine nucleotides, GTP and GDP. They are the crucial in cell-to-cell communications and act as molecular switches, regulating signal transfer from cell surface to effector mechanisms inside the cell. G-proteins are present in virtually all eukaryotic cells. They regulate a myriad of metabolic, hormonal, neural, sensory and developmental functions and at least 9 different heterotrimeric G-proteins have now been identified (Dolphin, 1991; Spiegel, 1990).

Mechanism of Activation and Deactivation of G-proteins

In the resting state , the G-protein exists as an unattached $\alpha\beta\gamma$ trimer, with GDP occupying the site on the α subunit. On activation G-protein undergoes cyclical activation/deactivation under the influence of GTP and the agonist stimulated receptor. G-protein mediated signal transduction is initiated when an

extracellular first messenger (a hormone, growth factor or neurotransmitter) binds to a specific receptor on the cell surface. The receptor configuration is temporarily altered, presumably involving the cytoplasmic domain of the receptor, causing it to acquire high affinity for αβγ. Association of αβγ with the receptor causes the bound GDP to dissociate and to be replaced with GTP (GDP/GTP exchange), which in turn causes the dissociation of α-GTP from the βγ subunits. α-GTP is the active form of the G-protein, which diffuses in the membrane towards membrane-bound effectors (enzymes, ion channels) and can associate with them causing activation or inactivation as the case may be. This process is terminated and deactivation of G-protein initiated when the hydrolysis of GTP to GDP occurs through the intrinsic GTPase activity of the α subunit. Both trimeric α-GTP-βγ and monomeric α-GTP are assumed to be active GTPase (Brown and Birnbaumer, 1988). The resulting α-GDP dissociates from the effector and reassociates with βγ, completing the cycle.

The Adenylate Cyclase/Cyclic AMP System

Cyclic AMP is a nucleotide synthesized within the cells from ATP by the reaction of the enzyme adenylate cyclase. It is produced continually and inactivated by hydrolysis to 5-AMP, by the action of a family of enzymes known as phosphodiesterases. Many different drugs, hormones and neurotransmitters produce their effects by increasing or decreasing the catalytic activity of adenylate cyclase and thus raising and lowering the concentration of cyclic AMP within cells. Cyclic AMP regulates a large variety of cellular functions including, metabolism, cell division, cell differentiation, ion transport, ion channel function, contraction, secretion etc. All these functions are brought about by a common mechanism, namely the activation of various protein kinases by cyclic AMP. These enzymes catalyze the phosphorylation of serine and

threonine residues in different cellular proteins, known as substrate proteins, using ATP as the source of phosphate groups and thereby regulate their function. The phosphorylated substrate proteins return to the basal state by the action of specific phosphatases. As described above, the adenylate cyclase is under the control of G-proteins and the specific receptors on the cell membrane. A schematic representation of the whole process is shown in Fig. 9.

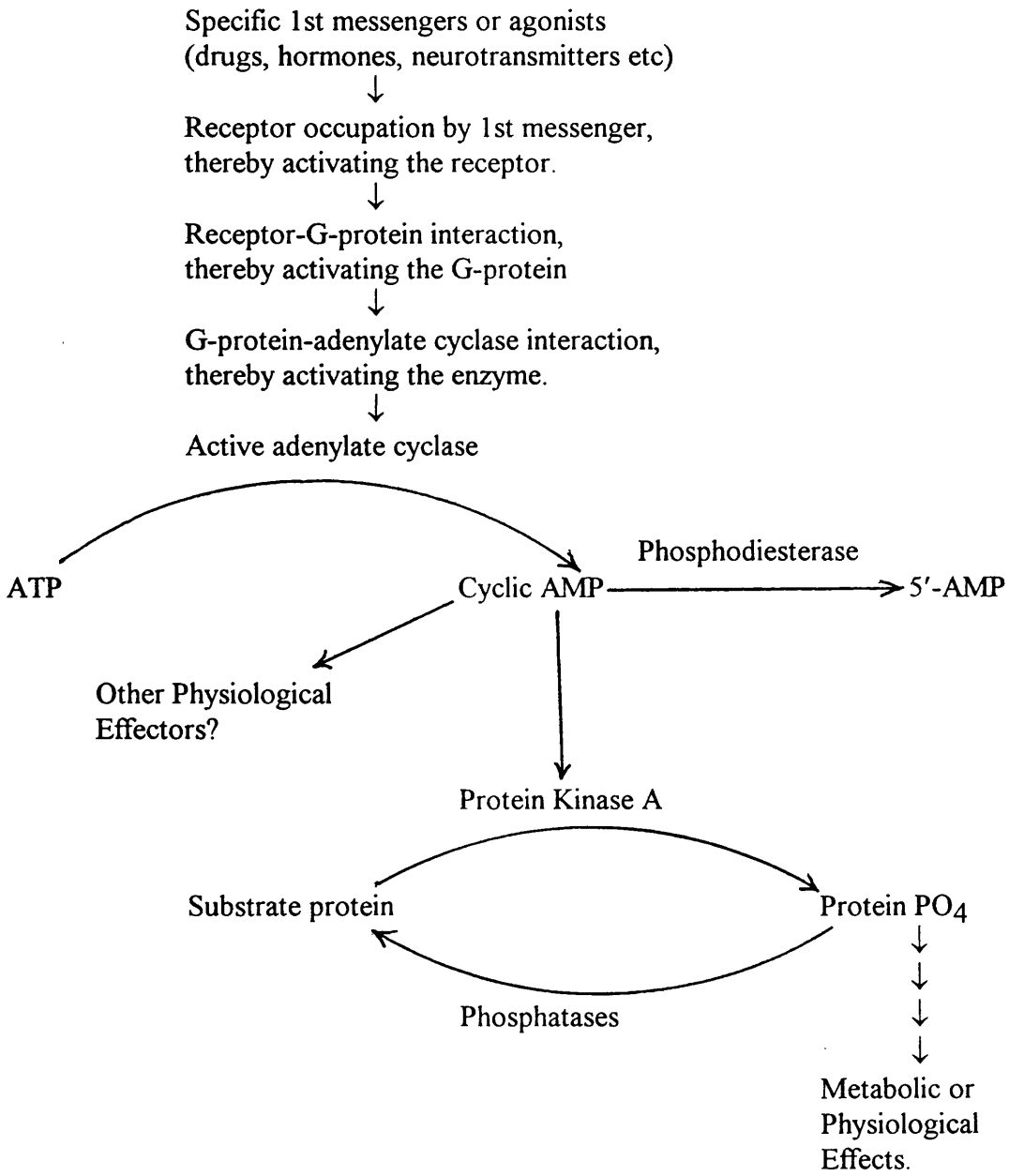

Fig. 9. Schematic representation of adenylate cyclase/cyclic AMP-mediated signal transduction mechanism.

The Guanylate Cyclase/Cyclic GMP System

Cyclic GMP is now known to be a second messenger in diverse tissues including the cardiovascular system (Walter, 1989), other smooth muscle and neutrophils (Wolfe et al., 1989), brain (Levitt et al., 1984) and CE (Korenfeld and Becker, 1989; Nathanson, 1987; Mittag et al., 1987). Thus cyclic GMP is receiving greater attention as an important second messenger in biological systems.

Unlike adenylate cyclase, which has only one form, there are two forms of guanylate cyclase:

(i) The Membrane Bound or Particulate Form

The activation and function of the membrane bound or particulate form of the enzyme is thought to be similar that of the adenylate cyclase/cyclic AMP system (Fig. 10).

(ii) The Cytoplasmic Form or Soluble Guanylate Cyclase

Nitric oxide (NO) is the only known activator of this enzyme (Murad et al., 1978; Bohme et al., 1978). The purified guanylate cyclase is a haemoprotein and interaction of NO with the haeme moiety is considered as the mechanism of enzyme activation. NO has now been found to be synthesized in various tissues (Moncada et al., 1991) including the much studied vascular endothelium (Palmer et al., 1987).

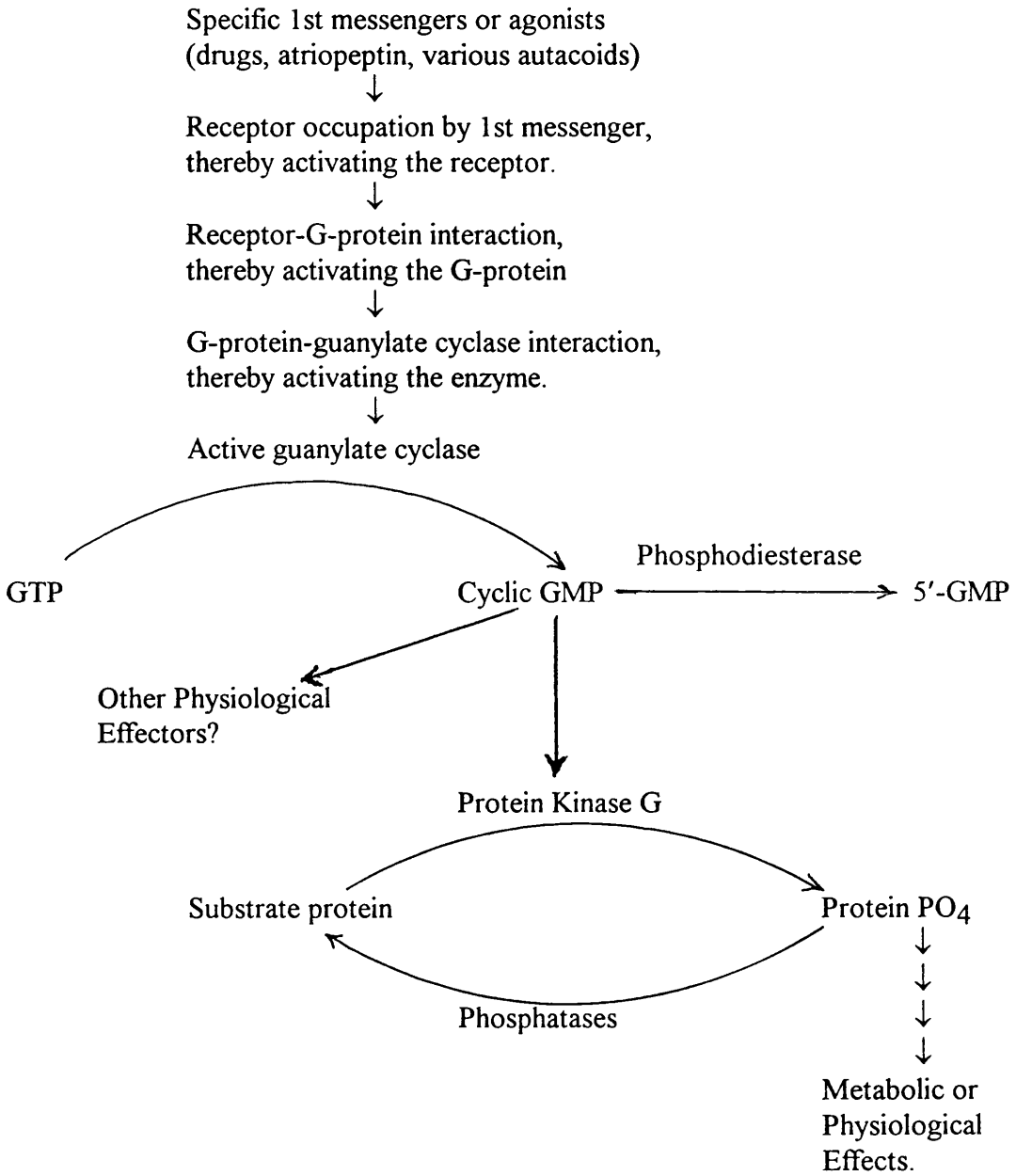

Fig. 10. Schematic representation of particulate guanylate cyclase/cyclic GMP-mediated signal transduction mechanism.

The cytoplasmic enzyme was found to be a heterodimer containing 2 subunits, of 70 and 82 KDa, each having a carboxy-terminal catalytic domain and unable to function as a monomer (Schulz, et al., 1989; Nakane et al., 1990). They are shown to contain haeme as prosthetic group and modulate their activity after binding to NO or other nitrogen-containing gaseous molecules derived from

arginine metabolism. In contrast, the plasma membrane form is a multidomain protein made from the association of a peptide receptor domain (e.g., atriopeptin receptor), a protein kinase-like domain and a guanylate cyclase domain (Chinkers et al., 1989).

Soluble guanylate cyclase-mediated signal transduction is initiated with the production of NO by the metabolism of L-arginine, the metabolic reaction being catalysed by nitric oxide synthase. NO then binds to the haeme moiety of the soluble guanylate cyclase, thereby activating it. Guanylate cyclase in turn produces cyclic GMP from GTP. The overall process can be shown as follows (Fig. 11.).

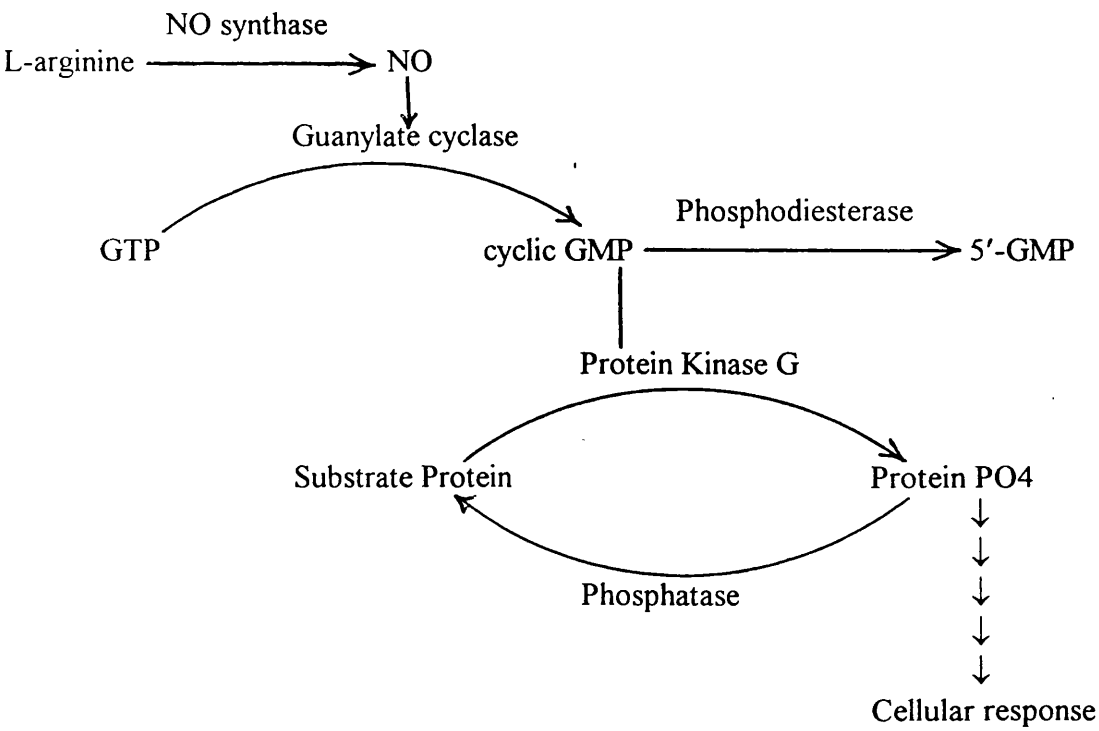

Fig. 11. Schematic representation of signal transduction mechanism by soluble guanylate cyclase/NO system.

Calcium-dependent Signal Transduction

Calcium-dependent receptor-mediated functions are regulated by a large number of messengers such as neurotransmitters, hormones and a wide variety of growth promoting factors. Calcium plays a more general and widespread role in cell function than do cyclic AMP and cyclic GMP and is now regarded as the most important signal transduction system.

Calcium mobilizing receptors affect intracellular Ca^{2+} levels via hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). Phosphatidyl inositol 4-phosphate (PIP) and PIP_2, collectively called polyphosphoinositides (PPI) are a class of minor membrane phospholipids. PIP_2 which has the phosphate group attached to the inositol ring plays a key role in their function. This phospholipid is the substrate for a membrane-bound enzyme, phospholipase C (PLC), which splits it into DAG and IP_3, both of which function as second messengers. The cycle is completed by the phosphorylation of DAG into phosphatidic acid (PA) and by a series of dephosphorylation reactions catalysed by intracellular phosphatases which dephosphorylate IP_3 ultimately to form inositol. Recoupling of PA with inositol then occurs to form phosphatidyl inositol once again. The process of activation of PLC by various agonists turns out to involve a G-protein and to be essentially identical to the mechanism of adenylate cyclase activation, although different G-protein subtypes are involved.

IP_3 and DAG synergistically mediate signal transduction in receptor mediated systems linked to Ca^{2+} mobilization. IP_3 has been shown to be involved in the release of Ca^{2+} from intracellular stores and DAG activates protein kinase C, which phosphorylates specific substrate proteins. Many of the cellular actions

of calcium are mediated by its binding to specific calcium-binding proteins, such as troponin, calmodulin etc., forming Ca^{2+}/Ca^{2+}-binding protein complex which in turn activate their specific protein kinases. In addition to calcium-binding proteins, Ca^{2+} also exerts some of its cellular functions in conjunction with lipids, such as phosphatidylserine. This Ca^{2+}/phosphatidylserine then activate the protein kinase. There are several Ca^{2+}/calmodulin-dependent protein kinases.

Possible Links Among Phospholipases

Recent evidence suggests that the same agonists which induce the hydrolysis of PI also induce hydrolysis of other phospholipids, particularly phosphatidylcholine (PC) that also produces DAG at a relatively later phase in the cellular response, and a possible role of phospholipase D in prolonging this DAG elevation has been postulated (Exton, 1990; Billah and Anthes, 1990). Substantial evidence is now available that the sustained elevation of PK_C activity is essential for subsequent responses such as cell proliferation and cell differentiation (Davis and Lipsky, 1988; Asaoka et al., 1991). In addition, phospholipase A_2 (PLA_2) is activated by most of the agonists which induce PI hydrolysis (Nakamura et al., 1993).

Intracellular Ca^{2+}

Intracellular signalling via changes in cytosolic free Ca^{2+}, $(Ca^{2+})_i$, plays a central role in cell regulation. Most $(Ca^{2+})_i$ signals are generated in part by enhanced Ca^{2+} influx. Furthermore, Ca^{2+} influx is required to maintain intracellular stores from which Ca^{2+} is mobilized for signalling. Ca^{2+} influx into cells can occur either during action potential or at resting potential.

Ca^{2+} Influx During Action Potential

The most prominent route for Ca^{2+} entry consists of voltage-gated Ca^{2+} channels, opened in a concerted manner during action potential conduction. Ca^{2+} enters the cells predominantly via L-type voltage-gated Ca^{2+} channels (high voltage-gated Ca^{2+} channels). Phosphorylation of the L-type channels by cyclic AMP-dependent protein kinase (PK_A) and protein kinase C (PK_C) (Chang et al., 1991) and direct receptor control of the same channel via G-proteins (Dolphin, 1991; Heschler et al., 1990) determine how much Ca^{2+} will enter during a single action potential, controlling, e.g. the number of channels which will open. Dihydropyridine-sensitive Ca^{2+} stimulation (through L-type channels) is achieved by the same α subunit that stimulates adenylate cyclase (Yatani et al., 1988; Brown and Birnbaumer, 1988)

Voltage Dependent Ca^{2+} Influx at Resting Potential

Ca^{2+} influx can be observed during slight depolarization in the range of the normal resting potential. It was found that slight sustained depolarizations (<10 mV) from resting potentials between -60 and -50 mV caused in part of the cell an inward current of minor amplitude (2 - 10 pA). This current was associated with a marked rise in $(Ca^{2+})_i$ in part of the cytosol.

Extrusion of Calcium from the Cytosol

Because a high concentration of ionised calcium is toxic (Rasmussen and Barret, 1984) the mobilized Ca^{2+} is subsequently extruded from the cells. Cytosolic Ca^{2+} levels are maintained by various regulatory mechanisms in the cell including: (a) the Ca^{2+}-dependent plasma membrane ATPase pump, (b) the

plasma membrane Na^+/Ca^{2+} exchange mechanism, (c) the mitrochondrial Ca^{2+} pump and (d) the ATP-dependent Ca^{2+} pump in the ER.

Conclusion

The brief outline described above represents only a very straightforward and simple understanding of the various signal transduction pathways in the cellular arena. In fact the processes are highly complicated and there are numerous interactions between the individual as well as among the different transduction pathways. The very large number of extracellular 1st messengers, their cell surface or intracellular receptors, the heterotrimeric composition of G-proteins with each subunit occurring in multiple forms and existence of different isoforms of effectors (e.g. adenylate cyclase, phospholipases, Ca^{2+} channels), different protein kinases (specific or non-specific) etc. contribute to this enormous functional diversity and complexity.

PHARMACOLOGICAL REVIEW OF OCULAR HYPOTENSIVE AGENTS

Ocular hypotensive agents used in glaucoma treatment and research can be divided into two broad divisions according to the nature of their effect in reducing IOP. One division include substances which decrease IOP by reducing AH formation, typical examples of which are the beta-adrenoceptor antagonists and carbonic anhydrase (CA) inhibitors. In addition to these, there are other types of drugs such as the experimental agents, forskolin (Caprioli and Sears, 1983, 1984; Caprioli et al., 1984; Sears, 1985) and cholera toxin (Gregory et at., 1981). The vasodilator hormone atriopeptin has been found to reduce IOP by reducing AH formation in rabbits (Korenfeld and Becker, 1989) and also

reportedly reduces IOP in glaucoma patients (Diestelhorst and Krieglstein, 1989). The cyclic AMP phosphodiesterase inhibitor, 8-pivaloyloxymethyl ester (POM-ester) of griseolic acid also has been reported to decrease IOP by reducing AH formation (Kiuchi et al., 1991).

Another group includes substances which produce their effect by increasing the outflow of AH. Well known examples are parasympathomimetic agents, adrenergic agonists such as adrenaline and its prodrug, dipivefrin (dipivalyl adrenaline), clonidine, apraclonidine and certain prostaglandins, especially PGF_2 α and its analogue, PhXA41 (Alm et al., 1993; Toris et al., 1993). The relatively specific α_2-adrenergic agonist, apraclonidine, however, is reported to reduce both AH formation and outflow resistance (Gharagozloo et al., 1988; Robin, 1988).

Beta-adrenergic Agents

There are a number of β-adrenergic agents, both agonists and antagonists (specific or non-specific), used in the field of glaucoma research, such as tazolol (β_1-agonist), metoprolol (β_1-antagonist), salbutamol, terbutaline (β_2-agonists), butoxamine (β_2- antagonist), isoprenaline (non-selective β-agonist), oxprenolol (non-selective β-antagonist) etc. β-adrenergic blocking agents are the most commonly prescribed drugs in glaucoma therapy. The agents currently in clinical use include timolol, levobunolol, metipranolol, carteolol and betaxolol. The first four are non-selective β-antagonists (i.e. have affinity to both β_1 and β_2- adrenoceptors) while betaxolol is a cardio-selective (β_1- selective) antagonist, cardioselectivity being highly dose-dependent (Lertora et al., 1975). All are used topically as eyedrops (Brooks and Gillies, 1992). There is now general agreement that they decrease IOP by reducing AH formation (Leopold and

Duzman, 1986; Sugrue, 1989; Hurvitz et al., 1991; Brooks and Gillies, 1992), but the mechanism by which they reduce AH formation is a matter of great controversy as will be reviewed in detail. The most logical and widely believed mechanism is the classical β-adrenergic antagonism, which by definition is to inhibit competitively the binding of endogenous adrenergic transmitters at β-receptors, thus preventing agonist induced activation of adenylate cyclase, blocking the resultant increase in cyclic AMP levels. This, it is supposed, causes the reduction of AH flow.

Carbonic Anhydrase Inhibitors

CA inhibitors are the next major group of antiglaucoma drugs affecting AH formation. Clinically they are used orally for long term treatment of primary and secondary open angle glaucoma (OAG). Examples of oral drugs are acetazolamide, methazolamide, ethoxzolamide and dichlorfenamide, which have been in use to treat glaucoma for over 3 decades (Woltersdorf et al, 1989). Recently, topical preparations of some novel CA inhibitors have been introduced. These include MK-927, MK-417, MK-507 (L-671, 152), L-662583, AHR-16329 (Lippa et al., 1988, 1992; Pfeiffer et al., 1990; Hurvitz et al., 1991; Sears et al., 1991; Brechue and Maren, 1993a,b). In general, the topically applied drugs are ampholytes with a basic amine and acid sulphonamide group, delivered as their hydrochloride salts. These drugs have relatively high water and lipid solubility which facilitate corneal penetration and reaching the ciliary process in concentrations sufficient to saturate the enzyme binding sites. CA inhibitors decrease the formation of AH, with no effect on outflow. The exact mechanism, however, is not known, but may involve decreasing sodium efflux into the AH, secondary to decreased formation of bicarbonate (Cole, 1977). All CA inhibitors contain a ring structure attached to a sulfonamide group (R-

SO_2NH_2); the sulfonamide moiety competes with carbonic acid for its site on CA (Maren, 1976) which catalyses the reaction, $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ and thus plays an essential role in ion transport across the CE by supplying these two ions.

Cholinergic Agents

Cholinergic agents are the oldest, effective, non-surgical therapy for glaucoma (Leopold and Duzman, 1986). They can be divided into two groups according to their mode of action - direct acting and indirect acting. The former act directly on muscarinic receptors, e.g. pilocarpine and carbachol. The indirect acting drugs block the enzyme acetylcholinesterase, allowing acetylcholine to accumulate at the parasympathetic nerve endings. Examples of this group of drugs include physostigmine, demecarium, echothiophate and isofluorophate. Physostigmine is short acting and the other three elicit a long-lasting inhibition of the enzyme. Among the cholinergics, pilocarpine is the most commonly used and extensively studied drug for the treatment of glaucoma. These drugs exert their IOP lowering effect by increasing the facility of outflow of AH through the trabecular meshwork, which occurs by causing contraction of the ciliary muscle, which pulls on the scleral spur (Kaufman and Barany, 1976; Zimmerman, 1981).

Adrenaline and Other Adrenergic Agonists

The mechanism whereby adrenaline lowers IOP appears to result from a combination of several factors none of which are unequivocally established. A key factor is that it activates both alpha and beta-adrenoceptors. Experimental evidence suggests that adrenaline increases outflow of AH (Sonntag et al., 1978; Townsend and Brubaker, 1980). Contradictory reports are available in

respect of AH formation - both an increase (Townsend and Brubaker, 1980; Nagataki and Brubaker, 1981; Schenker et al., 1981) and a decrease (Lamble, 1977). The outflow effect was originally attributed to the activation of α-adrenoceptors (Richardson, 1972) but the observation that this action of adrenaline can be attenuated by timolol indicates involvement of β-adrenoceptors (Cyrlyn et al., 1982; Erickson-Lamy and Nathanson, 1992). That these are β_2-adrenoceptors is supported by the fact that betaxolol (β_1-selective) failed to blunt the outflow effect of adrenaline (Allen and Epstein, 1986), and the highly specific β_2-antagonist, ICI118,551, completely blocked adrenaline's effect (Erickson-Lamy and Nathanson, 1992). The precise mechanism, however, is still unknown. Some experimental evidence suggests implications of prostaglandins or other cyclooxygenase products in the mechanism of action of adrenergic agonist (Camras et al., 1985; Anderson and Wilson, 1990) which is further supported by the fact that repeated administration of adrenaline is associated with an increase in the content of prostaglandin E in AH of rabbits (Miyake et al., 1988) and monkeys (Camras et al., 1987).

Recently-introduced selective α_2-adrenergic agonists, apraclonidine (Robin et al., 1987; Abrams et al., 1987; Brown et al., 1988; Hurvitz et al, 1991), B-HT 920 and UK 14304 (Burke and Potter, 1986; Steidle et al., 1987; Serle et al, 1991) have proved to be very potent ocular hypotensive agents. These drugs lower IOP mostly by reducing AH formation rather than increasing outflow (Krieglstein et al., 1978; Lee et al., 1984; Serle et al., 1991). However, clonidine decreases blood flow to the limbal blood vessels (Ralli, 1975; Bill and Heilmann, 1978) which could alter episcleral venous pressure. Although it does not alter tonographic outflow facility (Robin, 1988), it may have an effect on uveoscleral outflow. It has been shown that 1% apraclonidine decreases AH flow by approximately one-third in normal volunteers (Gharagozloo et al.,

1988), but about 50% of normal volunteers tested with 0.5% apraclonidine have IOP below presumed episcleral venous pressure of 8 mmHg (Abrams et al., 1989; Coleman et al., 1989a). Also no volunteer tested with 0.5% timolol attains such a low IOP (Coleman et al., 1989b). Apraclonidine's effect on AH formation is believed to be mediated by α_2-adrenergic receptors. Radioligand binding studies point to the presence of α_1 and α_2 adrenergic receptors in the iris-ciliary body, with the latter being more prevalent (Mittag and Tormay, 1985a; Mittag et al., 1985; Jumblatt et al., 1987; Mallorga et al., 1988). In functional studies it has been shown that stimulation of α_1-adrenoceptors in the rabbit ciliary process results in increased phosphatydylinositol turnover (Mallorga et al., 1988) whereas α_2-adrenoceptors are negatively coupled to adenylate cyclase in the rabbit iris-ciliary body (Mittag and Tormay, 1985b) and ciliary processes (Bausher et al., 1987; Kintz et al., 1988).

Prostaglandins

Lower doses of certain prostaglandins, especially $PGF_{2\alpha}$ and its analogue PhXA41, have a dramatic IOP-lowering effect in many species (Lee et al., 1984b; Goeneboer et al., 1989; Camras, 1993; Toris et al., 1993) including normotensives (Kerstetter et al., 1988; Lee et al., 1988; Villumsen and Alm, 1989), glaucoma patients (Camras et al., 1989; Villumsen et al., 1989) and subhuman primates (Camras and Bito, 1981; Crawford et al., 1987). These drugs lower IOP by increasing uveoscleral outflow (Crawford and Kaufman, 1987; Nilsson et al., 1989; Gabelt and Kaufman, 1989). The mechanism involved is a slow enzymic lysis of the connective tissue between the ciliary muscle bundles (Tamm et al., 1989) thus widening the connective tissue spaces through which AH leaves the anterior chamber by this route (Lutjen-Drecoll and Tamm, 1988).

Dopaminergic Drugs

Dopamine agonists, Ha117 (Potter et al,., 1993), bromocriptine and antagonists, haloperidol, moperol, metoclopramide and trifluperidol lower IOP while few agonist and antagonist increase IOP in laboratory animals (Chiou et al., 1989). Bromocriptine and pergolide have been demonstrated to lower IOP in normal (Mekki et al., 1983, 1984; Al-Sereiti et al., 1989) and glaucomatous humans (Lusting, 1983; Geyer et al., 1987). The function of dopaminergic drugs in lowering IOP remains largely unexplained. De Vries et al. (1986) reported that adenylate cyclase-linked dopamine receptors are present in both human and bovine ciliary body. Lograno et al. (1990) showed both functional and biochemical evidence for the presence of dopamine D_1 receptors in bovine ciliary body. However, the definitive presence and characterization of these receptors in the CE remains elusive (Wax, 1993).

Calcium Channel Blockers and Vasodilator Drugs

Systemic calcium channel (L-type) blockers such as verapamil and nifedipine have been shown to lower IOP, (Payne et al., 1990). The ocular hypotensive effect of topical verapamil and nifedipine (Segarra et al., 1986, 1993; Abelson et al., 1988) upon the human eye have also been demonstrated. Several nitrovasodilator drugs which mediate an increase in intracellular cyclic GMP concentration via activation of soluble guanylate cyclase, e.g. nitroglycerine (Nathanson, 1988), sodium nitroprusside and sodium azide (Karnezis and Murphy, 1988) have been reported to have an ocular effect.

Other IOP Lowering Agents

There is a wide variety of other agents which reportedly lower IOP in experimental animals or in man by various mechanisms. These include the 5-HT-antagonist, ketanserin (Costagliola et al., 1991), α-adrenoceptor antagonists, such as prazosin (Krupin et al., 1980b), yohimbine (Mittag et al., 1985), dibenamine, phentolamine, phenoxybenzamine and thymoxamine (Mittag, 1983), H_1-antihistaminines, such as antazoline and pyrilamine (Krupin et al., 1980c), topical corticosteroid (Becker, 1965) steroid blocker (Phillips et al., 1984; Tsukahara et al., 1986) etc.

Beta-adrenoceptor Antagonists

Since the introduction of timolol maleate for clinical use in 1978, after studies showed its efficacy as an ocular hypotensive agent (Zimmerman and Kaufman, 1977a, 1977b; Coakes and Brubaker, 1978; Sonntag et al., 1978), beta blockers have become the most popular therapeutic agents in the medical treatment of glaucoma. Phillips et al. (1967) were the first to report that topical administration of the beta-blocker propranolol reduced IOP. Since then many β -blockers have been introduced. The first preclinical evidence that timolol lowers IOP was reported by Vareilles et al. (1977) who demonstrated that 0.5% and 1.5 % solution of (-)-timolol significantly lowerd IOP in rabbits with an experimentally induced form of open angle glaucoma (OAG), utilizing α-chymotrypsin. This form of OAG is most likely due to impaired outflow facility (Sears and Sears, 1974). Also the pathological changes of this type of glaucoma resemble those of COAG, i.e. progressive cupping of the optic nerve head (Best et al., 1975). The effects of this group of drugs upon AH dynamics has been well established both clinically (Zimmerman, 1977b; Keats and Stone, 1984; The

levobunolol study group, 1989; Brooks and Gillies, 1992) and experimentally in several species in vivo (Vareilles et al., 1977; Nathanson, 1981a; Potter, 1981; Schenker et al., 1981; Liu et al., 1983; Bar-Ilan, 1984; Sugrue et al., 1985; Alkondon et al., 1986). Their IOP lowering effect is known to be due to a reduction of AH secretion (Liu and Chiou, 1981; Schenker et al., 1981; Vareilles and Lotti, 1981; Brubaker et al., 1982; Miichi and Nagataki, 1983; Bartels, 1988). The subcellular mechanism by which they lower AH formation is still a matter of controversy (Leopold and Duzman, 1986; Sugrue, 1989). Most of the existing data support the notion that the reduction of AH flow is a local action of the drug upon the eye, because the reduction in IOP is greater in the treated than in the contralateral, untreated eye following topical administration of a beta-adrenergic antagonist (Katz et al., 1976; Zimmerman and Kaufman, 1977a). It has been suggested that the effect in the treated eye is a local phenomenon and that the effect in the untreated eye is a result of systemic absorption. On the other hand, the drugs may act at a site either within or via the CNS (Neufeld et al., 1983). It has also been suggested that timolol may act to reduce AH secretion by reducing the levels of melatonin in the iris and ciliary body (Rhode et al., 1985). Indeed, orally administered melatonin reportedly lowers IOP of healthy volunteers (Sample et al., 1988). However, levels of melatonin in the iris and ciliary body of rabbits and chicks are unaltered by topical timolol (Rhode and Chiou, 1987). There is also some indication that timolol may antagonise dopaminergic function resulting in reduction of blood flow to the ciliary body and hence a reduction is AH secretion (Watanabe and Chiou, 1983). Macri and Cevario (1978a) studied AH and blood flow dynamics in the cat eye and concluded that various pharmacologic agents decrease AH formation by producing vasoconstriction of afferent ciliary process vessels, which might consequently decrease AH ultrafiltration. However, the hypothesis of ultrafiltration through the CE has now been almost abandoned (Bill, 1975; Sears,

1991). Also, no correlation has been found between ocular blood flow and changes in IOP using dopamine agonists and antagonists and various other antiglaucoma drugs including β-blockers (Chiou et al., 1989, 1990; Chiou and Chen, 1992). In a recent study it has been shown that most antiglaucoma drugs including the β-antagonists, timolol, levobunolol and betaxolol, enhance ocular pulsatile blood flow (Hong and Chiou, 1993). However, Chiou and Chen (1993) reported that the β-blockers L-timolol, levobunolol, metipranolol and betaxolol all decreased ocular blood flow in ocular hypertensive rabbits.

Attempts have been made to explain the ocular action of β-blockers on the basis of presence and distribution of β-adrenergic receptors in the CE. Radioligand and enzymatic studies showed presence of β-adrenoceptors in the anterior segment of the eye (Neufeld and Page, 1977; Bromberg et al., 1980). The vast majority of these receptors in man and animals have been shown to be β_2-subtype (Trope and Clark, 1982; Schmitt et al., 1984; Elena et al., 1984b; Mittag and Tormay, 1985a; Wax and Molinoff, 1987). β_2-adrenoceptor radioligand binding sites have been found in the NPE cells of the bovine ciliary process (Polansky et al., 1985). β_2-adrenoceptors have also been visualized in rabbit ciliary process epithelial cells by in vitro autoradiographic technique (Elena et al., 1987). Presence of adenylate cyclase with pharmacological characteristics indicative of β_2-adrenoceptor linkage has been found in the epithelial cells of the rabbit (Nathanson, 1980) and human (Nathanson, 1981b) ciliary process. Others confirmed the presence of β_2-adrenoceptor-adenylate cyclase system both in rabbit ciliary process (Cepelic and Cernohorsky, 1981; Patkama et al., 1986) and in the NPE cells of the bovine ciliary process (Elena et al., 1984a). Adenylate cyclase on the plasma membrane of rabbit NPE has been visualised by electron microscopy (Tsukahara and Maezawa, 1978).

In spite of the convincing evidence of the presence of predominantly β_2-adrenoceptors and adenylate cyclase in the CE of both man and animals, results of a large number of experiments show that the β-antagonists may not act on AH dynamics through β_2-adrenoceptor blockade (Liu and Chiou, 1981; Chiou, 1983; Watanabe and Chiou, 1983; Lotti et al., 1984b; Chiou et al., 1985). At present a considerable body of experimental evidence has been accumulated in support of the hypothesis that β-adrenoceptor antagonists reduce AH formation via antagonism of conventional β-adrenoceptors and also in support of the hypothesis that they may act by mechanisms other than through conventional β-adrenoceptors.

Evidence Supporting Classical β-adrenoceptor Mechanisms

A large number of studies on AH dynamics (reviewed by Potter, 1981; Potter and Rowland, 1981) suggest that AH secretion by the ciliary process epithelial cells is modulated by β-adrenergic receptors. The β-agonist isoprenaline stimulates cyclic AMP synthesis and increases AH formation and timolol blocks this effect and decreases AH secretion in the monkey eye suggesting the effect is mediated by β-adrenoceptors (Nathanson, 1881a; Miichi and Nagataki, 1983). The relatively selective β_2-agonist, terbutaline, increases AH formation by about 100% in cynomolgus monkey, whether the drug is administered intravenously or intracamerally and this effect was completely abolished by timolol given by either route (Nilsson et al., 1990).

Timolol is a potent non-selective β-adrenoceptor antagonist (Scriabine et al., 1973) which is not, however, noted for any other pharmacological activities shown by a number of β-adrenergic antagonists, such as intrinsic sympathomimetic activity, membrane stabilizing activity or local anaesthetic

activity (James, 1989; Chrisp and Sorkin, 1992). It has also been reported that the ocular hypotensive effect of timolol is not elicited by CA inhibition (Liu et al., 1980), ATPase inhibition (Watanabe and Chiou, 1983) nor prostaglandin blockade (Rowland and Potter, 1981). However, timolol is one of the most effective and widely studied agents known to affect AH secretion and IOP. The drug is a very effective displacer of radioligand binding to β_2-binding sites in membrane fractions of both iris-ciliary body from humans (Wax and Moniloff, 1987) or rabbits (Neufeld and Page, 1977; Schmitt et al., 1984) or ciliary processes from rabbits (Bromberg etal., 1980), sheep (Trope and Clark, 1982) or bovine (Elena et al., 1984b). Comparable activity was observed for inhibition of adenylate cyclase in ciliary process from human (Nathanson, 1980) and rabbits (Nathanson, 1980; 1985a) and NPE cells of the bovine ciliary process (Elena et al., 1984a).

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 reduce AH formation and IOP implies that endogenous adrenergic tone stimulating β-adrenoceptors exists in the eye. It has been observed that the rate of AH flow in humans undergoes a circadian rhythm characterised by a higher flow during working hours (highest being in the morning) and lower flow during sleep (Reiss et al., 1984). AH formation in human volunteers is decreased by approximately 45% during sleep (Reiss et al., 1984) and, in contrast to day time, 0.5% timolol is devoid of any ocular hypotensive effect (Topper and Brubaker, 1985). These observation suggest that endogenous adrenergic activity during the day stimulates AH 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 AH formation in volunteers during the day, but significantly increased during sleep (Larson and Brubaker, 1988). AH

flow stimulation during sleep has also been found with adrenaline (Topper and Brubaker, 1985) and terbutaline (Gharagozloo et al., 1988). These observations along with obliteration of the circadian cycle in human by timolol (Topper and Brubaker, 1985) can be best explained by classical β-adrenergic blockade. The melatonin release during sleep which was previously reported to be the cause of reduced production of AH during sleep (Samples et al., 1988) has been contradicted by Koskela and Brubaker (1991) by comparing aqueous flow during nocturnal sleep in the dark and during nocturnal sleep under bright light and by measuring aqueous flow during a short period of sleep in a dark room during the day.

Rabbits have also a circadian pattern of AH flow being highest during the dark and lowest during the light phase of the cycle (Rowland et al., 1986; Smith and Gregory, 1989). Yoshitomi et al. (1991) measured concentrations of catecholamines and cyclic AMP in the AH of rabbits entrained to 12h dark and 12h light. Aqueous nor-adrenaline and cyclic AMP were significantly higher in the dark. Superior cervical ganglionectomy reduced the aqueous noradrenaline concentration during both light and dark phases and superior cervical ganglionectomy or pretreatment with 0.1% timolol totally eliminated the dark phase increase in cyclic AMP. Thus increased adrenergic activity during the dark is the cause of increased cyclic AMP production and timolol antagonised its production. Linner and Prijot (1955) noted that cervical sympathetic ganglionectomy resulted in reduced AH flow. Superior cervical ganglionectomy or preganglionic section of the cervical sympathetic trunk blunted the dark phase increases in IOP (Gregory et al., 1985; Braslow and Gregory, 1987; Liu and Dacas, 1991), AH flow (Yoshitomi and Gregory, 1991), AH nor-adrenaline concentration (Liu and Dacas, 1991; Yoshitomi et al, 1991) and AH cyclic AMP (Yoshitomi et al, 1991).

Evidences Against the Involvement of Conventional β-adrenergic Receptors in AH Formation

Topically applied timolol can block β-adrenoceptors in the eye as evidenced by the attenuation of the ability of topical isoprenaline to increase both the IOP following water loading and the enhancement of production of cyclic AMP in the AH of rabbit (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 different mechanisms are involved in both phenomenon. This is supported by the observation that the effectiveness of various β-antagonists in lowering IOP in rabbit models of ocular hypertension did not correlate with their ability to block β-adrenoceptors (Bonomi et al., 1979).

Studies with specific β_1 and β_2-adrenergic agonists and antagonists for their effects on AH dynamics in cat showed that tazolol (β_1-agonist) reduce formation of AH more than outflow, while metoprolol (β_1-antagonist) reduce outflow more than formation, both salbutamol (β_2-agonist) and butoxamine (β_2-antagonist) inhibited AH formation and outflow to an equal extent (Chiou et al., 1985). The relatively selective β_2-agonists, salbutamol, terbutaline and soterenol which are used systemically for the treatment of asthma, have also been shown to lower IOP in monkeys (Langham and Biggs, 1974) and humans (Paterson and Paterson, 1972; Wettrell et al., 1977). Their IOP lowering effect accounted to be mainly a result of reduction of AH production; however, an increase in outflow might also play an important role (Kaham, 1981). It is an obvious paradox that both β-agonists and antagonists lower AH production.

Betaxolol is a β_1-specific adrenergic antagonist. Like timolol, it also has no partial agonist activity (Boudot et al., 1979) and has little or no local anaesthetic or membrane-stabilizing activity (Cavero and Lefevre-Brog, 1983). Betaxolol reduces IOP by reducing AH formation without affecting outflow (Reiss and Brubaker, 1983; Allen et al., 1986). Although β_2-adrenoceptors greatly predominate in the ciliary processes, the ocular hypotensive activities of betaxolol and timolol, both at 0.5%, in glaucoma patients were viewed as being comparable (Berry et al., 1984; Levy et al., 1985; Stewart et al., 1986). More recent studies, however, suggest that the ocular hypotensive effect of betaxolol is rather less than that of timolol (Allen and Epstein, 1986; Feghali et al., 1988; Vogel et al., 1989; Collignon-Brach, 1992), but both drugs are in common clinical use (Brooks and Gillies, 1992).

Both D-and L-isomers of timolol are almost equipotent in decreasing AH flow and IOP in various species including man (Liu and Chiou, 1981; Liu et al., 1983; Keats and Stone, Richards and Tattersfield, 1987; 1984; Mills et al., 1988). But in vivo and in vitro experiments show adrenergic receptors have specific affinity toward L-isomers. From direct receptor binding studies it has been demonstrated that L-isomers of timolol and propranolol have much higher affinity (40 fold and 281 fold, respectively) than D-isomers toward β-adrenergic receptors in iris-ciliary body preparations (Chiou et al., 1985).

Timolol and other β-adrenoceptor antagonists inhibit the production of intracellular cyclic AMP and lower AH production and IOP. However, agents such as cholera toxin (Gregory et al., 1981) and forskolin (Caprioli and Sears, 1983; Caprioli et al., 1984; Sears, 1985; Bartels et al., 1987; Shibata et al., 1988) both stimulate the production of cyclic AMP, yet they also lower IOP by

reducing AH flow and without affecting outflow facility (Caprioli et al., 1984; Lee et al., 1984a).

The β-adrenoceptor antagonist sotalol reportedly inhibits the production of intracellular cyclic AMP, as do all other β-adrenoceptor antagonists. Sotalol, however, when applied topically at 2% concentration to patients with COAG, has no effect upon IOP (Merte and Styz, 1983). Alprenolol effectively lowered IOP but exhibited no activity in antagonizing the effect of isoprenaline on either IOP or cyclic AMP. Oxprenolol, on the other hand, had little or no ocular hypotensive activity but was very effective in antagonizing both actions of isoprenaline. All of these agents penetrate the anterior chamber readily (Schmitt et al., 1981a).

Another β-adrenoceptor antagonist, carteolol, which is used to lower IOP in patients with COAG, reportedly has 1.67 times 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).

The fact that topically applied (+)-timolol can lower IOP of both rabbits (Share et al., 1984) and man (Keats and Stone, 1984; Mills et al., 1988) has been used to support the contention that the ocular hypotensive action of timolol is not mediated via β-adrenoceptors (Liu and Chiou, 1981; Chiou et al., 1985). Both timolol and its (+)-enantiomer were found to be equally effective in reducing AH production when perfused intracamerally in the anaesthetized cat eye. However, only one concentration of each agent was employed (Liu and Chiou, 1981). Both enantiomers were also found to be equipotent after topical administration in a model which indirectly measured AH secretion in conscious

rabbits (Chiou, 1983b). The ability of topically administered (+)-timolol to lower the elevated IOP of α-chymotrypsin-treated rabbits is somewhat less than that of timolol (Share et al., 1984). Timolol is slightly more potent than its (+)-enantiomer in blunting the water-load induced increase in IOP in pigmented rabbits (Liu et al., 1983). (+)-timolol is effective at 1% in lowering the IOP of glaucoma patients (Keats and Stone, 1984). The instillation of solutions of (+)-timolol ranging from 0.25 to 2% has been found to lower IOP of glaucoma patients but all these concentrations of (+)-timolol were less effective than 0.25% timolol (Mills et al., 1988).

In contrast to all these observations relating to ciliary β-receptors, (+)-timolol, at 1%, has some extraocular β-adrenoceptor blocking activity 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 and 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 iris-ciliary body preparation (Liu et al., 1983). (+)-timolol is about one-third as potent as timolol in displacing ^{3}H-dihydroalprenolol binding to iris-ciliary body tissue, reducing AH formation and lowering IOP of α-chymotrypsin-treated hypertensive eyes (Share et al., 1984). The (+)-enantiomer is fifty to ninety times less potent than the (-)-enantiomer in antagonizing the effects of isoprenaline on pulmonary and atrial β-adrenergic receptors (Share et al., 1984). The inhibitory effects of both enantiomers of timolol upon isoprenaline-induced stimulation of adenylate cyclase in the rabbit ciliary process are also comparable (Nathanson, 1988c). In water-loaded pigmented rabbits both enantiomers blunt the peak increase in IOP, the (-)-enantiomer is only slightly more effective (Liu et al., 1983).

Horner's syndrome is the clinical equivalent of superior cervical ganglionectomy and can occur spontaneously in humans or secondary to an underlying disorder, for example, migrainous neuralgia, or a tumour (such as lymphoma). AH secretion in such patients is normal however. The suppression of AH flow following 0.5% timolol is the same in such individuals as in the normal population (Wentworth and Brubaker, 1981). This suggests a post synaptic locus of action for 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 was completely prevented by superior cervical ganglionectomy (Colasanti and Trotter, 1981, 1983). The fact that the effect of 2% timolol was only partially suppressed makes these observations difficult to interpret . Adrenergic innervation is required for timolol to block the increase in IOP following water loading of normotensive, pigmented rabbits, as indicated by the lack of effect in denervated eye (Liu et al., 1984). Thus a paradox exists between clinical and preclinical findings, possibly resulting from species differences.

Timolol reduces AH formation in the bovine arterially perfused eye preparation (Wilson et al., 1993) which lacks intact adrenergic innervation and therefore has no sympathetic tone for a β-adrenergic antagonist to block, further suggesting the mechanism of action of timolol upon AH formation is not mediated via a classical β-adrenoceptors. In the same preparation, it has also been shown that no correlation existed between the levels of ciliary cyclic AMP and AH production using timolol, terbutaline, forskolin and 8-bromo cyclic AMP (Shahidullah and Wilson, 1992). Thus the available experimental and clinical evidence suggests that the β-adrenoceptors in the ciliary body are unconventional in some ways. Much work requires to be done to resolve these paradoxes and to fully explain the mechanism of action of timolol in the eye.

Atrial Natriuretic Peptides

Atrial natriuretic peptides (AP), also called atriopeptins or cardiopeptins, are a family of closely related peptides containing 21 to 28 amino acid residues which are synthesized and released by atrial myocytes (de Bold, 1985; Needleman and Greenwald, 1986) in response to distension of the atria (e.g., by an increase in blood volume). The AP have a number of functions, which have the effect of powerful vasodilatation and an increase in renal sodium excretion (Currie et al., 1983; Maack et al., 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 localized in ocular tissues (Stone, 1987).

AP receptors are associated with the membrane-bound form of the enzyme guanylate cyclase (Winquist et al., 1984; Waldman et al., 1984; Hamet et al., 1986) and are found in high concentrations in rabbit CE (Bianchi et al., 1986). There are several reports of decreasing IOP after intravitreal (Sugrue and Viader, 1986; Nathanson, 1987; Mittag et al., 1987a; 1987b), intravenous (Sasaki et al., 1986) and intracameral (Sugrue and Viader, 1986) administration of AP. Intravenous injection of human AP also reported to lower IOP in glaucomatous patients (Diestelhorst and Krieglstein, 1989). There is evidence that a decrease in basal and stimulated adenylate cyclase activity occurs (Bianchi et al., 1986) and an increase in guanylate cyclase activity results when ciliary processes are exposed to physiological concentrations of AP (Nathanson, 1987; Mittag et al., 1987a, 1987b). These observations have been supported by Korenfeld and Becker (1989) who demonstrated that intravitreal injection of AP reduces AH formation and IOP and increases cyclic GMP in the iris-ciliary body preparation.

It has been proposed that the biochemical mechanism by which AP reduces AH formation and IOP is the activation of particulate guanylate cyclase and consequent increase in intracellular cyclic GMP (Korenfeld and Becker, 1989; Fawcett and Wilson, 1989). This was supported by the finding that 8-bromo cyclic GMP (an analogue of cyclic GMP, more resistant to hydrolysis by phosphodiesterases than cyclic GMP) lowers IOP when given topically in albino rabbits (Becker, 1990) and subconjuctivally in pigmented rabbits (Busch et al., 1992). It has been shown that AP decrease IOP in isolated arterially perfused bovine eye (Millar et al., 1990).

Nitrovasodilators

The evidence for the effects of this class of drugs upon IOP is much less abundant. However, there are reports that certain nitrovasodilators, such as sodium nitroprusside (Nathanson, 1987; Nathanson, 1992) and nitroglycerine (Nathanson, 1988, 1992) caused a marked decrease in IOP in rabbits. Sodium nitroprusside and sodium azide are shown to lower IOP in bovine perfused eye (Millar and Wilson, 1991). These agents are activators of soluble guanylate cyclase.

Sodium azide is also thought to directly stimulate intracellular soluble guanylate cyclase and also promotes release of endothelium-derived relaxing factor - EDRF (Furchgott and Zawadzki, 1980) from blood vessel endothelia. EDRF is now thought to be NO (Palmer et al., 1987). Sodium nitroprusside stimulates guanylate cyclase but has no effect upon secretion of EDRF. Topical administration 1% or 2% solution of sodium azide or sodium nitroprusside to rabbits, however, was found to increase IOP in a dose-dependent manner (Krupin et al., 1977). The same experiments also showed that the AH outflow,

systemic blood pressure and heart rate were not affected by these drugs, suggesting an increase in AH flow as the mechanism of increasing the IOP. By contrast in arterially perfused bovine eye, however these drugs reduce IOP (Millar and Wilson, 1991). In a recent study, soluble guanylate cyclase has been characterized in transformed human NPE and sodium nitroprusside had been shown to increase cyclic GMP by about 40 to 60 fold in these cells (Danziger et al., 1993) supporting possible involvement of soluble guanylate cyclase in mediating AH formation.

Calcium Channel Blockers

The calcium ion has now been established as an important second messenger which modifies a variety of cellular functions, such as secretion, contraction and proliferation (Rasmussen, 1986; Carafoli, 1987). Recently the subject of possible roles for Ca^{2+} in the CE has attracted the attention of many researchers. Using spectrophotometry, Lee et al. (1989) reported calcium changes in SV40 transformed human ciliary non-pigmented (8-SVHCE) cells with various agonists. Ca^{2+}-calmodulin and protein kinase C-dependent phosphorylation have been reported in rabbit ciliary process and 8-SVHCE cells (Yoshimura et al., 1989; Lopez-Briones, 1990). The presence of a calcium signalling system in cultured non-transformed human and rabbit NPE cells has been confirmed by Ohuchi et al. (1992). Recently, Mittag et al. (1993) reported a calmodulin-activated adenylate cyclase in rabbit ciliary process. Jacob (1991) identified a low-thresold T-type calcium channel in bovine CE cells. With regard to outflow apparatus, it has been shown that removal of Ca^{2+} from the anterior chamber of the eye of rabbit and cynomolgus monkey by intracameral infusion with both calcium-free aqueous-like solutions or calcium-chelating agents, such as EDTA or EGTA, causes a marked increase in outflow facility and a large fall

in IOP (Kaye et al., 1968; Bill et al., 1980). This presumably reflects the fact that calcium has a role in maintaining the structural integrity of many tissues, probably including the trabecular meshwork and the inner wall of Schlemm's canal.

It seems obvious from all this experimental evidence that both the AH inflow and outflow pathways have calcium-dependent components. Consequently, drugs like verapamil or other calcium channel blockers could be used to manipulate AH inflow or outflow to lower IOP, particularly in view of the fact that these drugs have the ability of causing generalized vasodilatation and an improvement of blood supply to the organ concerned. Discovery of drugs which can lower IOP as well as improve ocular blood flow is an important strategy in glaucoma research. Improved blood supply to the optic nerve and retina is an important therapeutic aim to reverse or at least to stop the progressive damage of the retina and the optic nerve - the principal pathology of glaucoma which renders the eye blind.

Despite these promising aspects, little work has so far been done on ocular effects of calcium channel blockers. Also the few available reports are disappointingly conflicting. Garrido (1984) and Segarra et al. (1986; 1993) suggest that topical application of verapamil and nifedipine lower IOP in conscious normotensive rabbits. Green and Kim (1977) showed that intravenous perfusion of verapamil causes a fall in IOP in anaesthetized rabbit and more recently Payne et al. (1990) have shown that intravenous verapamil and nifedipine caused a significant reduction of IOP in conscious rabbits. On the contrary, the results of Beatty et al. (1984) suggest that verapamil, nifedipine or diltiazem cause an increase in IOP in conscious rabbits and men. Similary, Schnell (1975) found that although a single dose of nifedipine causes a reduction

of IOP in glaucomatous men, repeated oral doses have no effect on these patients or normal subjects. On the other hand, Kelly and Walley (1988) found that oral or intravenous nifedipine had no effect at all on IOP in normal human volunteers. In a recent study, Netland et al. (1993) showed that calcium channel blockers have no effect in open angle glaucoma but are useful in preventing visual field loss in patients with low-tension glaucoma.

The mechanism of ocular effect of verapamil is not known. From data on the pharmacological interactions between verapamil and prostaglandins, Green and Kim (1977) have proposed that verapamil reduced AH formation by acting on the uveal vessels, by a vasodilator effect on the efferent uveal vessels. However, the actions of prostaglandins on the eye are complex and recently it has been shown that they reduce IOP by increasing uveoscleral outflow (Crawford and Kaufman, 1987; Nilsson et al., 1989; Gabelt and Kaufman, 1989). Consequently, conclusions based on interactions between verapamil and prostaglandins can not be utilized to infer the sites and mechanism of action of verapamil. In any case, the dilatation of efferent vessels is unlikely, because it is now well established fact that Ca^{2+}-channel blockers cause generalized vasodilation, but do not affect the veins much (Soward et al., 1986). However, relaxation of afferent ciliary vasculature may cause local reduction of blood pressure and comcomitant improvement in blood flow. Again it has been shown that the effects of drugs on ocular blood flow are not likely to cause changes in the IOP (Chiou et al., 1989, 1990; Chiou and Chen, 1992, 1993). Tonographic data from Seggara et al. (1993) suggest that both verapamil (3.85±0.18 to 1.85± 0.19 $\mu l.min^{-1}$) and nifedipine (3.63±0.23 to 1.24±0.44 $\mu l.min^{-1}$) reduce outflow of AH in rabbits when applied topically. Beatty et al. (1984) found a small insignificant increase in outflow facility and an apparent increase in AH inflow after the topical use of verapamil in rabbits and human. The discrepancy

between these two reports using the same drug in the same species and utilizing the same route of administration is difficult to explain. Different doses and technique used might be accounted.

Isolated Arterially Perfused Eye

The isolated arterially perfused eye has become a frequently used model in the investigation of ocular functions (Niemeyer, 1981). The earliest efforts to establish such an in vitro model was made by Seaman and his coworkers (Seaman et al., 1965; Tazawa and Seaman, 1972; Tazawa et al., 1971) utilizing bovine eye and subsequently by Macri and Cevario utilizing cat eye (Macri and Cevario, 1973; 1974a; 1974b; 1975). Since then considerable success has been demonstrated in studying physiology and pharmacology of AH dynamics using isolated perfused eye either from cat (Macri and Cevario, 1978a, 1978b; Macri et al., 1980; Van Alphen and Macri, 1981) or from rabbit (Kodama et al., 1983, 1985; van Pinxteren and van Alphen, 1985). The isolated outflow pathway from bovine (Erickson-Lamy et al., 1988) and human eye (Erickson-Lamy et al., 1991; Erickson-Lamy and Nathanson, 1992) have also been adapted for physiological studies. The whole bovine eye was perfused by Kishida et al. (1985), who rejected it as unsuitable for the study of AH dynamics. The benefits of its size, unique vascularization and ready availability led us to investigate the bovine perfused eye further and to establish it as a useful model for studying AH dynamics (Wilson et al.,1993). This model also allows rapid sampling of substantial amounts of tissues for biochemical analysis (Shahidullah and Wilson, 1992) and for culturing the CE cells in vitro for subcellular studies (Shahidullah and Wilson, 1993). It is very encouraging to note that recently de Coo et al. (1993) succeeded in keeping the isolated arterially perfused bovine eye metabolically viable for up to 9 days.

Aims of the Project

It has been noted, while reviewing the various antiglaucoma drugs, that none of the important and widely used drugs has been established on a firm molecular basis of their mechanism of action on AH formation. In the case of adrenergic drugs, particularly the extensively used β-blockers, no correlation has been found between their physiological response and underlying biochemical effect either using glaucoma patients or normal volunteers or experimental animals. Recently, there have been some interesting new developments in the biochemistry of ciliary tissues, such as demonstration of the presence of serotonergic (Tobin et al., 1988; Tobin and Osborne, 1989; Barnett and Osborne, 1993) and purinergic receptors (Wax et al, 1993) in the iris ciliary-body preparation and CE respectively. Activation of ciliary process adenylate cyclase by calmodulin (Mittag et al., 1993), demonstration of soluble guanylate cyclase in human NPE cells (Danziger et al., 1993), T-type Ca^{2+} channel in bovine CE (Jacob, 1991) have been reported. There are also promising new agents to investigate, such as 5-HT receptor antagonists, atriopeptin and nitrovasodilators (which produce their effect by activating soluble guanylate cyclase) which reportedly lower IOP.

Extensive study of these agents, to relate their physiological and biochemical effects on the target tissues is necessary. This demands an effective in vitro experimental model in which AH dynamics and their pharmacological manipulation can be studied. In vivo work involves the influence of the CNS as well as hormonal and cardio-vascular systems which often complicate the interpretation of results. With this general end in view, the aims of the present project were:

1. To develop the isolated arterially perfused eye as an in vitro model for studying AH dynamics and their pharmacological manipulation, including the development of a fluorescein dilution method for measuring AH formation rate. To achieve this and to prove the validity of the model three approaches were undertaken.

    a) Testing the model using standard antiglaucoma drugs, timolol and a carbonic anhydrase inhibitor (MK-927), both known to reduce AH formation.

    b) Testing the model by using a wide range of arterial flow rates and correlating the observed IOP and perfusion pressures. This may confirm that AH formation in this preparation results from active secretion and not by some passive filtration process.

    c) Testing the metabolic activity of the perfused eye by measuring the O_2 consumption and CO_2 elaboration.

2. To study the mechanism of action of timolol by measuring the relative effects on IOP, AH formation and ciliary cyclic AMP accumulation of a variety of agents.

3. To confirm whether AP and sodium azide lower IOP in the bovine perfused eye by reducing the AH formation rate and to test the involvement of cyclic GMP in the mechanism of these agents.

4. To extend the study of cyclic nucleotides as putative ciliary second messengers to the next biochemical level by using selective inhibitors of protein kinase A and protein kinase G.

5. To develop a method of culturing bovine CE, in order to open up further possibilities of pursuing these post receptor mechanisms to the ultimate conclusion; in particular to study the possible involvement of intracellular calcium ions.

METHODS

MATERIALS AND METHODS

The Bovine Perfused Eye Preparation

Bovine eyes obtained from the abattoir were transported to the laboratory at ambient temperature and maintained at room temperature for usually about 10 to 15 min (needed for dissection and cannulation) until the start of arterial perfusion at 37°C. The ambient temperatures during transportation were in the range of 2 to 17°C depending on the season. The eyes were not transported 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 on commencing perfusion, would be slowed considerably by cooling the whole eye in ice. Immediately after transportation to the laboratory, the excess adnexal tissue was trimmed away from the eye but care was taken not to damage blood vessels running over the posterior surface of the globe. 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 at a point proximal to the heavy pigmentation which appears in the arterial wall before it pierces the sclera. This portion of the artery is quite elastic, offers easy cannulation and withstands the necessary manipulation.

After cannulation, the eye was placed in a warming jacket maintained at 37°C (Fig. 12). The exposed surface of the eye was kept moist by wrapping with surgical gauze soaked in Krebs' solution at 37°C and then covered with an insulated plastic cup. The cannulated 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; KH_2PO_4,

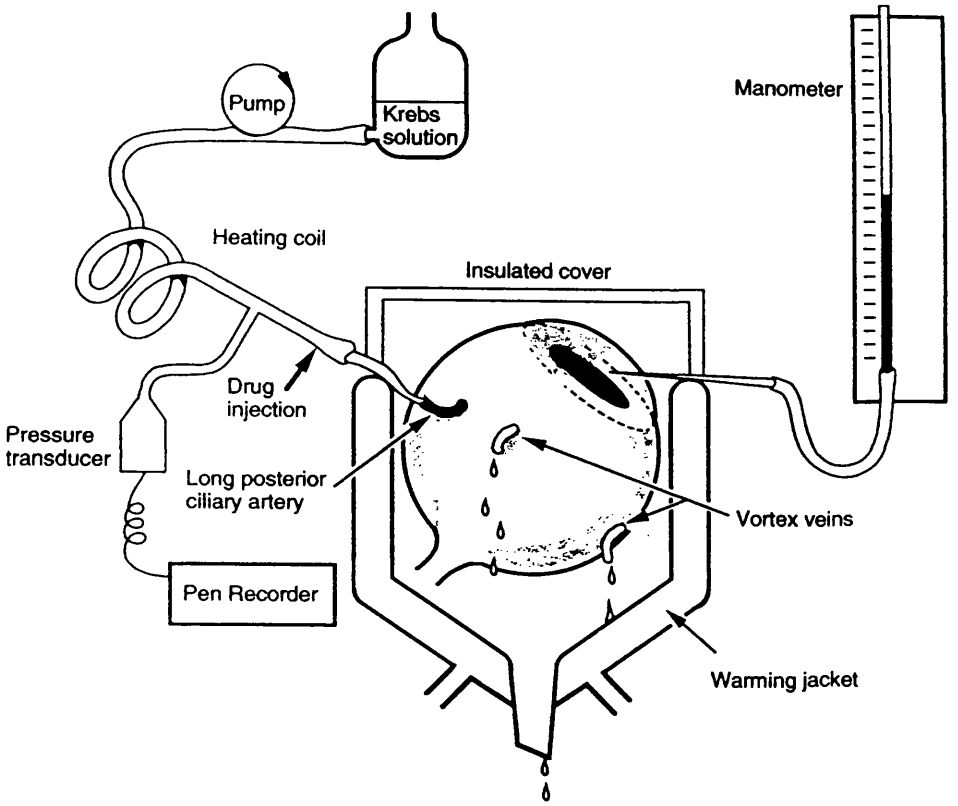

Fig. 12. The bovine isolated eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under conditions of constant flow rate. Perfusion pressure and IOP were both monitored as shown.

1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; ascorbate, 0.05. The pH of the solution was adjusted to 7.4 by bubbling with O_2 containing 5% CO_2. The flow was induced by using a Watson-Marlow peristaltic pump fitted with either a 502S or a 503S pumphead. Arterial perfusion pressure was recorded via a tranducer and Linseis pen recorder (Fig. 12). Flow was commenced at approximately 0.2 $ml.min^{-1}$ and increased usually in 10 increments to 2.25 - 2.5 $ml.min^{-1}$, over a period of approximately 50 min. However, in some eyes which showed low perfusion pressure, optimum flow rate was reached much earlier than this, sometimes within 30 min of the start of perfusion. During this period perfusion pressure often fluctuated, usually due to poor alignment of the cannula with the artery, but was not allowed to exceed 100 mmHg. This was achieved either by correcting the arterial alignment with the cannula or by adjusting the flow rate or by both. After approximately 50 min, when AH secretion had started and the globe had become firm, the anterior chamber was cannulated with a 23G needle and connected via silicon rubber tubing (i.d. 0.8 mm) either with a water manometer or via a T-piece with both a water manometer and the cuvette of a fluorimeter for the purpose of determining either IOP (Fig. 12) or AH flow rate respectively (Fig. 13).

Measurement of IOP

For this purpose the anterior chamber was cannulated with a 23 gauge needle and connected via a fine silicon rubber tube to a water manometer of internal diameter 1 mm (Fig. 12). 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 of 95 to 165 mmH_2O (7 to 12 mmHg), and a stable arterial perfusion pressure within the range of 20 to 60 mmHg, after an equilibration period of a further 30 to 60 min, were accepted for study. At this

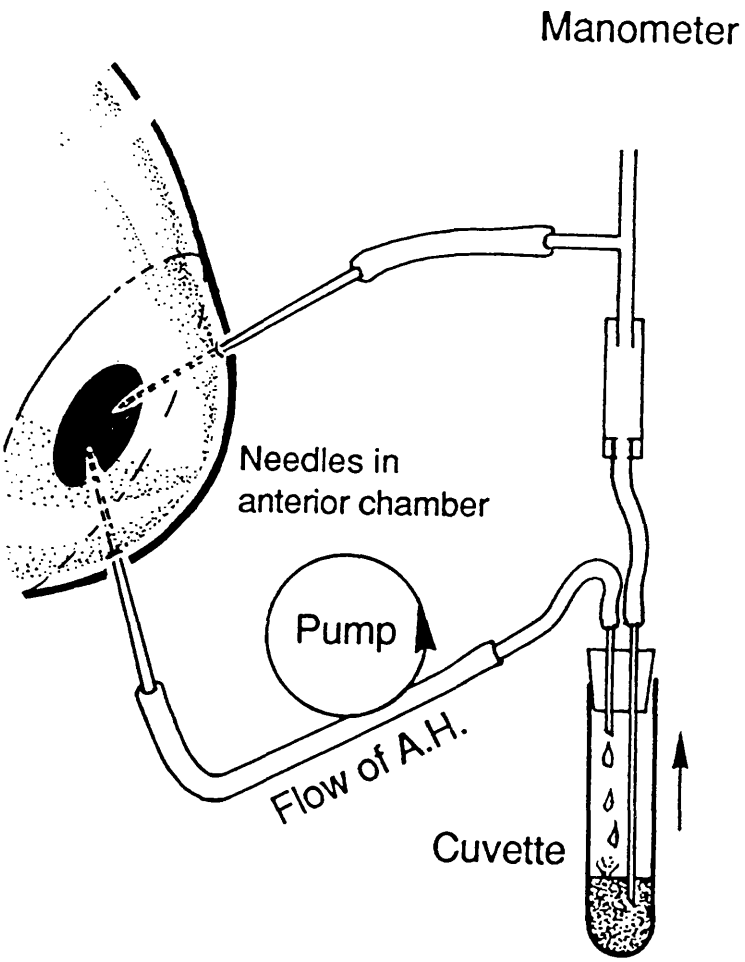

Fig. 13. Anterior chamber perfusion system used for determination of AH formation rate. Fluorescein dissolved in aqueous humour substitute was pumped from the anterior chamber through a circuit consisting of fine tubing and a cuvette, where fluorescence was monitored.

point bolus doses of drugs or the appropriate vehicle were injected, in volumes of 3 to 10 µl using a microsyringe, into the perfusate immediately proximal to the arterial cannula, and their effects on IOP or arterial perfusion pressure were measured and recorded. Before injecting the drug or the vehicle, it was ascertained that the IOP did not fluctuate by more that 1 mmH_2O for a period of at least 15 min. The time of injection was designated as zero time.

Measurement of AH Formation Rate

The anterior chamber was cannulated first with one 23 gauge needle. This was connected by a thick-walled silicon rubber tubing (i.d., 0.8 mm; wall thickness 1.8 mm) through a Watson-Marlow peristaltic pump (Model 502S and 503S) to the cuvette of a spectrophotofluorimeter (Aminco-Bowman or a Perkin-Elmer LS-3), returning to the anterior chamber via a second 23 gauge needle and a T-piece (Fig. 13); the latter was connected to a water manometer. The points of the needles in the anterior chamber were kept widely separated to optimise mixing.

The system of tubing and cuvette was filled with a known volume (1.06 ml in case of Aminco-Bowman spectrophotofluorimeter and 1.34 ml in case of Perkin-Elmer LS-3 spectrometer) of an aqueous humour substitute (AHS) comprising (mM): NaCl, 110; KCl, 3.0; $CaCl_2$, 1.4; $MgCl_2$, 0.5; K_2HPO_4, 0.9; $NaHCO_3$, 30; glucose, 6.0; ascorbate, 3.0 (Barany, 1964); and fluorescein sodium 2.7 µM. The AHS was bubbled with O_2 containing 5% CO_2, before mixing with fluorescein, for 8 min, at which time the pH was 7.6. After recording the initial fluorescence of this solution, the pump was started and the fluid was circulated through the anterior chamber at a rate of 0.25 ml.min^{-1}. With the establishment of the circulation, AH from the anterior chamber started mixing with the AHS and fluorescein in the cuvette and tubing system. As aqueous

passed through and mixed with the fluorescein, its concentration fell and was recorded from the fluorimeter every 5 min. The fluorescence was recorded at an excitation wavelength of 470 nm and an emission wavelength of 515 nm. When mixing was completed (which never took more than 20 min), a steady state was established, and from then on dilution of fluorescein in the cuvette was only due to newly formed AH.

Emission by fluorescein varies considerably with pH. Aqueous pH was measured at the end of some of the 2-3 hour experimental periods and compared to the pH of the mixture of AH plus AHS and fluorescein at the start of the experiment. IOP was also monitored from the water manometer to exclude eyes showing exceedingly high pressure (above 165 mmH_2O), which was taken as a sign of breakdown of the blood-aqueous barrier (Wilson, Shahidullah and Millar, 1993).

The AH formation rate was estimated by the fluorescein dilution technique, with the usual assumption that the marker leaves the anterior chamber only by bulk flow. When the rate of formation is constant, a plot of $\log_e$ of fluorescein concentration against time is a straight line whose slope gives the rate constant for AH formation (K_{out}.min^{-1}). The rate constant for AH formation is a function of the rate of disappearance of fluorescein from the anterior chamber and at a steady IOP and constant anterior chamber volume, is directly proportional to the rate of formation of AH. Regression lines representing the decay of fluorescence in the aqueous were constructed using the ANOVA statistical package provided by Minitab. The K_{out} value for a period of 30 min, after initial 20 min of mixing time, was used as the "same eye" control value to compare against any subsequent drug-induced change. For this, the appropriate vehicle was injected into the perfusate immediately behind the arterial cannula,

as in the case of IOP, just after completion of the 20 min mixing period. In most experiments, however, drugs or vehicle were injected into separate eyes.

The K_{out} value was also used to find, by extrapolation, the fluorescence value (F_m) at zero time, corresponding to the value which would have pertained if complete and hypothetical instantaneous mixing had occurred. This extrapolation allowed estimation of the anterior chamber volume. For this calculation it was assumed that the rate of AH formation, i.e. the $K_{out}.min^{-1}$ value, for the 30 min control period was similar to that for the preceding 20 min mixing period.

Anterior chamber volume at the beginning of each experiment was thus measured by extrapolation of the decay curve for fluorescence. At the end of some experiments, the chamber volume was also measured by withdrawal of AH through a lateral corneal puncture, using a preweighed disposable plastic syringe fitted with 23 gauge needle as described by Vogh et al. (1989). The rate of AH formation, in terms of absolute units, could them be calculated as the product of ($V_{ac} + V_s$) and $K_{out}.min^{-1}$. The relationship which allows this calculation is:

$$F_o \times V_s = F_m (V_s + V_{ac})$$ where,

F_o represents the initial fluorescence in the cuvette and tubing system prior to cannulation, F_m is the fluorescence at the start of anterior chamber perfusion if instantaneous mixing were achieved, V_s is the volume of cuvette and tubing system, V_{ac} is the volume of anterior chamber and, as mentioned above, F_m was obtained by extrapolation and thus:

$$V_{ac} = \frac{V_s (F_o - F_m)}{F_m}$$

Testing the Integrity of Blood-aqueous Barrier of the Isolated Eye Perfused with Krebs' Solution

As one of the procedures used to validate the perfused eye preparation, various arterial flow rates ranging from 1.5 $ml.min^{-1}$ to 3.5 $ml.min^{-1}$ were used. Preparations were set up and arterial perfusion carried out at the standard flow rate of 2.25 $ml.min^{-1}$ (which was used throughout all other experiments). Upon establishing that IOP and perfusion pressure were stable, the recordings were continued up to 40 min. Then the flow rate was changed to 1.5 $ml.min^{-1}$ and at this new flow rate the IOP and perfusion pressure were recorded for another 40 min. Similar observations of 40 min duration were also made for each flow rate of 2.5 $ml.min^{-1}$ and 3.5 $ml.min^{-1}$ respectively on the same eye. Variation of perfusion pressure and IOP were then statistically analysed using Student's t-test.

Measurement of O_2 and CO_2 Tension

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 fresh perfused eyes. These were compared with corresponding data from bovine eyes which had been stored at 4°C for at least 24 hour (after intially perfusing with non-oxygenated Krebs' solution for 30 min in order to remove blood from the vasculature which may interfere with subsequent perfusion). Both fresh and dead eyes were arterially perfused for 90 min at 37° C as described above and comparison was made of the gas tensions of the perfused fluid and the perfusate emerging from the vortex veins. O_2 and CO_2 were measured using a pH/Blood Gas Analyzer (Instrumentation Laboratory Systems, 1302).

Effects of Timolol and MK-927 on AH Formation

In order to confirm that it is possible to demonstrate significant and reproducible ocular effects of standard drugs, on the bovine isolated perfused eye preparation, a single bolus dose of timolol, and 3 different doses of the CA inhibitor, MK-927, were tested in respect of AH formation. In these experiments separate eyes were used for studying control and drug effects. Both the drugs were dissolved in freshly prepared Krebs' solution. The dose used for timolol maleate was 10 nmol and for MK-927 was 100, 10 or 1 nmol. After drug or vehicle (in these cases Krebs' solution) injection, the fluorescence reading were monitored for 120 min. The volume of the bolus injection was 10 μl in both cases. Statistical comparison of values of $K_{out}.min^{-1}$ were made by analysis of variance (ANOVA) and for values of $\mu l.min^{-1}$ by two-tailed Student's t-test for unpaired data using Minitab.

Effects of Terbutaline, Forskolin and 8-bromo Cyclic AMP on AH Formation

Terbutaline, forskolin and 8-bromo cyclic AMP were tested for their effects on AH formation. The dose used for terbutaline and forskolin was 30 nmol and for 8-bromo cyclic AMP was 100 nmol. The vehicle for terbutaline and 8-bromo cyclic AMP was Krebs' solution. Forskolin was dissolved in dimethylsulfoxide (DMSO). Drug solution or vehicle in volumes of 3 to 10 μl was injected into the arterial perfusate as before. The fluorescence readings after drug injection were taken for 120 min in case of terbutaline and 90 min for the other two agents. $K_{out}.min^{-1}$ values were calculated and analysed statistically as before using analysis of variance (ANOVA).

Effects of Terbutaline and Sodium Azide on IOP

Single bolus doses of 30 nmol of terbutaline and 10 nmol of sodium azide, were also investigated for their effect on IOP. The vehicle for each drug was Krebs' solution and a group of vehicle-treated eyes was used as control for both drugs. Upon establishing the stable IOP for 15 min as described above, the drug or the vehicle (both in a volume of 10 μl) was injected. IOP was monitored for 90 min after injection of drug or vehicle. The regression line for IOP values at 5 min intervals was constructed for each eye and the statistical comparison was made on the mean slope of control or treated group by ANOVA.

Effect of Verapamil on IOP

Three doses of verapamil, were investigated to observe any dose-dependent response. The drug was dissolved in 50% DMSO. The doses used were 1, 10 and 100 nmol. Volume of the bolus injection was 10 μl for each of the doses. Statistical comparison between the control and treated groups was made by ANOVA.

Effect of Atriopeptin on AH Formation

A 50 pmol dose of atriopeptin, was also investigated in respect of AH formation. Same-eye as well as different-eye controls were used for the vehicle (water). The drug solution or vehicle was injected in a volume of 5 μl. In the case of the group where same-eye controls were used, after the initial 20 min of mixing, vehicle was injected and then after a further 30 min, the drug was injected. For the different-eye control group the same protocol was maintained as for other

drugs. Calculation of K_{out} values and statistical analysis was done as described for timolol.

Effect of Specific Inhibitors of Cyclic AMP-dependent (KT-5720) and Cyclic GMP-dependent (KT-5823) Protein Kinases on Terbutaline and Sodium Azide-Induced Reduction of IOP and AH Formation

Determination of Effect on IOP

After the establishment of 15 min of stable IOP, the specific protein kinase inhibitor (KT-5720 in case of terbutaline and KT-5823 in case of sodium azide) was injected as a bolus dose of 10 nmol. After 15 min the second drug (30 nmol of terbutaline and 10 nmol of sodium azide) was injected. IOP was monitored for 105 min from the time of first injection. A similar but cross over study on IOP was also conducted to investigate the effect of KT-5823 (cyclic GMP-dependent protein kinase inhibitor) on terbutaline's response and KT-5720 (cyclic AMP-dependent protein kinase inhibitor) on the effect of sodium azide.

Determination of Effect on AH Formation

The effects of KT-5720 on the reduction of AH formation by terbutaline and of KT-5823 on the suppression of AH formation by sodium azide were studied. Here also same-eye and different-eye controls were used. After 20 min of aqueous mixing, the appropriate vehicle in appropriate volume was injected into the arterial perfusate. After 30 min of control period the specific inhibitor of protein kinase (KT-5720 in case of terbutaline and KT-5823 in case of sodiun azide) was injected. After another 15 min 30 nmol of terbutaline or 10 nmol of

sodium azide was injected. Measurement of K_{out} values and statistical anlysis were made as before.

Determination of Ciliary Cyclic Nucleotides Concentrations (Cyclic AMP and Cyclic GMP).

In order to determine the effects of timolol, terbutaline and forskolin upon cyclic AMP and the effect of atriopeptin on cyclic GMP in the CE, three types of tissue preparation were used. These were (1) the superficial scrapings of the CE obtained after drug challenge from the eyes perfused as in the case of IOP and AH formation experiments, (2) incubation with the drug of the ciliary process tips excised from fresh eyes and (3) incubation with drug of the CE cells obtained by tissue culture in the laboratory. In the case of terbutaline, all three types of tissue preparation were used for determining its effect on cyclic AMP. In the case of atriopeptin, only the cultured CE cells and in all other cases the scrapings of CE cells from the perfused eye were used.

Collection of Tissue Samples from Perfused Eyes

Perfused eye preparations were set up as in the case of IOP experiments and allowed to achieve stability. Eyes showing stable IOP and perfusion pressure were then challenged with either the drug or the vehicle in appropriate doses and volume into the perfusate through the rubber tubing immediately behind the arterial cannula. The perfusion was subsequently allowed to proceed for precisely 3 min, whereupon the eye was taken off the perfusion system and rapidly dissected through the posterior pole to remove the vitreous, lens and lens capsule. A portion of the sclera along with choroid and retina was also dissected out to facilitate manipulation. The remaining anterior portion of the

eye was then inverted over a plasticine sphere, which filled the curvature of the depressed cornea in the anterior chamber, and was immediately immersed in liquid nitrogen for precisely 20 s in order to freeze the ciliary processes and stop any chemical or enzymically catalysed reaction. This dissection and subsequent procedures took approximately 3 min, giving a total drug exposure time of approximately 6 min. After removal from the liquid nitrogen, the anterior eye portions were placed in a freezer at -30°C for about 30 min. They were then removed from the freezer and the CE cells were scraped very superficially from the processes along the region of the pars plicata by means of a sharp stainless steel spatula. While scraping, care was taken to harvest as little as possible of the underlying stromal tissue. The scraped tissue was collected into 0.5 ml of TCA (6%, w/v) in Eppendorf tubes. Scrapings from each eye were collected in separate Eppendorf tubes and stored at -30°C until required for analysis.

Collection of Samples from Fresh Eyes

Fresh eyes were dissected from the posterior pole as described above. After removal of vitreous, lens and lens capsule, the tips of the ciliary processes were cut with fine scissors and harvested in Eppendorf tubes containing 1 ml of saline. Unlike the ciliary processes in rabbit and human, the bovine processes are well developed and project freely for a distance of about 3 mm over the iris, the so-called operculum or corpora nigra. Thus the tips of the processes can be cut without any contamination from the adjacent tissues. The Eppendorf tubes with the cut ciliary processes were incubated at 37°C. After about 10 min, when the temperature of the contents in the Eppendorf tubes was equilibrated at 37°C, drug solutions or vehicle were added. Drug solutions were added to the Eppendorf tubes to the desired concentrations. After addition of drugs or vehicle the incubation was continued for a further 12 min. The fluid in the

Eppendorf tubes was then removed with a Pasteur pipette and 0.5 ml of 6% TCA was added to stop any enzymic reaction. The tubes were then stored at -30°C until required. Cut tips were used only for terbutaline experiments. Four concentrations of terbutaline (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}M) were used.

Collection of Samples in Case of Cultured CE Cells

Bovine CE cells were cultured and maintained as described in page 92-95. Only the 1st and 2nd passage cells were used for investigating drug effects on cyclic nucleotides. For this purpose, cells were grown in 25 cm^2 GIBCO culture flasks. At confluence, the culture medium was decanted and the cells were washed two times with normal saline (pH 7.4). The cells in each flask were then incubated with 5 ml of the same saline solution for about 10 min to equilibrate the temperature at 37°C. After addition of drug or vehicle, the flasks were incubated for another 6 min. The fluid from the flask was decanted and 0.5 ml of 6% TCA was spreaded over the cell layer to stop enzymic reaction. The cells were then scraped with a cell scraper and collected along with the TCA into Eppendorf tubes using a Pasteur pipette. Cells from separate flasks were collected in separate Eppendorf tubes and stored at -30°C until needed.

Preparation of Cyclic Nucleotide Extract for RIA

The previously collected raw samples in the Eppendorf tubes (for all three types) were thawed in a water bath at 50°C for 10 min, then sonicated for 5 - 10 min in a Camlab Transsonic T310 sonicator bath, mixed well with a vortex mixer and frozen again at -30°C for 20 min. The above sequence of procedures (freeze-thaw-sonication-vortexing) was carried out 5 times altogether. The purpose of this was to break open all of the cells in the tissue. At this stage, either ^{3}H-

cyclic AMP for cyclic AMP RIA or ^{3}H-cyclic GMP for cyclic GMP RIA (e.g., 0.072 pmol of ^{3}H-cyclic AMP, equivalent to 4440 dpm) was added to each sample in order to determine the recovery efficiency of the extraction process. This amount was subtracted while calculating the total amount of cyclic nucleotide in the samples.

The homogenate in Eppendorf tubes was then spun in a microcentrifuge (10,000 g, for 5 - 10 min). The supernatant was transferred to a 3 ml glass test tube containing 1.6 ml of 0.5 M (22.5%, v/v) tri-N-octylamine (TNO) in Freon. The resultant immiscible layers were mixed thoroughly on a vortex mixer for 4 × 5s and centrifuged briefly (1000 g, 3 min) to break the emulsion and to clarify the separate phases. The top layer was transferred to a 4cm column of well washed (with distilled water, 10 times) Dowex-50W cation exchange resin (dry mesh 200 - 400 in case of samples for cyclic AMP assay; and 50 - 100 for cyclic GMP assay). The resin columns were made using 155 mm disposable Pasteur pipettes with a glass wool plug. While transferring the supernatant, great care was taken to avoid contamination of the aqueous layer from the underlying Freon layer. This was very important because it was observed that a slight contamination seriously hampered the recovery of nucleotides through the column. This was conveniently done using a glass Pasteur pipette and suction bulb. A further 0.2 ml of 6% TCA was added to each pellet remaining in the Eppendorf tubes which were then vortex mixed to break up the pellets. A micro-homogenizer pestle was used at this stage, when needed, in order to assist in disrupting the pellets. The washed pellets were then spun down once again (10,000 g, 5 min). The supernatant was transferred to the same 3 ml glass tubes containing TNO in Freon, vortex mixed, centrifuged and the aqueous layer transferred to the same resin column. The pellets in the Eppendorf tubes at this stage were stored at -30 °C for protein assay. Cyclic nucleotide was then eluted from the column by

washing 10 times with 0.5 ml of distilled water with minimum disturbance of the resin. The first three washings were discarded as dead volume of the column and 4th to 10th washings were collected as eluate in 10 ml glass test tubes. The eluates were dried overnight under a stream of air and were stored at 4°C for subsequent assay.

The dead volume and eluate volume of the column were previously determined using standard ^{3}H-cyclic AMP or ^{3}H-cyclic GMP in TCA and were found to be 2.5 ml and 3.5 ml respectively. 0.5 ml of TCA was mixed with 0.1ml of ^{3}H-cyclic AMP (concentration was 3.6 pmol.ml^{-1}) and the mixture was then added to 1.6 ml TNO in Freon in a 3 ml glass test tube. The tube was vortex mixed for 20 s and centrifuged for 2 min (10,000 g). 0.4 ml of supernatant was then transferred to the Dowex-50W cation exchange resin column and the resulting eluate collected in a 25 ml plastic vial for liquid scintillation counting. The remaining 0.2 ml of supernatant in the tube containing the TNO in Freon was then transferred to the same column and the eluate collected in another counting vial. The column was washed 15 times with 0.5 ml of distilled water and the eluate of each washing was collected in separate counting vials. 10 ml of Ecoscint scintillation cocktail was added to each of the 17 vials and the β-radiation counted in a Packard 2000CA Tri-Carb Liquid Scintillation Counter. The liquid scintillation counter printout included an automatic correction for counting efficiency.

Determination of Recovery Efficiency of Nucleotide from the Extraction Process

The dried tissue extracts in 10 ml glass test tubes were redissolved in 500 µl of Na-acetate buffer (pH 6.2) supplied with the cyclic AMP or cyclic GMP

radioimmunoassay kit. From each tube a 100 µl aliquot was taken in a scintillation counting vial and 10 ml of scintillation cocktail and 0.5 ml of distilled water were added to it. The contents were mixed well by shaking and the beta-radiation was counted by the Liquid Scintillation Counter (Packard TriCarb 2000A). Two empty vials containing only distilled water and scintillation cocktail and two total count vials containing 0.072 pmol of ^{3}H-cyclic AMP (or in case of cyclic GMP assay 0.018 pmol of ^{3}H-cyclic GMP) were also counted for use as background and total counts respectively. The recovery efficiency for each extraction was then calculated.

Radioimmunoassay (RIA)

The basic principle of RIA first described by Yalow and Berson (1971) consists of competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. If increasing amounts of unlabelled antigen (i.e., in standards or unknown samples) and a fixed amount of labelled antigen (i.e., tracer) are allowed to react with a constant amount of antibody, a decreasing amount of labelled antigen is bound to the antibody. This relationship can be expressed as a standard curve and the amount of unlabelled antigen in a sample can be determined by interpolation from this curve. This reaction can be represented schematically as follows:

Labelled Antigen + Specific Antiboby → Labelled Antigen-Antibody Complex

Ag* Ab Ag*Ab

+

Unlabelled Antigen (In standard solutions or unknown samples)

Ag

↓

Unlabelled Antigen-Antibody Complex

AgAb

RIA for Nucleotides (Cyclic AMP and Cyclic GMP)

Determination of nucleotides was adapted from the procedure of Steiner et al (1972) using New England Nuclear RIA kits (DuPont). In their original work Steiner et al. (1972) reported that cyclic nucleotide substituted at 2′-O-position has a higher affinity for the antibody and thus displaces the [^{125}I]-labelled derivative better than the unsubstituted cyclic nucleotide. Following this, Cailla et al (1973) described a procedure for succinylating tissue extract of cyclic AMP yielding 2′-O-succinyl cyclic AMP and increasing the sensitivity of the assay by 100-fold. Harper and Booker (1975) and Fradson and Krishna (1976) showed that a similar result could be achieved by acetylating the sample with acetic anhydride to give 2′-O-acetyl cyclic AMP or 2′-O-acetyl cyclic GMP. Thus both standards and samples were acetylated with acetic anhydride to increase the sensitivity of the assay.

The experimental protocol for both cyclic AMP and cyclic GMP assay were as per instructions for acetylated samples included in the kits. All standards and samples were run in duplicate.

Analysis of Radioactivity

All tubes were counted for 5 min in a gamma counter (Packard Cobra Auto Gamma, 5003). The analysis of radioactivity in tubes was controlled by an IBM-computer with a user-defined RIA protocol. Each assay run included duplicates for total counts, blanks, standards and unknowns (samples). The standard curve was plotted with the characteristics of a bound fraction RIA curve with a negative slope. The concentration for each unknown sample was automatically calculated by interpolation from the standard curve and the values

were printed. From the printed value, the total amount of nucleotide in each sample was calculated. The nucleotide added for recovery estimation was subtracted to find the actual amount of nucleotide present in the sample.

Protein Assay

The amount of protein in the pellets was determined by the Lowry (1951) method. The pellets in the Eppendorf tubes were transferred to 10 ml glass test tubes. This was achieved very conveniently by addition of 1 ml of N-NaOH to each Eppendorf tube allowing a few minutes to soften the pellets and then transferring with a disposable glass Pasteur pipette. Another 4 ml of N-NaOH was added to each tube and the tubes were heated in a water bath at 60° C with occasional shaking for about 2 h or until all pellets had dissolved. Three convenient aliquots from each samples were transferred to separate 10 ml glass test tubes and N-NaOH was added to each aliquot to yield a final volume of 300 µl. A blank and 5 standards, each in duplicate were prepared in 10 ml glass test tubes using bovine serun albumin (BSA) solution in distilled water ($1mg.ml^{-1}$) as follows (Table 4):

Then at 15 s intervals, 3 ml of solution C (Lowry et al., 1951) were added. The tubes were mixed and left at room temperature for 15 min. 1N-Folin-Ciocalteu reagent was then added to each tube at 15 s intervals, mixed well and left for 30 min. After this time and at 15 s intervals, absorbance of each aliquot was measured at 750 nm (E_{750}) using a Cecil Spectrophotometer. A standard curve was constructed with values for protein (in micrograms) present in the standards plotted along the abscissa and the corresponding E_{750} values along the ordinate. The standard curve was constructed using the computerised statistical package Minitab. Protein in each aliquot was calculated from the regression equation

obtained by using the Minitab package. The results were expressed as mg of protein per sample.

Table 4.

Tubes	BSA (µl)	N-NaOH (µl)
Blank	0	300
*S1	30	270
S2	60	240
S3	90	210
S4	120	180
S5	150	150

* S = Standard

The results for cyclic AMP were expressed as pmol.mg^{-1} protein and for cyclic GMP as fmol.mg^{-1} protein. All results were analysed using MINITAB and statistical significance was tested by using the two-tailed Students t-test for unpaired data.

Culturing Ciliary Epithelial Cells

Two methods of isolation for primary cultures of CE cells have been adopted: one using the arterially perfused eye as described above and another by cutting the tips of ciliary processes from unperfused eyes.

Isolation and Culture of Cells Using Perfused Bovine Eye

The perfusion procedure was essentially similar to that described for IOP experiments except that the system was sterile and 3 distinct perfusates were used instead of modified Krebs' solution. The eyes were initially perfused with Dulbecco's modification of Eagle's medium (DMEM) supplied with 100% O_2 (instead of previously used mixture of 95% O_2 and 5% CO_2, because CO_2 influences the pH of the Hepes bufferred DMEM used) for about 30 min while raising the flow rate to 1.5 $ml.min^{-1}$ with a view to open up the ciliary vasculature as well as supplying nutrients and oxygen. The second perfusion was for exactly 12 min with a Ca^{2+}-free buffer (pH 7.4) comprising (mM): NaCl, 142; KCl, 13.41; Hepes 4.82 and EDTA, 0.25. Finally, the eyes were perfused with collagenase A (Boehringer) solution (0.1%) in a buffer (pH 7.6) comprising (mM): NaCl, 66.73; KCl, 13.41; Hepes, 3.84 and $CaCl_2$, 4.82; for 20 min, at a flow rate of 0.22 $ml.min^{-1}$ (reduced to economize on collagenase). The eyes were immediately removed from the perfusion system and all subsequent operations were carried out in a flow hood (Flow Laboratories).

The remaining extraocular tissues were cleaned and the eyes were washed 3 times with a sterile saline containing gentamycin (200 $\mu g.ml^{-1}$). The eyes were dissected from the posterior pole and the vitreous and lens along with its capsule were carefully removed. The partially digested CE sheets were then picked up with a round tipped wet Pasteur pitette and collected in sterile centrifuge tubes containing DMEM with newborn calf serum (NCS) and foetal calf serum (FCS) (10% of each). The sheets of cells thus collected were disrupted by squirting gently with the Pasteur pipette without allowing formation of air bubbles. Squirting was done in 3 other subsequent tubes transferring each time the sediment from the preceding tube. This helped to subject the isolated cells to a

minimum of stress. The undisrupted sediment in the last tube was removed and cells in all the tubes were centrifuged (in an IEC Centra-8R Centrifuge, model 100-240, at 700 rev.min^{-1} for 4 min at 4°C) and resuspended for washing in DMEM. Finally, the cells were centrifuged and resuspended in 2 ml of DMEM containing 10% NCS, 10% FCS and gentamycin (200 μg.ml^{-1}) - this will be referred to as complete medium throughout the rest of the text. The cells were then seeded in a 80 cm^2 culture flask (GIBCO) containing 15 ml of complete medium and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. The medium was changed the day following the initial isolation and every 2 - 3 days subsequently. Confluence was reached by 7 - 8 days. Cells from 2 perfused eyes were usually enough for an 80 cm^2 flask.

Isolation and Culturing of Cells Using Unperfused Eyes

Both fresh eyes just after transportation to the laboratory at room temperature and eyes collected and maintained on ice were found to be equally suitable for this purpose. However, usually eyes maintained on ice were used in the present study. The extraocular tissues were cleared off and the eyes were washed 3 times in saline containing gentamycin (200 μg.ml^{-1}) before dissection. All work was then carried out aseptically in a flow hood. The eyes were dissected from the posterior pole as described before and inverted on a plasticine sphere which fits the curved space formed by the inverted cornea. The vitreous was first removed by pushing from the side with a curved forceps keeping the lens intact in its position. The lens along with its capsule was then removed by cutting the zonular fibres with fine sharp scissors. This revealed the ciliary processes lying on the iris. The free suspended portion of the tips were cut with fine scissors and collected in centrifuge tubes containing DMEM and gentamycin (200 μg.ml^{-1}). The cut tips were then tranferred to a large petri

dish containing 20 ml of the same Ca^{2+}-free solution used in the case of perfused eyes and incubated under constant and mild shaking (Luckham Rotatest Shaker, R100/WT) for 25 min. The tips were then picked up with a wet, sterile and round-tipped Pasteur pipette and transferred to another large petri dish containing 15 ml of the same 0.1% collagenase solution used for the perfused eye and incubated under constant shaking for a further period of 30 min. After 30 min of incubation, 3 ml of a mixture of NCS and FCS (1:1) was added to the petri dish to neutralise the enzyme. The partially digested tips were then transferred to a centrifuge tube containing DMEM and gentamycin and disrupted by squirting with the Pasteur pipette. Squirting was done in 3 other subsequent tubes transferring each time the sediment from the preceding tube. This helped to subject the isolated cells to a minimum of stress. The undisrupted sediment in the last tube were removed and the cells in all the tubes were then centrifuged (in an IEC Centra-8R Centrifuge, model 100-240, at 700 rev.min-1 for 4 min at 4 °C) washed with DMEM and finally centrifuged and resuspended in 2 ml of complete medium. The cells were then seeded in 80 cm^2 flasks (one flask for cells from 2 eyes). Incubation and all other tasks were done as in the previous case. The cells reached confluence in 7 to 8 days.

Trypsinization and Propagation of Cells

At confluence the cells were washed 2 times with 20 ml of sterile saline solution and incubated with 10 ml of trypsin-EDTA solution (1×, prepared in modified Puck's saline, GIBCO) at 37°C for 3 - 4 min. The cells were easily detached upon gently shaking the flask 3-4 times. Trypsin was immediately neutralized with 3 ml of a mixture of NCS and FCS (1:1). The cells were centrifuged and supernatant discarded. Cells were suspended and washed with DMEM and finally centrifuged, supernatant discarded and resuspended in 5 ml of complete

medium. Cells were passaged in 10 GIBCO culture flasks (25 cm^2) each containing 5 ml of complete medium. Confluence was usually reached after 3 to 4 days. Cells were propagated and maintained up to 10 passages without any obvious sign of change of growth rate but only cells from 1st and 2nd passages were used in the present study.

Effect of Terbutaline on Cyclic AMP in Cultured CE

Four concentrations (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}M) of terbutaline were tested for its effect on cyclic AMP content in the cultured CE cells. The vehicle was Krebs's solution. Only the 1st passage cells were used. The procedure was similar to that described for the collection of samples in case of cultured cells for RIA .

Effect of Atriopeptin on Cyclic GMP in Cultured CE

First and second passage cells were used. Three concentrations (10^{-6}, 10^{-7} and 10^{-8}M) were used for 1st passage cells and two concentrations (10^{-6} and 10^{-7}M) for the 2nd passage cells. The drug was dissolved in distilled water. The procedure was similar to that described for collection of cultured cells for RIA.

RESULTS

RESULTS

Development of the Isolated Arterially Perfused Eye as a Model for Studying AH Dynamics

The isolated arterially perfused eye has been developed as a useful in vitro model for studying AH dynamics (Wilson, et al., 1993) in which the AH formation rate in terms of both rate constant (defined as $K_{out}.min^{-1}$) and absolute units ($\mu l.min^{-1}$) can be determined directly by circulating the anterior chamber with a fluorescein solution. The slope of the regression line (a plot of log_e of fluorescein concentration in the anterior chamber against time) representing the decay of fluorescein from the anterior chamber is the rate constant for AH formation, $K_{out}.min^{-1}$. The anterior chamber volume can be determined by a simple formula as described in the Methods section. The rate of AH formation in absolute terms can then be calculated by multiplying the $K_{out}.min^{-1}$ value by the sum of the volumes of anterior chamber and tubing system.

The anterior chamber volume was determined in 54 eyes by the fluorescein dilution technique and also in 10 eyes by the direct method as described by Vogh et al. (1989). The mean volume with the fluorescein dilution technique was 1.69 ± 0.03 ml (mean $\pm$ SEM) with a maximum of 2.10 ml and minimum of 1.23 ml. The mean total (anterior + posterior) chamber volume was 2.0 ± 0.13 ml by the direct method. The mean normal $K_{out}.min^{-1}$ was found to be 0.0045 ± 0.0001 (n=51), the mean normal AH formation rate was 12.9 ± 0.44 $\mu l.min^{-1}$ (n=11) and the mean normal IOP was 9.37 ± 0.14 mmHg (n=88).

Effects of Different Flow Rates on IOP in Arterially Perfused Eye

The isolated eye was tested using four different arterial flow rates ranging from 1.5 $ml.min^{-1}$ to 3.5 $ml.min^{-1}$, and then correlating flow rates with the observed IOP and perfusion pressure. No correlation has been found between the flow rates and the IOP. The results are shown in table 5.

Table 5. Variation of Perfusion Pressure and IOP Due to Changes in Flow Rate During Perfusion of the Isolated Eye.

Perfusion Flow Rate ($ml.min^{-1}$)	Perfusion Pressure (mmHg)	IOP (mmHg)
2.25	37.3 ± 3.1	9.40 ± 0.44
1.50	28.4 ± 2.7*	8.95 ± 0.41
2.50	42.0 ± 3.7	9.08 ± 0.34
3.50	50.0± 4.6*	9.97 ± 0.41

Each eye was perfused with Krebs' solution for 40 min in turn at each of the flow rates shown. Each value is a mean ± SEM of the results from 8 experiments. Significance of difference from pressure during perfusion at 2.25 $ml.min^{-1}$: *$p < 0.05$.

Metabolic Activity of Bovine Perfused Eye

Metabolic activity of bovine eye during perfusion was determined by comparing the O_2 consumption and CO_2 elaboration by the living and the dead (frozen) eyes. The perfused eyes show a considerable metabolic activity as shown in table 6.

Table 6. Metabolic Activity of the Bovine Isolated Perfused Eye.

	n	CO_2	O_2
Fresh eyes	12	16.2 ± 0.7	163 ± 7.4
Eyes stored at 4° C	15	10.1 ± 1.5**	129 ± 8.0**

The O_2 utilization and CO_2 production of the bovine perfused eye, calculated by measuring O_2 and CO_2 tension (mmHg) in the perfusion fluid entering the ciliary artery and leaving the vortex veins. Each value is a mean ± SEM of the number (n) of experiments shown. Significance of difference from fresh eyes: ** $0.01 > p > 0.001$.

Influence of pH on Fluorescence

It has been found that the pH of the solution of AH and fluorescein sodium has a tremendous influence on its fluorescence. There appears to be a direct proportionality of pH and fluorescence. Thus to exclude any variation due to pH, we measured the pH of a mixture of 2 ml (approximate anterior chamber volume) of fresh AH and 1.06 ml (vol. of the cuvette and the tubing system) of

fluorescein solution after gassing as previously described. The pH of the chamber fluid after the end of some experiemts was also measured and compared with that of the mixture prior to perfusion. No variation of pH was observed (Fig. 14).

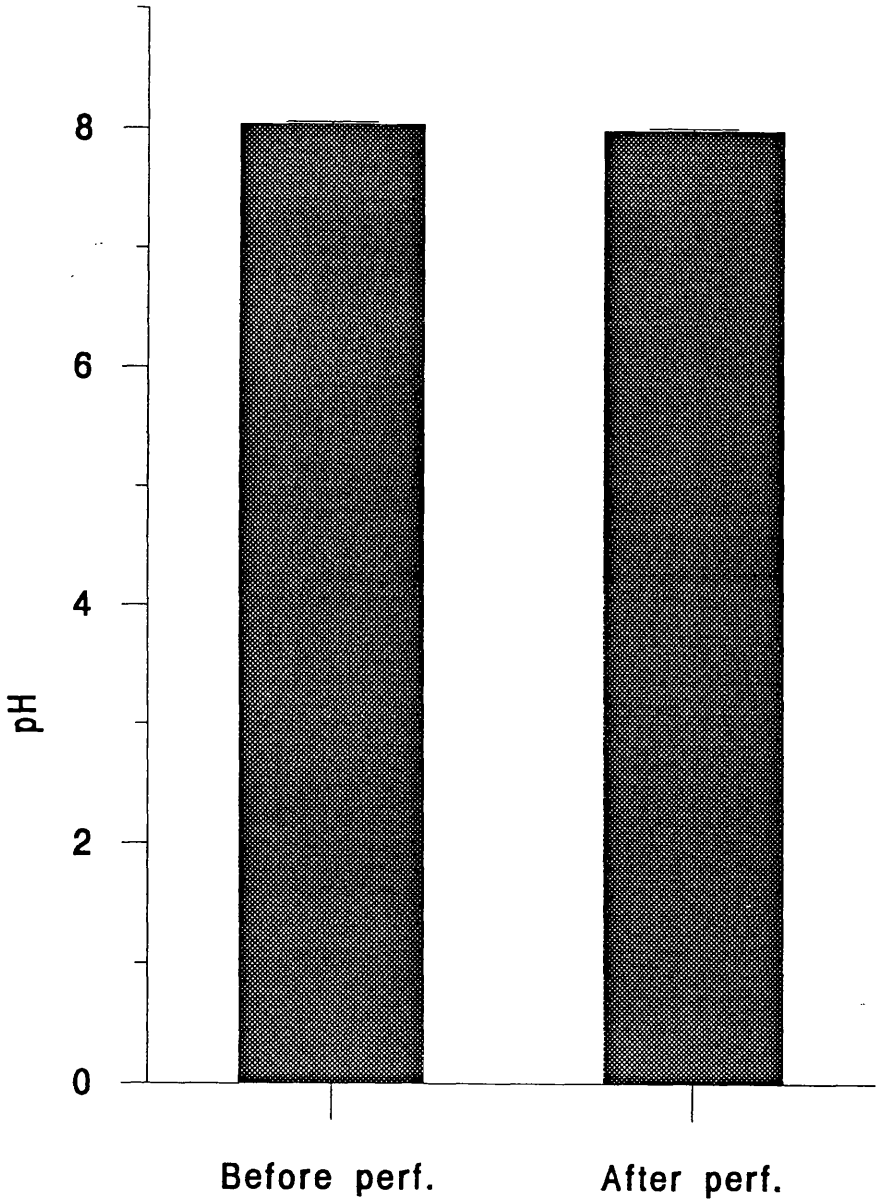

Fig. 14. Histogram showing pH of anterior chamber fluid before and after perfusion of the isolated bovine eye.

Effects of β-adrenoceptor Drugs on AH Formation

The effects of a single bolus dose of terbutaline (30 nmol) or timolol (10 nmol) on AH formation were studied for a period of 2h. Both terbutaline and timolol caused a significant reduction of AH formation compared to the control group as shown in table 7.

Table 7. Effect of Timolol, Terbutaline or MK-927 on AH Formation

Treatment	Dose (nmol)	n	K_{out} ($min^{-1} \times 10^4$)	V_{ac} (ml)	Rate ($\mu l.min^{-1}$)
Control (Krebs)	—	11	46 ± 1.2	1.75 ± 0.05	12.9 ± 0.44
Timolol	10	14	40 ± 1.1**	1.67 ± 0.05	10.9 ± 0.30**
Terbutaline	30	7	38 ± 1.7**	1.73 ± 0.05	10.7 ± 0.54**
MK-927	100	9	35 ± 2.8***	1.67 ± 0.07	9.4 ± 0.54***
MK-927	10	7	39 ± 2.2**	1.63 ± 0.08	10.4 ± 0.50**
MK-927	1	6	41 ± 1.0*	1.68 ± 0.08	11.1 ± 0.51*

The effects of timolol, terbutaline or MK-927 on AH formation in the bovine isolated perfused eye, shown both as $K_{out}.min^{-1}$ and rate (the product of $K_{out}.min^{-1}$ and $V_{ac} + V_s$). V_{ac} = anterior chamber volume, V_s = volume of cuvette and tubing system. Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences from control: * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

Effect of CA Inhibitor, MK-927, on AH Formation

The effects of three doses, viz, 100, 10 or 1 nmol of MK-927, on AH formation were studied. All three doses caused a significant reduction in AH formation and also in a dose-dependent manner (Table 7)

Effect of Timolol, Terbutaline or Mk-927 on IOP

The IOP was found to be reduced sigficantly with all three drugs and in the same doses tested for AH formation. Table 8 shows the results in terms of mean slopes for treated and control groups.

Table 8.

Treatment	Dose (nmol)	n	Slopes (Mean ± SEM)
Control	—	11	0.0082 ± 0.0018
Timolol	10	14	-0.0108 ± 0.0019****
Terbutaline	30	7	-0.0086 ± 0.0017****
Mk-927	100	9	-0.0036 ± 0.0021***
Mk-927	10	7	-0.0043 ± 0.0023**
Mk-927	1	6	-0.0052 ± 0.0032**

The effects of bolus doses of timolol, terbutaline and MK-927 on IOP in the bovine isolated perfused eye, shown as the mean slope of the regression lines drawn on IOP vs time (120 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences from control: ** $0.001 < p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Effect of Forskolin and 8-Bromo Cyclic AMP on AH Formation

Forskolin, which is a powerful stimulator of adenylate cyclase and 8-bromo cyclic AMP, a cell permeable analogue of cyclic AMP both failed to influence AH formation in isolated arterially perfused eye (Table 9)

Table 9.

Treatment	Dose (nmol)	n	K_{out} ($min^{-1} \times 10^4$)
Control (DMSO)	—	10	40 ± 1.8
Forskolin	30 nmol	10	39 ± 2.0 n.s.
Control (Krebs)	—	8	44 ± 1.5
8-Bromo cAMP	100 nmol	7	43 ± 2.1 n.s.

Effects of forskolin and 8-bromo cyclic AMP on AH formation in the bovine isolated perfused eye, shown as $K_{out}.min^{-1}$. Each value is a mean ± SEM of the number (n) of experiments shown. Significance of differences from control: n.s. not significant; *** $p < 0.001$.

Effects of Atriopeptin and Sodium Azide on AH Formation

The vasodilator drugs atriopeptin and sodium azide both caused a significant reduction of AH formation (Table 10)

Table 10.

Treatment	Dose	n	K_{out} ($min^{-1} \times 10^{-4}$)
Control A	—	8	47 ± 3.0
Control B	—	8	48 ± 1.3
Atriopeptin	50 pmol	8	29 ± 1.9***
Control A	—	6	45 ± 3.8
Control B	—	11	46 ± 1.2
Sodium Azide	10 nmol	6	24 ± 2.2***

Effects of atriopeptin and sodium azide on AH formation in the bovine isolated perfused eye, shown as $K_{out}.min^{-1}$. Each value is a mean ± SEM of the number (n) of experiments shown. Control A = same eye control; control B = different eye control. Significance of difference from control: *** $p < 0.001$.

Effect of KT-5720 on Terbutaline-induced Reduction of AH Formation

KT-5720, a specific inhibitor of protein kinase A caused abolition of the AH reducing effect of terbutaline (Table. 11).

Table 11.

Treatment	Dose (nmol)	n	K_{out} ($min^{-1} \times 10^4$)
Control (KT-5720 in DMSO)	10	7	48 ± 2.9
Terbutaline+KT-5720	30 + 10	7	46 ± 2.3$^{n.s.}$

Blunting by KT-5720 of terbutaline's effect on AH formation in the bovine isolated perfused eye, shown as $K_{out}.min^{-1}$. Each value is a mean ± SEM of the number (n) of experiments shown. Significance of difference from control (same eye): $^{n.s.}$ not significant.

Effects of KT-5720 and KT-5823 on IOP-Reducing Effect of Terbutaline

KT-5720, a specific inhibitor of cyclic AMP-dependent protein kinase, completely abolished the IOP-reducing effect of terbutaline while KT-5823, a specific inhibitor of cyclic GMP-dependent protein kinase, was ineffective in blunting terbutaline's effect on IOP. The results are shown in table 12.

Table 12.

Treatment	Dose (nmol)	n	Slope (mean ± SEM)
Control (DMSO)	—	8	0.0067 ± 0.0041
Terbutaline	30	7	-0.1822 ± 0.0248***
KT-5720 + Terbutaline	10 + 30	7	-0.0128 ±0.0133n.s.
KT-5823 + Terbutaline	10 + 30	8	-0.2482 ± 0.0216***

Effects of KT-5720 and KT-5823 on the IOP-reducing effect of terbutaline in the bovine perfused eye, shown as the mean slope of the regression lines drawn on IOP vs time (90 min). Each value is a mean ± SEM of the number (n) of experiments shown. Significance of differences from control: n.s.-not significant; *** $p < 0.001$.

Effects of KT-5823 and KT-5720 on IOP-Reducing Effect of Sodium Azide

KT-5823, a specific inhibitor of cyclic GMP-dependent protein kinase abolished the IOP-reducing effect of sodium azide while KT-5720, the specific PK_A inhibitor, was ineffective (table 13).

Table 13.

Treatment	Dose (nmol)	n	Slopes (mean ± SEM)
Control (DMSO)	—	7	0.0192 ± 0.0016
Sodium Azide	10	7	-0.2702 ± 0.0223****
KT-5823 + Sodium Azide	10 + 10	7	-0.0167 ± 0.0032n.s.
KT-5720 + Sodium Azide	10 + 10	6	-0.2537 ±0.0195****

Effects of KT-5823 and KT-5720 on IOP-reducing effect of sodium azide in the bovine perfused eye, shown as the mean slope of the regression lines drawn on IOP vs time (90 min). Each value is a mean ± SEM of the number (n) of experiments shown. Significance of differences from control: n.s.not significant; **** $p < 0.0001$.

Effect of Verapamil on IOP

Verapamil, a calcium channel blocker, produced a dose-dependent reduction in IOP (table 14).

Table 14.

Treatment	Dose (nmol)	n	Slope (mean ± SEM)
Control (50% DMSO)	—	10	0.0112 ± 0.0045
Verapamil	100	8	-0.2321 ± 0.0112****
Verapamil	10	6	-0.2108 ± 0.0132****
Verapamil	1	7	-0.1505 ± 0.0069***

The effects of bolus doses of verapamil on IOP in the bovine perfused eye, shown as the mean slope of the regression lines drawn on IOP vs time (120 min). Each value is a mean ± SEM of the number (n) of experiments shown. Significance of differences from control: *** $p < 0.001$; **** $p < 0.0001$.

Effects of Timolol, Terbutaline or Forskolin on CE Cyclic AMP in the Perfused Eye.

In the perfused eye, at the same doses which produced a significant reduction in AH formation rate, neither timolol nor terbutaline produced any effect on ciliary cyclic AMP. Even at a higher dose level , terbutaline was entirely without any effect on ciliary cyclic AMP. Forskolin, on the other hand, produced a highly significant rise in ciliary cyclic AMP at a dose which produced no effect on AH formation. The results are shown in table 15.

Table 15.

Treatment	**Dose (nmol)**	**n**	**Cyclic AMP (pmol.mg^{-1} Protein) (mean ± SEM)**
Control (Krebs)	—	20	9.8 ± 0.5
Timolol	10	10	9.8 ± 0.4$^{n.s.}$
Terbutaline	30	9	8.7 ± 0.4$^{n.s.}$
Terbutaline	100	9	9.3 ± 0.9$^{n.s.}$
Control (DMSO)	—	10	8.4 ± 0.3
Forskolin	30	10	12.6 ± o.8***

Effects of timolol, terbutaline or forskolin on ciliary cyclic AMP in the bovine perfused eye shown as pmol of cyclic AMP.mg^{-1} of protein. Each value is mean ± SEM of the number (n) of experiments shown. Significance of differences from control: $^{n.s.}$ not significant; *** $p < 0.001$.

Effect of Terbutaline on Ciliary Cyclic AMP in Excised Ciliary Processes of Fresh Bovine Eyes.

When incubated with tips of ciliary processes cut from freshly collected eyes, terbutaline produced a dose-dependent increase in ciliary cyclic AMP as shown in table 16.

Table 16.

Treatment	Dose (M)	n	Cyclic AMP (pmol.mg^{-1} Protein)(mean ± SEM)
Control (Krebs)	—	6	9.4 ± 0.9
Terbutaline	10^{-7}	6	9.6 ± 0.5$^{n.s.}$
Terbutaline	10^{-6}	6	14.1 ± 1.2*
Terbutaline	10^{-5}	6	15.1 ± 1.1**
Terbutaline	10^{-4}	6	21.4 ± 1.6***

Effects terbutaline on cyclic AMP in the ciliary processes of bovine eye shown as pmol of cyclic AMP.mg^{-1} of protein. Each value is mean ± SEM of the number (n) of experiments shown. Significance of differences from control: $^{n.s.}$ not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effect of Terbutaline on Cyclic AMP in Cultured CE of Bovine Eyes

In the cultured ciliary epithelial cells terbutaline also produced a marked and dose-dependent increase in ciliary cyclic AMP (Table 17).

Table 17.

Treatment	Dose (M)	n	Cyclic AMP (pmol.mg^{-1} Protein) (mean ± SEM)
Control (Krebs)	—	12	48.9 ± 2.6
Terbutaline	10^{-7}	10	52.2 ± 6.8n.s.
Terbutaline	10^{-6}	10	85.8 ± 3.7***
Terbutaline	10^{-5}	10	142.2 ± 7.8****
Terbutaline	10^{-4}	12	357.0 ± 21.5****

Effect of terbutaline on cyclic AMP in cultured CE (1st passage) cells of bovine eye shown as pmol of cyclic AMP.mg^{-1} of protein. Each value is mean ± SEM of the number (n) of experiments shown. Significance of differences from control: n.s. not significant; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

Effect of Atriopeptin on Cyclic GMP Levels in Cultured CE

Incubation (6 min) of cultured CE cells with atriopeptin produced a dose-dependent increase in cyclic GMP as shown in table 18.

Table 18.

Treatment	Dose (M)	n	First Passage Cells	Second Passage Cells
Control (water)	—	9	64 ± 7	67 ± 7
Atriopeptin	10^{-8}	9	66 ± 5 n.s.	—
Atriopeptin	10^{-7}	9	135 ± 18**	78 ± 8 n.s.
Atriopeptin	10^{-6}	9	174 ± 13***	141 ± 18**

Effects of atriopeptin on cyclic GMP in cultured CE (1st and 2nd passage) cells of bovine eye shown as fmol of cyclic GMP.mg^{-1} of protein. Each value is mean ± SEM of the number (n) of experiments shown. Significance of differences from control: n.s. not significant; ** $p < 0.01$; *** $p < 0.001$.

DISCUSSION

DISCUSSION

Isolated Arterially Perfused Eye as a Model for AH Dynamics

Studies of isolated arterially perfused eye preparations are rare in the literature. Considerable success has been demonstrated in studying physiology and pharmacology of AH dynamics using isolated cat (Macri and Cevario, 1978a; 1978b; Macri et al., 1980; van Alphen and Macri, 1981) or rabbit (Kodama et al., 1983, 1985; van Pinxteren and van Alphen, 1985) eyes. Facility of outflow in the perfused anterior segment of bovine (Erickson-Lamy et al., 1988) and human (Erickson-Lamy et al., 1991; Erickson-Lamy and Nathanson, 1992) eyes has been measured.

Tazawa and Seaman (1972) described a technique for recording the electroretinogram utilizing the perfused extracorporeal bovine eye. In a preceding study the same group (Seaman et al., 1965) confirmed the viability and metabolic activity of the tissue of the perfused eye as evidenced by oxygen utilization, carbon dioxide elaboration, glucose consumption and the blockade of these activities by cyanide. 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. (1985) judged this preparation to be unsuitable for the investigation of AH dynamics. However, the delayed commencement of perfusion, within 2 h post mortem, may explain their dissatisfaction with their results. The present results indicate that delayed commencement of perfusion beyond 1 h after slaughter considerably reduces the probability of achieving a stable IOP and perfusion pressure. However, no correlation was found between success rate and

postmortem time when not exceeding 50 min. In a very recent study de Coo et al. (1993) reported that using properly oxygenated serum-free MEM with full precautions for sterility, the bovine arterially perfused eye could be kept viable for 5 days. The same study also showed that with addition of some essential serum substitutes to the perfusate, viability could be extended up to 9 days.

We have demonstrated that the isolated arterially perfused eye is an effective, cheap, quick and humane model for studying the physiology and pharmacology of AH dynamics and the mechanisms of various antiglaucoma drugs (Wilson et al., 1993). Clearly any drug which affects IOP in isolated eye is doing so by a local mechanism and is unaffected by the absence of blood, hormones and CNS influences. The model showed its usefulness in all three different approaches undertaken to prove its validity. It showed a reduction in AH formation and IOP in response to two antiglaucoma drugs, namely timolol and MK-927, the latter producing a dose-dependent response with the use of very modest doses. Significant response to reasonable doses of standard drugs is of prime importance in any pharmacological studies.

The blood-aqueous barrier is an important anatomical and physiological entity involved in AH production. It is constituted by the tight junctions of the NPE cells and the nonfenestrated endothelium of iris capillaries. In an effort to determine the effectiveness of the blood-aqueous barrier in isolated perfused bovine eye we used four different flow rates: 2.25, 1.5, 2.5 and 3.5 $ml.min^{-1}$. The resulting perfusion pressure and IOP were then compared. It was found that when perfusion pressure rose by 76%, IOP increased by less than 10% indicating that the barrier effectively prevents ultrafiltration. It was particulary important to establish this fact since the preparation is maintained with a perfusate having no oncotic pressure. This clearly implies that production of

aqueous in the perfused bovine eye is an active secretory process. This conclusion is supported by the substantial arterio-venous differences measured for O_2 and CO_2 (Table 6). Estimation of O_2 consumption and CO_2 production by perfused fresh and dead eyes showed a significant net consumption of O_2 and elaboration of CO_2 only by the fresh eyes indicating that the perfused eyes were metabolically viable.

Attainment of steady-state IOP is another important criterion shown by the perfused eye. In the present study, the mean steady-state IOP in 88 eyes, measured in 12 different sets of experiments, was found to be 9.37 ± 0.14 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, the value is equivalent to 18.37 mmHg in vivo, if we also assume an episcleral venous pressure of 9 mmHg in the living animal. This value is in reasonable agreement with the report by Woelfel et al. (1964) that in the calf, IOP in vivo is 16.4 ± 0.6 mmHg. The steady-state IOP in these 12 sets of experiments was found to be very reproducible and were not statistically different among the different sets of experiments.

The water manometer was chosen as the method of measurement of IOP in this preparation since the movement of the manometer fluid column in mmH_2O is considerably greater than the corresponding change in mmHg (there is a 13.6 fold difference). Superficial tonometry does not guarantee as reproducible results as anterior chamber cannulation. Electronic measurement and recording might well involve baseline drift in excess of 1 mmHg over a 1 to 2 h 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 of IOP being measured.

The constant flow rate method of perfusion was adopted as the preferred method. Earlier work with the bovine perfused eye (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 values and a rather lower proportion of eyes whose IOP remained stable during the predrug part of the experiment. The mean starting IOP was 7.26 ± 0.16 mmHg (n=26) when perfusion was carried out under conditions of constant pressure (Wilson et al., 1993), whereas in the constant flow method, the mean starting IOP was found to be 9.37 ± 0.14 (n=88).

After arterial cannulation, some eyes were rejected due to failure to achieve adequate perfusion. This was judged from the following criteria: that there should be at least two vortex veins flowing; that there should be no rise in perfusion pressure above 100 mmHg during any stage of the perfusion; that the perfusion pressure should not display major fluctuation (for example, due to an air bubble). Other eyes were rejected due to failure to achieve a stable IOP, or an IOP which rose by more than 0.1 $mmH_2O.min^{-1}$. Additionally, in the predrug part of the experiment, perfusion pressure and IOP should equilibrate within a period of 90 min after commencement of perfusion. In the case of anterior chamber perfusion, for estimation of AH formation rate, stability in IOP was sometimes not achieved, perhaps due to the turbulence. In these cases eyes showing IOP greater than 12.5 mmHg were rejected. The overall rejection rate on account of all criteria was found to be 34% (Wilson et al., 1993). While this rejection rate is high, only a very small number of eyes (less than 6%) were rejected after drug injection, usually due to sudden appearance of an air bubble in the perfusate. Hence, rejection did not significantly bias the pharmacological results. The high rejection rate in the predrug part of the experiment, can be

accounted for at least in part by damage during enucleation at the abattoir by unskilled operators. Also from our experience, we found that a considerable percentage of bovine eyes are diseased - injury or infection of the cornea, cataract, and perhaps an abnormality in the outflow channels.

In the present experiments, there was no obvious correlation between post-mortem interval and the success rate of the experiment when the interval between death and start of perfusion was within 50 min. Macri and Cevario (1973), utilizing the perfused cat eye, showed that perfusion just 10 min after enucleation compared with perfusion 3-4 h after enucleation, had produced no significant difference in pharmacological responses. Thus, in the present preparation, starting perfusion within 50 min of slaughter of the animals seems quite reasonable.

Wilson (1988), describing the constant pressure perfusion of the bovine eye, inferred that 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 perfusate 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 dextran and albumin in the perfusate and therefore, the avoidance of any problems of drug binding and consequent diminution of pharmacological effect. 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 barrier for the purpose of study of IOP. Wilson et al. (1993) reported that a correlation exists between a continually rising IOP and leakage of albumin into the chamber.

In contrast to the two posterior ciliary arteries in living animals, only one long posterior ciliary artery was cannulated to perfuse the entire ciliary body. In mammals, the two posterior ciliary arteries (lateral and medial) anastomose and form the major iridic circle (Ruskell, 1964). From the circle, arteries run forward to supply the ciliary processes and iris. In the bovine the major iridic circle is called the greater arterial circle and is very well developed (Prince et al., 1960). Thus cannulating one of the long posterior ciliary artery in the bovine eye allows physiological circulation to the anterior uvea. This has been confirmed by perfusing fluorescein into one long posterior ciliary artery while observing the ciliary body, exposed by dissection. If one ciliary process at the side opposite to that of the perfusing artery is now cut, fluorescein is immediately seen emerging from the incision (Wilson, personal communication). Kodama et al. (1983) conducted experiments in isolated rabbit eyes perfusing in one series through ophthalmic arteries and in another series one of the two long posterior ciliary arteries (the ophthalmic artery bifurcates to form the two long posterior ciliary arteries) and found no significant differences in results in the two series of experiments.

Tazawa and Seaman (1972) reported that the tissue of an enucleated bovine eye will gradually degenerate and lose all physiologic function, so an enucleated eye should be properly perfused with a flow rate providing sufficient nutrients. In the present study the constant arterial flow rate was 2.25 $ml.min^{-1}$. An estimation of total blood flow rate in the human eye is approximately 0.6 $ml.min^{-1}$ (Silver et al., 1989). The literature about the perfusion flow rate of bovine eyes is ambiguous. Tazawa and Seaman (1972) used a flow rate of 1 $ml.min^{-1}$. Imaizumi et al. (1976) used a flow rate of 2 $ml.min^{-1}$. Rover et al. (1978) used 2.5 $ml.min^{-1}$ and Kishida et al. (1985) used 2.0 - 2.5 $ml.min^{-1}$. Recently, de Coo et al. (1993) used a flow rate of only 0.66 $ml.min^{-1}$ and

succeeded in keeping the eye metabolically viable for about 5 days. The literature shows that when the flow rate is changed, the perfusion pressure will change, as well as the physiological parameters of the eye (Niemeyer et al., 1982). To determine an appropriate flow rate, we studied four different rates ranging from 1.5 to 3.5 $ml.min^{-1}$ and compared the IOP due to each of the flow rates. No significant variation of IOP was found using these rates. Thus consulting the literature and our experimental result we concluded that a flow rate of 2.25 $ml.min^{-1}$ would be an appropriate one.

Fluorescein was used as the marker for estimation of AH formation rate. The ideal marker should conform to at least 2 criteria: that it should be an inert compound with high molecular weight or ionization so that it leaves the anterior chamber only by bulk flow and does not diffuse into the ocular tissues and that it should produce fluorescence directly proportional to its concentration which is detectable by a fluorimeter. Fluorescein appears to be ideal in respect of both criteria and it has been used by other investigators for this purpose (Jones and Maurice, 1966; Nagataki, 1975).

Measurement of volume of the anterior chamber (V_{ac}) from the fluorescence decay curve indicated that the volume of distribution of the marker was 1.69 ml. The chamber volume of 2.00 ml measured directly at the end of each experiment includes the posterior chamber contents, which presumably were little penetrated by fluorescein, since newly formed aqueous is continuously flowing toward the anterior chamber through the narrow slit under the iris. Since the bovine anterior chamber is quite deep, a posterior chamber contribution of 15% to the total chamber volume seems reasonable and in agreement with the reported values. Siedel (1921) found that the volume of posterior chamber fluid in the cat is about 14% of the volume of the anterior fluid. Copeland and Kinsey

(1950) found that it is about 20% in the rabbit while Prince (1964) reported that in rabbit the posterior chamber contributes about 18% of the total chamber volume. If this is true, it confirms that little tissue adsorption of fluorescein occurred during the perfusion.

We have found that the mean rate constant for AH formation ($K_{out}.min^{-1}$) in 51 control eyes drawn from 6 different series of experiments was 0.0045 ± 0.0001 (mean ± SEM). A mean $K_{out}.min^{-1}$ value of 0.0042 has been reported in 15 human volunteers of which 3 were normal and 12 were ocular hypertensive without any visual field loss (Yablonski et al., 1978) which is quite in agreement with our result. In contrast the K_{out} values in the isolated arterially perfused cat eye and rabbit eye were found to be 0.0088 (Macri and Cevario, 1973) and 0.018 (Kodama et al., 1983) respectively. Other reported values of rate constant range from 0.0085 to 0.028 for the living rabbit eye and from 0.0092 to 0.015 for the living human eye (Cole, 1984). No reported data are available on K_{out} values either for the living or the enucleated and perfused bovine eye. The lower K_{out} value could be interpreted as a species difference, as a reflection of the much larger eye or it could be that the ciliary cells are in some way compromised by the perfusion process. Since the metabolic requirements of the aqueous are more a function of the internal surface area of the anterior chamber than of its volume, the lower surface/volume ratio of the large eye may only require a lower turnover of AH. In any event, this low K_{out} value will not affect the pharmacological studies with the system because it will apply to both the control and treated eyes. We have already demonstrated this fact by studying the effects of standard drugs, timolol and MK-927 on K_{out} value using the newly developed fluorescein dilution technique.

The magnitude of inhibition of AH formation by timolol in the bovine perfused eye was rather small (about 13%). However, Vareilles et al. (1977) reported about 10.5% reduction of IOP in unanaesthetized rabbit with normal IOP. A timolol-induced suppression of AH formation ranging from 13% to 48% in normal human eye has been reported by Coakes and Brubaker (1978). In contrast, greater effects (52%) in the isolated arterially perfused rabbit eye (Kodama et al., 1985) and (about 70%) in human in vivo (Yablonski et al., 1978) have been reported. This may imply that this drug is less effective in the bovine than in other species tested, or that it has more than one mechanism of action in other species. It is also possible that its effect somehow cannot be fully expressed in an eye whose secretory function may have been compromised by non-blood perfusion and perhaps by anoxia. While MK-927 also induces a decrease in formation rate which is smaller than might be expected from experiments in rabbit and man (Sugrue et al., 1988; Wang et al., 1991), it is possible that doses greater than 100 nmol might have produced larger effects in our preparation. Topper and Brubaker (1985) showed that timolol and acetazolamide are effective in reducing AH formation during the day, while they are ineffective during the night when sympathetic tone and CNS activity is at a minimum. In the isolated perfused bovine eye preparation, the lack of sympathetic and CNS tone, may be responsible for the smaller drug effect.

The advantages offerred by this preparation as a model of AH dynamics lie in the convenience of easily obtained and inexpensive tissue. Bovine eyes from the abattoir afford the opportunity for research on ocular physiology and pharmacology without the necessity of killing animals for experimental purposes. Public concern over this issue was reported by Fox (1984) and has been growing ever since.

In the case of the isolated arterially perfused eye, analysis of the pharmacological mechanism is simplified by the absence of systemic circulatory and neuronal effects and the absence of unpredictably fluctuating arterial and venous pressures. The standard topical route of administration of drugs to the eye is fraught with high and unpredictable losses to the general circulation, via the AH, and the conjunctival, scleral and nasal vasculature. The intra-arterial route of drug administration affords much more predictable delivery to the site of action and speed of drug response than does the topical route. If a known concentration of drug is perfused (instead of injecting a bolus dose) then the drug concentration in the vicinity of the target cells (CE) will be much more accurately known. This avoids the use of the large doses of drug which are required for corneal and conjunctival absorption, e.g. the standard ocular dose of timolol in man is approximately 1000 nmol. By comparing the concentration of drug required to alter AH formation with that required to alter biochemical parameters (such as cyclic nucleotide concentrations) we can advance our understanding of drug mechanisms.

The bovine perfused eye preparation therefore lends itself to studies of drug mechanisms on AH dynamics especially where the drug may have effects on the blood vessels or on the blood-aqueous barrier. The preparation has already provided data on the relationship between aqueous formation and ciliary cyclic AMP (Shahidullah and Wilson, 1992) and on the mechanisms by which atriopeptin and other vasodilator drugs lower IOP (Millar and Wilson, 1991)

The system gives experimental control over the perfusion medium and may allow study of the dependence of AH formation on individual ions etc. Also in this preparation both control and test data can be obtained from the same eye thus avoiding variation from eye to eye. In the present study, data obtained in

two sets of experiments both using same-eye control and different-eye control shows that same-eye control data may be equally reproducible. However, before drawing a definite conclusion, further experimental evidence is necessary.

The preparation is also very suitable for isolating and culturing CE cells. In the present study, two new methods of isolating and culturing CE cells have been established using collagenase as the digesting enzyme.

The bovine perfused eye has also been used to analyse drug-induced changes in regional blood flow using labelled microspheres (Millar et al., 1992).

Mechanisms of Action of Timolol and Terbutaline

In the eye, β_2-adrenoeceptors are present predominantly in the CE, which is the tissue responsible for AH formation. Since timolol is a β-adrenergic blocker (McDevitt, 1979), it is reasonable to deduce that it may reduce AH formation and hence lower IOP through β-adrenergic blockade. It has been well documented that β-adrenoceptors in the CE are coupled to the adenylate cyclase system (Nathanson, 1980, 1981b; Cepelic and Cernohorsky, 1981; Elena et al., 1984a; Palkama et al, 1986). The classical biochemical mechanism is that occupation of the receptor by an agonist causes its stimulation which in turn activates, via G_S protein, the membrane-bound enzyme adenylate cyclase. Adenylate cyclase then catalyzes the hydrolysis of ATP to produce cyclic AMP, widely recognised as a second messenger. On this basis the β-adrenergic antagonists will inhibit the agonist-induced tonic production of cyclic AMP in the CE. Hence it has been concluded that cyclic AMP in the CE is involved in modifying AH dynamics and hence IOP (Nathanson, 1980; Cepelic and Cernohorsky, 1981; Mitttag and Tormay, 1985b).

In the present study we have tried to explore the mechanism of action of timolol and terbutaline by relating cyclic AMP content of CE to the AH formation rate and IOP, using the isolated arterially perfused bovine eye as a model which has already been shown to be a very useful preparation for this sort of purpose (Wilson et al., 1993). It would appear from our data that the cyclic AMP content of CE has no relevance to the formation of AH. A preliminary communication of these results has been made (Shahidullah and Wilson, 1992). We used a number of drugs to achieve this conclusion. The drugs used were timolol, a beta-adrenergic antagonist, terbutaline, a beta-adrenergic agonist, forskolin, a potent non-adrenergic stimulator of adenylate cyclase and 8-bromo cyclic AMP, a cell-permeable analogue of cyclic AMP. The various drugs were used either because they were known to decrease AH formation or because they were likely to alter cyclic AMP levels or both. For cyclic AMP estimation in CE we used three different types of tissue samples. These includes scraped ciliary processes obtained from a perfused eye challenged with the appropriate bolus doses of either drug or vehicle, excised ciliary processes and cultured CE incubated with drug or the vehicle.

The data shows that in our preparation either 10 nmol timolol or 30 nmol terbutaline produces a highly significant reduction of AH formation as measured by the fluorescein dilution technique. Reduction of AH formation by timolol has been reported in anaesthetized cat (Liu and Chiou, 1981), in unanaesthetized owl monkeys (Bartels, 1988), in normal human subjects (Coakes and Brubaker, 1978), in ocular hypertensive human subjects (Yablonski et al., 1978) and in patients with COAG (Brubaker et al., 1982). Although controversy exist as to the cellular mechanism of action, it is now established that timolol and other β-blockers, such as levobunolol (Yablonski et al., 1987), metipranolol (Sugrue et al., 1985a), carteolol (Araie and Takase, 1985), betaxolol (Reiss and Brubaker,

1983) etc. produce their ocular hypotensive effect by reducing AH formation. Thus significant reduction of AH formation in isolated bovine eye is in the line of general agreement.

Surprisingly, terbutaline, a relatively selective β_2-adrenoceptor agonist also reduced AH formation in our preparation. The literature on the effect of terbutaline and other β-adrenoceptor agonists on AH formation and IOP is conflicting. Nilsson et al. (1990) found 100% increase in AH formation by terbutaline in cynomolgus monkeys. Isoprenaline stimulates cyclic AMP synthesis and increases AH formation in the monkey eye (Nathanson, 1980; Miichi and Nagataki, 1983). The β-agonists salbutamol and metaproterenol have also been found to increase AH formation in man (Araie and Takase, 1981; Coakes and Siah, 1984) and monkeys (Miichi and Nagataki, 1983). In contrast, several investigations showed that terbutaline, salbutamol or soterenol reduce IOP in monkeys (Langham and Biggs, 1974) and humans (Paterson and Paterson, 1972; Wettrell et al., 1977). They postulated that the reduction in IOP was largely due to a reduction in AH formation. Using a number of selective β_1 and β_2-adrenoceptor agonists and antagonists, Colasanti and Trotter (1981) concluded that β_2 stimulation is a especially effective way of lowering IOP. However, Brubaker and Gaasterland (1984) were unable to demonstrate either a lowering of IOP with isoprenaline nor an effect of isoproterenol on the flow of AH in the normal eye.

The conflicting results described above may be, in part, due to species difference. There is marked species difference in the distribution of sympathetic nerves (Laties and Jacobowitz, 1966; Ehinger, 1971) as well as of α-and β-adrenergic receptors in the anterior segment of the eye (van Alphen et al., 1965). This anatomical variation makes it difficult to extrapolate results obtained in one

species to another. However, some of these variable findings can not be accounted for by species difference. For example, terbutaline, isoproterenol, salbutamol and metaproterenol are all β-adrenergic agonists and expected to increase adenylate cyclase activity to stimulate cyclic AMP production and yet some of them cause increase (Araie and Takase, 1981; Coakes and Siah, 1982) and some of them cause a decrease (Paterson and Paterson, 1972; Wettrell et al., 1977) in AH formation in man. At present there is no explanation of these variable effects.

As expected, 10 nmol bolus dose of timolol produced no effect on CE cyclic AMP content. Phylactos (1986) showed that timolol in moderate concentration (10^{-7} to 10^{-5} M) could not inhibit adenylate cyclase activity in rabbit iris-ciliary body membrane rich fraction but higher concentrations (10^{-2} to 10^{-4} M) had some inhibitory effect. An antagonist can abolish the effect of an agonist but itself does not usually produce any effect. Thus failure of a small dose of timolol to inhibit adenylate cyclase activity, particularly in a preparation which does not have any adrenergic tone, is quite understandable. The same dose produced a significant reduction of AH formation in the preparation indicating that a mechanism other than antagonism at β-adrenoceptor is involved.

More surprisingly, terbutaline also failed to raise ciliary cyclic AMP levels at the same dose of 30 nmol which produced a significant reduction of AH formation rate. Even at a higher dose level (100 nmol) terbutaline was entirely ineffective in stimulating ciliary cyclic AMP accumulation. It is, however, impossible to relate bolus doses to the concentrations with any accuracy. Perfusion of drug instead of bolus dose would answer this question. When excised ciliary processes from fresh eyes were incubated with terbutaline, it produced a concentration-dependent increase in cyclic AMP with concentrations of 10^{-6},

10^{-5} and 10^{-4} M. The same concentrations also produced more pronounced and concentration-dependent increases in cyclic AMP in cultured 1st passage CE cells. With the largest concentration used (10^{-4} M), there occurred about 800% increase in ciliary cyclic AMP. As expected, the baseline value was also higher (48.9 pmol.mg^{-1} protein) compared to the scraped and excised ciliary processes where the baseline values were 9.8 and 9.4 pmol.mg^{-1} protein respectively. In the case of scraped and excised ciliary processes, the lower baseline value of cyclic AMP per unit of TCA-precipitated protein can be reasonably explained as the contamination of CE cells by the underlying connective tissue. Connective tissue is likely to have a higher proportion of proteins than epithelial cells. There is no published data regarding the cyclic AMP content of bovine CE. A baseline value of 9.4 pmol.mg^{-1} of protein has been reported in excised ciliary processes of rabbit (Bausher et al., 1987). A basal value of 32.2 pmol.mg^{-1} protein in rabbit iris-ciliary body has been reported by Bartels et al. (1980). Thus the baseline values obtained in scraped and excised ciliary processes as well as in cultured CE of the present experiment are in good agreement with the reported values.

Bausher et al. (1987) found that isoprenaline, produced a biphasic dose-response curve for cyclic AMP accumulation; stimulation at low concentration (0.1 to 1.0 μM) and inhibition at high concentration (10 to 1000 μM). A similar biphasic response was also observed with terbutaline, a relatively more β_2-selective agonist, but the concentrations of agonist required for stimulation of and for inhibition of cyclic AMP production were shifted to higher values. Increase in aqueous cyclic AMP concentration in rabbit by terbutaline (Rowland and Potter, 1979) and isoprenaline (Bausher et al., 1987) and in bovine CE (Elena et al., 1984a, b) has been reported. Stimulatory effects of salbutamol on adenylate cyclase in the rabbit iris-ciliary body homogenate and its suppression by timolol

was also reported (Phylactos, 1986). On the basis of the presence of predominantly β_2-adrenergic receptors in the CE of man and animals (Nathanson, 1980, 1981b; Mittag and Tormay, 1985a) including bovine (Elena et al., 1984a, b; Polansky et al., 1985), these increases in cyclic AMP by the β-adrenoceptor agonists and inhibition by the antagonist, timolol, seem quite straightforward and unsurprising. However, if we seek relevance of such changes in adenylate cyclase/cyclic AMP to IOP and AH formation then the picture suddenly becomes much more complex. The available literature in this respect is very conflicting. Some studies have shown that an increase in cyclic AMP is associated with a decrease in AH flow and IOP (Gregory et al., 1981; Caprioli et al., 1984; Sears, 1985; Bartels et al., 1987; Mittag et al., 1993); some showed an increase in cyclic AMP causes an increase in AH inflow and IOP (Nathanson, 1981; Miichi and Nagataki, 1983; Rowland et al., 1986; Larson and Brubaker, 1988; Nilsson et al., 1990; Yoshitomi et al., 1991) and still others showed no relevance (Rowland and Potter,1979; Schmitt et al., 1981; Sears, 1984; Liu and Dacus, 1991).

In the present experiments failure to stimulate cyclic AMP accumulation in perfused eyes with 30 and 100 nmol of terbutaline, might be due to inadequate drug concentration. However, in the bovine excised ciliary processes and cultured CE the minimum concentration of terbutaline required to stimulate cyclic AMP accumulation was 10^{-6}M compared to 10^{-4}M in the case of excised ciliary processes of rabbit (Bausher et al.,1987). Because bolus doses of terbutaline were used in the perfused eye, we did not know the exact concentration of drug in the vicinity of the CE. However, the lower dose (30 nmol), also in bolus injection, produced marked reduction in AH formation. Thus it appears that terbutaline can stimulate cyclic AMP production quite efficiently in higher concentration, but the concentration which is needed to

reduce AH formation is much less. This indicates that there is no causal relationship between terbutaline's effect on AH formation and its ability to stimulate ciliary cyclic AMP production in the bovine perfused eye.

Forskolin has been shown to cause marked increase in cyclic AMP concentration in the AH, prolonged reduction of IOP and decreased AH formation in vivo in rabbit (Bartels et al., 1987). Decreased AH formation was also reported in cynomolgus monkeys (Lee et al., 1984a) and in human (Burstein et al.,1984). In vitro forskolin activates adenylate cyclase of crude particulate homogenates prepared from cultured human CE or from dissected CE processes of rabbit or human eyes (Caprioli et al., 1984). In the isolated bovine eye preparation it produced a highly significant increase in ciliary cyclic AMP in a bolus dose of 30 nmol. The same dose, however, completely failed to affect the AH formation rate in the preparation thus providing further evidence that cyclic AMP content of CE has no relevance to the AH formation rate in the bovine perfused eye. To confirm this result, we used a 100 nmol bolus dose of 8-bromo cyclic AMP in an effort to influence AH formation. It also failed to influence the AH formation rate in the preparation.

Timolol, a classic β-adrenoceptor antagonist, which is well known for its ocular hypotensive effect, and powerful β-antagonistic effect, also produced a significant reduction of AH formation in the isolated perfused bovine eye. Timolol, as would be expected, was found to have no effect on CE cyclic AMP content. Thus in the present study we manipulated either AH formation or ciliary cyclic AMP by using four different drugs, yet found no correlation between the AH formation and ciliary cyclic AMP content.

In the isolated eye preparation there is no endogenous β-adrenergic agonist, such as noradrenaline or adrenaline, which are released from the sympathetic nerve endings and adrenal medulla in vivo. By definition, an antagonist would be expected to have no effect in the absence of an agonist. Thus timolol suppression of AH flow in the isolated eye preparation is further evidence of a mechanism other than antagonism of the conventional β-adrenoceptor. There is a considerable body of evidence that timolol decreases AH formation by some mechanism other than β-adrenergic blockade (Liu and Chiou, 1981; Watanabe and Chiou, 1983; Keats and Stone, 1984; Chiou et al., 1985; Yorio, 1985; Mills et al., 1988). Studies with specific β_1 and β_2-adrenergic agonists and antagonists (Chiou et al., 1985) showed that the adrenergic receptor mechanism does exist in the eye tissue but that it does not control IOP or AH formation. Recent studies in rabbits by Liu and Dacus (1991) also refute the involvement of β-adrenoceptor activity and of cyclic AMP in the circadian fluctuation of IOP. Santafe et al. (1993) showed that in the conscious rabbit, both isoprenaline and timolol reduced IOP independently by reducing AH formation. When they used different doses of isoprenaline in the presence of a fixed concentration of timolol, the dose-response curve for isoprenaline showed a synergistic action of the two drugs. This strongly supports the view that timolol and isoprenaline both act, wholly or at least in part, through mechanisms unrelated to conventional β-adrenoceptors in the ciliary processes.

The above discussion makes it clear that there is no simple causal relationship between the ability of these various drugs to alter gross cyclic AMP levels in the CE and their ability to decrease AH formation in the bovine eye.

In contrast to this conclusion, when we used the specific inhibitors of cyclic AMP-dependent protein kinase (PK_A) to blunt the AH and IOP-reducing effect

of terbutaline we got results which suggest that cyclic AMP-stimulated protein kinase is involved in the mechanism by which terbutaline lowers AH formation and IOP. When a 30 nmol dose of terbutaline and a 10 nmol dose of the specific PK_A inhibitor (KT-5720) were used together, terbutaline failed to reduce both AH formation and IOP. On the other hand when we tried specific cyclic GMP-dependent protein kinase inhibitor (PK_G, KT-5823) with terbutaline, we found that PK_G inhibitor had no effect on terbulatine's IOP-lowering effect. This suggests that terbutaline does not reduce AH formation and IOP by increasing cyclic GMP levels in CE, which is in contrast to the report by Rowland and Potter (1979) who found a significant increase in cyclic GMP in AH. However, they admitted that due to experimental error, they could only estimate cyclic GMP in 4 of their samples. Thus their experimental data is not very reliable. Also cyclic GMP levels in the AH may not necessarily reflect the cyclic GMP levels in the CE cells. Nevertheless, our finding that terbutaline can still lower IOP in the presence of PK_G inhibitor, constitutes a form of control result which strengthens the inpact of the experiment in which the terbutaline's effect was blocked by the PK_A inhibitor. Thus, in the present investigation, only inhibition of PK_A was effective in blocking terbutaline's effect on AH formation and IOP. However, in view of the number of experimental results which show no relevance of cyclic AMP to AH formation, this single piece of experimental evidence cannot be used to reverse the conclusion. Further experiments to this end are necessary.

There may be other explanations for the effect of the PK_A inhibitor on the terbutaline response. It may be that the increase in cyclic AMP by terbutaline which is capable of influencing AH dynamics is so tiny that it could not me measured by the RIA used in the present experiments. There is also possibility of the existence of functional compartmentalization of cyclic AMP in bovine CE,

as reported in other tissues. For example, although it is generally accepted that activation of steroid synthesis in Leydig cells is mediated through the accumulation of intracellular cyclic AMP in response to luteinizing hormone/chorionic gonadotropin, there are discrepancies in the dose required to stimulate steroid biosynthesis or to activate the accumulation of cyclic AMP (Hunzicker-Dunn and Birnbaumer, 1985; Themmen et al., 1985; Sullivan and Cooke, 1986). It has also been demonstrated that there was no association between cyclic AMP accumulation and steroid synthesis in response to low levels of adrenocorticotropic hormone in isolated adrenal cells (Beall and Sayers, 1972; Sayers et al., 1972). Thus steroid accumulation can be increased by levels of tropic hormone which results in no increase in intracellular cyclic AMP. It was postulated that a functional compartmentalization or coupling of cyclic AMP generated through tropic hormone receptors to specific cyclic AMP dependent protein kinases occurs in the cells (Mendelson et al., 1975; Dufau et al., 1978) Douglas and Chaudhary (1990) showed in mouse Leydig tumour cells that cyclic AMP generated via human chorionic gonadotropin stimulation, whilst able to generate similar amounts of progesterone, does not stimulate the synthesis of the same proteins as does cyclic AMP added exogenously or generated through indiscriminate activation of adenylate cyclase. Similar functional compartmentalization of cyclic AMP has been reported in rat hepatocytes (Yamatani et al., 1987), where two types of hormone-sensitive (glucagon and secretin sensitive) adenylate cyclase were demonstrated and the suppressive effect of insulin is specific only for the glucagon-sensitive adenylate cyclase. It may be that in the bovine CE the cyclic AMP synthesis stimulated by forskolin or the exogenous cyclic AMP analogue has no effect on AH secretion whereas the cyclic AMP stimulated by terbutaline, although not detectable in the present experiment, is an important modulator of AH formation.

It is also possible that there is a critical area within the CE cell which controls the function of the ion-transport mechanism upon which AH production relies; one might postulate that a significant change in cyclic AMP level within those critical areas may be enough to alter AH formation, yet may be so small in relation to the total cyclic AMP content of the cell that no overall rise in cyclic AMP is measurable.

It has long been known that cyclic AMP modulates cell function in various tissues by activating specific protein kinases. Attempts to identify the proteins phosphorylated in CE have not yielded information about how AH flow might be modified via protein kinases. It is known by histochemical studies that both adenylate cyclase and Na,K-ATPase are located in the same membrane component of the rabbit ciliary processes (Palkama and Uusitalo, 1970; Uusitalo and Palkama, 1970; Tsukahara and Maezawa, 1978; Sweadner and Goldin, 1980; Palkama et al., 1981) and both enzymes participate in the formation of AH (Bonting and Becker, 1964; Simon et al., 1962; Uusitalo et al., 1982). The interdependence of the enzymes has been emphasized by Uusitalo et al. (1985) who observed that the activity of adenylate cyclase in the iris-ciliary body was stimulated, whereas that of Na,K-ATPase was reduced, in response to intravenous injection of ouabain. This has been strongly supported by the recent findings that Na,K-ATPase activity was reduced by more than 50% by the catalytic subunit of PK_A (Delamere et al., 1990). The same study also showed that neither cyclic AMP nor the enzyme alone altered Na,K-ATPase activity. Subsequently, it has been shown that preincubation of CE with forskolin plus isobutylmethylxanthine also reduced ouabain-sensitive ATPase activity (Delamere and King, 1992). Indirect immunofluorescence (Coca-Prados and Lopez-Briones, 1987) as well as histochemical and ultrahistochemical (Eichhorn et al., 1990) studies showed that basolateral surfaces of both non-

pigmented and pigmented CE cells of bovine eye contain Na,K-ATPase. Adenylate cyclase activity in the bovine pigmented and non-pigmented CE cells has been demonstrated (Elena at al., 1984a). Thus similar interdependence of adenylate cyclase and Na,K-ATPase may also play a role in the bovine eye.

These findings are compatible with the idea that terbutaline may decrease AH formation via a cyclic AMP-mediated mechanism involving PK_A, though it would seen that in the bovine eye this occurs at concentrations of terbutaline too low to induce any measurable change in cyclic AMP content of the ciliary processes.

The above conclusion still leaves a large question mark over the mechanism of timolol.

Atriopeptin and AH Formation

Previous studies showed that atriopeptin (AP) lowered IOP in rabbits (Mittag et al., 1987a; Nathanson, 1987), glaucoma patients (Diestelhorst and Krieglstein, 1989) and in the bovine arterially perfused eye (Millar et al., 1990). AP-activated guanylate cyclase activity has been demonstrated in the ciliary processes of the eye (Nathanson, 1987; Nathanson et al., 1987; Fawcett and Wilson, 1989). Activators of soluble guanylate cyclase such as nitroglycerine, sodium nitroprusside etc. have been shown to reduce IOP (Nathanson, 1988b, 1992). Topical application of 8-bromo cyclic GMP, a membrane permeable cyclic GMP analogue, also lowers IOP in rabbits (Becker, 1990), although Busch et al. (1992) found that subconjunctival, but not topical, cyclic GMP reduced IOP in the rabbit. Evidence that this fall in IOP is due to a reduction in AH formation is scanty. Neither the reported increase in cyclic GMP in iris-ciliary

body (Korenfeld and Becker, 1989) nor in the ciliary process homogenates (Nathanson, 1987) localizes this effect to the secretory epithelium. The guanylate cyclase involved could have been located in the vascular tissues within the ciliary process.

In the present study we showed that in the bovine perfused eye, AP lowers IOP by reducing AH formation. We also showed that AP increases cyclic GMP levels in cultured CE cells in a concentration-dependent manner. Culture of CE offers the opportunity to localize receptors. Thus the present investigation advances our knowledge of AP that it acts directly to reduce AH formation to an extent which is consistent with its much-reported ability to lower IOP; that AP receptors are present on cultured CE cells; and that these AP receptors are coupled to the guanylate cyclase/cyclic GMP system.

In the isolated bovine eye preparation, a very moderate dose of AP (50 pmol) produces a marked reduction (38%) in AH formation over a period of 60 min. This finding confirms the observation of Korenfeld and Becker (1989) in the living anaesthetized rabbit, who found a 20% reduction in AH formation following intravitreal injection of APIII, over a period of 80 min. The decreased response in their experiment may be due to intravitreal administration of drug. Intra-arterial administration in our preparation is probably the more physiological, since AP that reaches the eye most likely originates in the atrial myocytes. Although extra-atrial production of AP has been reported, such as in the kidney, adrenal medulla, pituitary (McKenzie et al., 1985; Gardner et al., 1986) no mRNA transcripts of AP were found in the anterior uvea (Stone, 1987). The quantifiable levels of AP found in anterior uvea (Stone and Glembotski, 1986) probably represent ocular tissue binding of cardiac APs. Decreased response may also be due to the markedly depressed baseline AH formation in

animals after systemic anaesthesia. A species difference could also account for the greater response in the bovine eye. For example, in the cynomolgus monkey AP causes an increase rather than a decrease in AH formation (Samuelsson-Almen et al., 1991).

It has been reported that intravenous infusion of AP in the living animal causes a fall in mean arterial blood pressure (Samuelsson-Almen et al.,1991) which may trigger an activation of the sympathetic nervous system. An effect of AP on AH dynamics in the living animal thus may be secondary to β-adrenoceptor activation and hence an increase in cyclic AMP levels in the target tissue. In the isolated eye preparation the ciliary body is devoid of sympathetic nervous tone and other systemic effects. Thus a decrease in AH formation in the isolated eye supports the theory that activation of ciliary guanylate cyclase is the main mechanism of AP in lowering IOP.

Several investigators (Bianchi et al., 1986; Sugrue and Vlader, 1986) suggested that AP receptors in the rabbit ciliary process are negatively coupled to adenylate cyclase. Negative coupling of AP receptors has also been demonstrated in other tissues, such as in adrenal cortical membrane (Anand-Srivastava et al., 1985) and in pituitary tissue (Anand-Srivastava et al., 1985). Bianchi et al. (1986) argued that the decrease in IOP is due to decreased levels of cyclic AMP which is effected secondary to increased cyclic GMP in response to AP. In contrast to their result, Mittag et al. (1987a) were unable to demonstrate any effect of AP on ciliary process adenylate cyclase activity in the same homogenates or particulate preparations where it activated guanylate cyclase. They showed that particulate cyclic AMP phosphodiesterase activity was stimulated by low doses (1 - 5 μM) of cyclic GMP in the ciliary process. Thus, AP, acting via a guanylate cyclase, has the potential to regulate phosphodiesterase activity and indirectly decrease

cyclic AMP levels in ciliary process. However, in the bovine eye, we have convincingly shown that cyclic AMP levels in the CE bear no relationship with AH formation. So a decrease in ciliary cyclic AMP is unlikely to be the cause of reduction of AH formation in the bovine isolated eye in response to AP. Increased cyclic GMP production by AP appears to be the most likely mediator of reduction in AH secretion. Also the concentration-dependent increase in cyclic GMP in cultured CE cells suggest that AP may act locally on the secretory cells to modify AH secretion via a cyclic GMP pathway perhaps by activating cyclic GMP-dependent protein kinase, particularly in view of the evidence involving azide and the selective inhibition of PK_G (see below).

Sodium Azide, IOP and AH Formation

In the isolated bovine eye preparation Millar and Wilson (1991) showed that a 10 nmol bolus dose of sodium azide caused a significant increase in ciliary cyclic GMP and a significant reduction in IOP. The same dose was shown to have no effect upon the ciliary vasculature (Millar and Wilson, 1991). They postulated that sodium azide might have reduced IOP by direct reduction in AH formation and that the ocular effect did not bear any relationship to its vascular effect. Other nitrovasodilators, such as nitroglycerin and sodium nitroprusside, which stimulate cyclic GMP synthesis by a family of soluble NO-activated guanylate cyclases (Chinkers and Garbers, 1992; Yuen and Garbers, 1992; Wong and Garbers, 1992) have also been shown to lower IOP (Nathanson, 1988b, 1992). That 8-bromo cyclic GMP, a membrane-permeable analogue, lowers IOP (Becker, 1990) suggests that cyclic GMP mediates the ocular hypotensive effect irrespective of whether its accumulation in the target tissue is stimulated either by AP receptors, which results in hormonally stimulated particulate guanylate cyclase or by activators of soluble guanylate cyclase, such as by

nitrovasodilators. Despite this apparent concordance, there are conflicting data in the literature, e.g. Nathanson (1992) concluded that nitroglycerin's site of action was at the outflow apparatus, not the ciliary process.

To address this, we investigated sodium azide for its effect on AH formation as well as on IOP. A specific inhibitor of cyclic GMP-dependent protein kinase (PK_G) was also used to blunt the IOP-lowering effect of sodium azide. Sodium azide is reported to directly stimulate an intracellular soluble guanylate cyclase as opposed to particulate guanylate cyclase stimulated by AP. It also promotes the release of the endothelial derived relaxing factor - EDRF (Furchgott and Zawadski, 1980). EDRF was subsequently identified as nitric oxide (Palmer et al., 1987). EDRF also stimulates an intracellular soluble guanylate cyclase.

A 10 nmol bolus dose of sodium azide produced a marked decrease in AH formation (46%) and IOP. A 10 nmol dose of PK_G inhibitor completely abolished the IOP-lowering effect of 10 nmol of sodium azide. To our satisfaction, when we used a 10 nmol bolus dose of specific PK_A inhibitor, we found that it completely failed to abolish the IOP-lowering effect of sodium azide. Thus it appears that activation specifically of PK_G is the mechanism of the reduction in IOP and AH formation in the isolated bovine eye.

Thus all experiments using AP and sodium azide indicate that they lower IOP by reduction of AH formation. This contradicts the previous findings that the physiological effects of AP and nitrovasodilator drugs in the eye differ in their mode of action - AP causes a decrease in secretion (Steardo and Nathanson, 1987; Korenfeld and Becker, 1989) with little effect on outflow, whereas the nitrovasodilators cause only a small effect on AH formation but a definite enhancing effect on outflow (Nathanson, 1992). A 46% reduction of AH flow

by 10 nmol sodium azide over a 60 min period undoubtedly proves that it lowers IOP by affecting secretion of AH. This difference in finding is very difficult to explain. It may reflect the difference of distribution of nitrovasodilator-sensitive guanylate cyclase in ocular tissues in different species. Predominant occurrence of such guanylate cyclase in the bovine CE cells may be the cause of marked reduction in AH formation due to sodium azide. Similarly predominant distribution of nitrovasodilator-sensitive guanylate cyclase in the rabbit trabecular epithelium may underly their effect on outflow of AH.

Verapamil and IOP

In the present study, intra-arterial administration of verapamil, a widely used Ca^{2+}-channel blocker, produced a dose-dependent decrease in IOP in the isolated perfused bovine eye. Reduction of IOP by topical application of verapamil in untreated ocular hypertensive volunteers has been reported (Abelson et al., 1988; Goyal et al., 1989). Topical verapamil and nifedipine lower IOP in conscious normotensive rabbits (Segarra et al., 1986). Intravenous perfusion of verapamil reduces IOP in anaesthetised rabbits and recently intravenous verapamil and nifedipine have been shown to lower IOP in conscious rabbits (Payne et al., 1990). All these findings are in agreement with our results. In contrast, the results of Beatty et al. (1984) in conscious rabbit and human suggest that topical verapamil, diltiazem and nifedipine increase IOP in both species. Monica et al., (1983) reported that oral nifedipine reduces IOP in the human, but Kelly and Wally (1988) found no effect of either oral or intravenous nifedipine on IOP in normal subjects. Schnell (1975) also showed that intravenous and oral nifedipine had no effect on IOP in normal volunteers or glaucomatous patients. Thus, the literature on ocular effects of verapamil and other Ca^{2+}-channel blockers is conflicting as well as scarce. Part of this

variability may result from the variable doses and routes of administration utilized by different workers. With such conflicting data in the literature, a dose-dependent reduction in IOP in the isolated eye preparation by small doses (1 to 100 nmol) of verapamil, is interesting and deserves further work to determine whether the effect is on AH formation.

The mechanism of ocular hypotensive effect of verapamil is, however, very difficult to explain, since the ocular effects of Ca^{2+}-channel blockers on AH formation is lacking and on outflow is scanty.

Recently it has been demonstrated that sympathetic nerve terminals in the rabbit ciliary body contain verapamil-sensitive channels which contribute to neurotransmitter release (Jumblatt, 1988), and thus suggesting an indirect means by which these drugs may influence IOP. In the isolated eye preparation such release of neurotransmitter would be very limited and can not account for a sustained reduction of IOP over a period of 2 h in response to a bolus dose used.

So far, the only important pharmacological effects of Ca^{2+}-channel blockers are confined to the cardiac and smooth muscles. The primary mechanism of action of this class of drugs is the blockade of membrane-bound calcium channels and an inhibition of extracellular Ca^{2+} influx. Ca^{2+} is an important intracellular messenger, often interacting with cyclic nucleotides to control a broad spectrum of physiological functions (Berridge, 1975) and is critical to many biological processes including muscle contraction, neurotransmitter release, enzyme activation and cellular secretion. For example, decreased intracellular free Ca^{2+} causes relaxation of smooth muscle and in vascular smooth muscle this causes vasodilatation and improved blood flow (Piepho, 1983).

Extrapolating from the action of Ca^{2+} in smooth muscle, we can argue that in the eye verapamil may cause relaxation of the ciliary muscle as a result of a reduction in intracellular Ca^{2+}. Relaxation of ciliary muscle should have produced relative closure of the trabecular meshwork, and thus hindered the AH drainage through the trabecular route. Decreased drainage and normal or increased AH formation can in no way produce decreased IOP. Thus it seems that the dose-dependent decrease in IOP in the isolated bovine eye is likely to be due to a reduction of AH inflow. Direct measurement of AH flow using verapamil and other Ca^{2+}-channel blockers might confirm this hypothesis (see future work).

There is little information available on the possible roles of Ca^{2+} in the CE. But presence of a calcium signalling system has been confirmed in the cultured nontransformed human and rabbit non-pigmented CE cells (Ohuchi et al., 1992). Although little information is available, preliminary data on the regulatory role of Ca^{2+} in the ciliary processes point to the possibility that the drugs which influence Ca^{2+} mobilization might affect the AH inflow by acting on electrolyte transport. For example, gap junctions have been described between non-pigmented and pigmented CE cells (Green et al., 1985). AH formation possibly could be modified by ion transport through the gap junctions between CE cells, which are known to be regulated by calcium ions (Loewenstein, 1981). Abelson et al. (1988) suggested that verapamil may interfere with these gap junctions, altering cellular permeability of the CE, and thus inhibiting the normal AH inflow.

It has been demonstrated that rabbit ciliary process membranes contain a calcium/calmodulin-regulated adenylate cyclase which is different from the adenylate cyclase regulated by stimulatory GTP-binding proteins, and thus

receptors which affect calcium mobilization may have the potential to regulate cyclic AMP levels in the ciliary processes and ciliary processes transport function (Tormay et al., 1987; Mittag et al., 1993). Therefore, calcium channel blockers may also alter the cyclic AMP content via an effect on calcium in the CE cells, and in this way decrease both AH inflow and IOP.

Compared to the cyclic AMP-dependent system, relatively little attention has been paid to calcium signal regulation with regard to electrolyte transport in the CE. However, Yoshimura et al (1989) has shown that rabbit ciliary process membranes contain a much higher activity of protein kinase C than of PK_A or other protein kinases. Although the physiological role of calcium-dependent protein phosphorylation has not been fully elucidated, the data of Yoshimura et al. (1989) suggest that drugs acting via calcium-dependent signal systems may exert a significant degree of control on cellular function in the ciliary process.

In conclusion, it appears that verapamil and other Ca^{2+}-channel blockers may have multiple sites and mechanisms of action in the eye through which they could affect AH dynamics. Thus extensive studies on this group of drugs is desirable to ascertain accurately their mechanism of action (see future work).

FUTURE WORK

FUTURE WORK

From our experimental data on IOP, AH formation and CE cyclic GMP, we postulated that both AP and sodium azide lower IOP by reducing AH formation and that these drugs act through cyclic GMP/guanylate cyclase system. Further work with other nitrovasodilators, such as sodium nitroprusside, nitroglycerine on both physiological (IOP, AH formation etc.) and biochemical (cyclic GMP levels of CE) parameters may confirm our hypothesis.

Results of experiments with timolol, terbutaline, forskolin, 8-bromo cyclic AMP and specific inhibitors of cyclic nucleotide-dependent protein kinases, on their effects on IOP, AH formation and cyclic AMP levels in CE cells, using three different types of tissue preparations (perfused eye, excised ciliary processes from fresh eyes and cultured CE), made it clear that there is no direct relationship between CE cyclic AMP content and AH formation or IOP. Our results suggest a functional compartmentalization of cyclic AMP in CE cell function. Further work in this respect with a large variety of agents which can activate the enzyme adenylate cyclase, such as catecholamines, glycoprotein hormones, organic fluorides, griseolic acid etc. might help to confirm this hypothesis.

We have confirmed, by incubating cultured CE cells with terbutaline and atriopeptin, that these cells retain their surface receptors. Jacob (1991) identified T-type Ca^{2+}-channel in bovine CE cells. Yoshimura et al (1989) showed that rabbit ciliary process membranes contain much higher activity of PK_C than PK_A and other kinases. We found a dose-dependent decrease in IOP by moderate bolus doses (1 to 100 nmol) of verapamil in the isolated arterially perfused eye. Thus Ca^{2+} may play an important role in CE function of AH

secretion. Future work involving microscale measurement of intracellular free Ca^{2+}, using a sensitive method, such as the fura-2 technique, described by Morgan and Morgan (1982) or Buchan and Martin (1992), would be a logical extension of the present work. Measurement of intracellular free Ca^{2+} in response to different Ca^{2+} channel blockers and other drugs having Ca^{2+} mobilizing ability, in cultured CE cells, and relating these biochemical events to their physiological effect, such as IOP and AH formation, would provide precise information about the role of Ca^{2+} in CE cell function.

More precise information concerning the concentration of drug actually present at the site of action, i.e. in the vicinity of CE, which is responsible for modulating physiological functions, would be obtained by administering the ocular hypotensive drugs at a fixed concentration in the perfusate, instead of bolus doses.

The CE appears to be a very complex functional entity and a site for a number of biochemical systems operating to modulate its function, such as the presence of cyclic AMP, cyclic GMP, Ca^{2+} messenger systems, serotonergic (Barnett and Osborne, 1993) and purinergic receptors (Wax et al., 1993) etc. Identification of calmodulin-activated adenylate cyclase in rabbit ciliary process (Mittag et al., 1993) and potentiation of agonist-induced stimulation of cyclic AMP by activation of PKC in cultured rat retinal PE (Nash et al., 1994), which is embryologically a close relative of CE, indicates some sort of cross-talk between different systems. Thus a future approach in studying the cross-talk between and among different biochemical systems could provide important information about the complex and poorly understood mechanisms of modulation of AH dynamics.

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APPENDIX

Appendix I

List of Abbreviations used in this thesis:

Abbreviation	Definition
ADP	Adenosine Diphosphate
AH	Aqueous Humour
ANOVA	Analysis of Variance
AP	Atriopeptin
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase
CAG	Closed-angle glaucoma
CE	Ciliary Epithelium
COAG	Chronic Open-angle Glaucoma
Cyclic AMP	3'5' Cyclic Adenosine Monophosphate
Cyclic GMP	3'5 Cyclic Guanosine Monophosphate
°C	Degrees Celcius
DAG	Diacylglycerol
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulphoxide

DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
fmol	femtomoles
g	Grammes
G-protein	Guanine Nucleotide-Binding Protein
GABA	Gamma Amino Butyric Acid
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
h	Hours
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IOP	Intraocular Pressure
IP_3	Inositol 1,4,5-Trisphosphate
Iu	International Unit
KDa	Kilodalton
M	Molar
mg	Milligrammes
min	Minutes
ml	Millilitres

mm	Millimetres
mM	Millimolar
mmH_2O	Millimetres of Water
mmHg	Millimetres of Mercury
mol	Moles
mV	Millivolts
μg	Microgrammes
μl	Microlitres
NCS	Newborn Calf Serum
ng	Nanogrammes
nmol	Nanomoles
NPE	Non-pigmented Ciliary Epithelium
OAG	Open-angle Glaucoma
PA	Phosphatidic Acid
pA	Picoamperes
PC	Phosphatidylcholine
PE	Pigmented Ciliary Epithelium
PIP	Phosphatidyl inositol 4-phosphate
PIP_2	Phosphoinositol 4,5-bisphosphate
PK_A	Cyclic AMP-dependent Protein Kinase
PK_C	Protein Kinase C

PK_G	Cyclic GMP-dependent Protein Kinase
PLA_2	Phospholipase A_2
PLC	Phosphilipase C
pmol	Picomoles
PPI	Polyphosphoinositides
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
s	Seconds
SEM	Standard Error of Mean
TCA	Trichloroacetic Acid
TNO	Tri-n-Octylamine

Appendix II

Materials Used:

Material	Source
Atriopeptin II (Rat sequence)	Sigma Chemical company
KT-5720 (PK_A inhibitor)	Calbiochem Corporation
KT-5823 (PK_G inhibitor)	Calbiochem Corporation
Collagenase A	Boehringer Mannheim
Gentamycin	Sigma (Cell Culture)
Dimethyl Sulfoxide(DMSO)	Sigma Chemical Company
Foetal Calf Serum	GIBCO, Life Technologies Ltd.
Newborn Calf Serum	GIBCO, Life Technologies Ltd.
Trypsin EDTA solution (1X)	GIBCO
DMEM	GIBCO
Verapamil Hydrochloride	Sigma Chemical Company
Timolol Maleate	Sigma Chemical Company
MK-927	Merk Sharp and Dohme Res. Lab.
Sodium Azide	BDH Chemicals Ltd.
Terbutaline Hemi-sulphate	Sigma Chemical Company
Cyclic AMP and cyclic GMP RIA Kit	DuPont, New England Nuclear
Forskolin	Sigma Chemical Company
8-bromo Cyclic AMP	Sigma Chemical Company

Appendix III

Solutions Used:

1. Solutions for Protein Assay

Solution C	98 parts of Solution A 2 parts of Solution B

These are prepared fresh and mixed just before use.

Solution A

Sodium Carbonate	2 g
Sodium Hydroxide (0.1 N)	add 100 ml.

Solution B

Cupric Sulphate	0.5 g
Sodium-potassium Tartrate	1.0 g
Distilled water	add 100 ml.

2. Buffers Used for Cell Culture

(a) X 10 Concentration of Ca^{2+}-free buffer

41.5 g NaCl
2.5 g KCl
12.0 g Hepes, dissolved in 500 ml of distilled water, pH adjusted to 7.4.

(b) X 10 Concentration of Collagenase Buffer

9.75 g NaCl
1.25 g KCl
6.00 g Hepes, dissolved in 250 ml distilled water, pH adjusted to 7.6
Add 12 ml of 1M Ca_2Cl, check pH again.