ADENYLYL CYCLASE: MEDIATOR OF THE INTRAOCULAR PRESSURE RESPONSE TO ADRENERGIC AGENTS

Experimental studies with a phosphodiesterase-inhibitor

Promotie commissie

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(ADENYLAAT CYCLASE: MEDIATOR VAN INTRAOCULAIRE DRUK EFFECTEN VAN ADRENERGE STOFFEN

Experimentele studies met een fosfodiesterase-remmer)

PROEFSCHRIFT

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Cover: Schematic representation for the proposed mechanism by which adrenaline and combinations between a selective α_1 and β_2 -adrenergic agonist reduce the intaocular pressure (see epilogue of this thesis, p 117).

Aan mijn ouders

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CONTENTS

Abbreviations

AC	adenylyl cyclase
AH	aqueous humour
ARVO	Association for Research on Vision and Ophthalmology
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BAB	blood aqueous barrier
cAMP	adenosine 3',5'- cyclisch monophosphate
cGMP	guanosine 3',5'-cyclisch monophosphate
CGRP	calcitonin-gene-related-peptide
C-value	coëfficient of outflow facility (expressed in $\mu l.min^{-1}.mmHg^{-1}$)
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DPE	dipivalyl epinephrine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(ß-aminoethylether)-N,N,N',N'-tetraaceticacid
EPI	epinephrine
EP-receptor	prostaglandin E receptor
FSK	forskolin
GDP	guanosine 5'-diphosphate
GDPßS	guanosine 5'-O-(2-thiodiphosphate)
G_i	inhibitory G-protein
G_s	stimulatory G-protein
GTP	guanosine 5'-triphosphate
HSA	human serum albumin
IBMX	3-isobutyl-1-methylxanthine
IBMX-AT	3-isobutyl-1-methylxanthine dissolved in 0.5% hydroxypropyl-methylcellulose (artificial tears)
IBMX-CD	3-isobutyl-1-methylxanthine dissolved in saline containing 2% ß-cyclodextrin
IBMX-ED	3-isobutyl-1-methylxanthine-ethylenediamine
IOP	intraocular pressure
IP_3	inositol triphosphate
ISO	isoproterenol
MABP	mean arterial blood pressure
NE	norepinephrine
NPE	non-pigmented epithelium (of ciliary processes)
NPY	neuropeptide Y
PBA	phenoxybenzamine
P_e	episcleral venous pressure
PG(s)	prostaglandin(s)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TM	trabecular meshwork
TML	timolol
VIP	vasoactive intestinal peptide

CHAPTER 1

GENERAL INTRODUCTION

Treatment of glaucoma focusses on reducing intraocular pressure (IOP) to prevent the loss of neuroretinal nerve fibres. The conservative management of glaucoma is directed toward pharmacological manipulation of the mechanisms that regulate intraocular pressure (IOP). The adrenergic nervous system is believed to play a major role in the maintenance of IOP homeostasis and agents that influence this system have been the subject of investigation for decades. Adrenergic antagonists, such as timolol, levobunolol and betaxolol, and adrenergic agonists, such as epinephrine (Diopine^{R}), have become pillars of glaucoma treatment.

The receptor concept is fundamental to adrenergic pharmacology. The binding site of a receptor recognizes a specific endogenous hormone that initiates a sequence of intracellular events, i.e. signal transduction. The signal transduction system, which is directly coupled to the receptor, can generate a "second messenger" molecule that evokes specific cellular responses. A few distinct second messenger systems have been proposed. Adenylyl cyclase (AC) is the most well-known second messenger system, which generates cyclic adenosine monophosphate (cAMP) as second messenger molecule. Adrenergic receptors have been subdivided into alpha and beta subclasses by Ahlquist (1948), and each subclass in turn is divided into two subtypes. AC has been found to be directly coupled to the β_2-adrenergic complex in a variety of tissues and species.

The AC second messenger system has long been considered to play an impotant role in the regulation of aqueous humour secretion by ciliary processes. Increasing evidence suggests that AC is also important for regulation of the outflow facility. Several drugs and agents that act on receptors coupled to AC in various tissues, e.g. ß-adrenergic agents such as epinephrine, affect the IOP, aqueous humour production and outflow facility.

This thesis focusses on the role of the AC/cAMP system in the response to adrenergic agents that reduce IOP.

The principal approach was to amplify the AC/cAMP signal of adrenergic agents pharmacologically by combination with the phosphodiesterase-inhibitor 3-isobutyl-1-methylxanthine (IBMX). IBMX is known to inhibit the activity of the enzyme

phosphodiesterase that metabolizes the second messenger molecule cAMP into inactive AMP.
The following physiological and biochemical parameters were studied in vivo: intraocular pressure, outflow facility, aqueous humour production, regional ocular blood flow; their correlation with aqueous cAMP levels was also assessed. In vitro, AC characteristics in trabecular meshwork membrane preparations were examined.

Intraocular pressure

The principal factors determining intraocular pressure are the rate of aqueous humour formation and resistence encountered in the outflow pathway. Aqueous humour from ciliary processes enters the posterior chamber via secretion, as a consequence of active ionic transport by non-pigmented epithelial cells (Kinsey, 1971; Maren, 1974), and by passive ultrafiltration which is determined by hydrostatic and colloid osmotic gradients (Barany, 1963; Macri and Cevario, 1974; Macri and Cevario, 1975; Bill, 1975; Sears et al, 1981). Aqueous humour passes from the posterior chamber through the pupil into the anterior chamber and then leaves the eye by two major routes, the trabecular meshwork and the uveoscleral pathway. The trabecular route is characterized by drainage of the aqueous humour across the inner wall of Schlemm's canal into collector channels, aqueous veins and finally the venous circulation. The bulk of the flow is through the trabecular meshwork. Uveoscleral drainage passes through the connective tissues between the muscle bundles of the iris root and ciliary muscle into the suprachoroidal space, then through the sclera and finally into the circulation (Bill, 1975). The uveoscleral pathway accounts for 25-60% of aqueous drainage in the monkey and man (Bill, 1989) and, under physiological conditions, is considerably lower in cats (20%)(Bill, 1966a) and rabbits (2%)(Bill, 1966b).

Adrenergic receptors

Adrenergic receptors are membrane-bound, localized postsynaptically and/or presynaptically and classified as α and ß-adrenoceptors (Ahlquist, 1948); α and ß-receptors are each divided into two subtypes, i.e. α_1 (postsynaptic), α_2 (presynaptic and postsynaptical) (Langer, 1974; Wikberg, 1978), $ß_1$ and $ß_2$ adrenoceptors (Lands, 1967). These receptors are bifunctional with a binding component and an effector component which induce a biological response. Basically, receptors can be studied in

two ways. The biological effect of the administration of agonists and antagonists on an intact (isolated) organ indicates the functional activity and efficacy of the receptor. Interpretation of the results, however, is complicated by transport and distribution of the drug as well as its interaction with surrounding tissues before it reaches the receptor and by biological effector responses, which consist of an unknown number of steps.
The second approach is measurement of radioactive ligand binding to a homogenate or slice preparation. Ligands (agonists) are molecules that bind to receptor binding sites. In this way the affinity for and localization of receptors in whole tissue, on cell membranes, in specific regions of cell membranes and subcellularly are investigated. Ideally, both approaches should be used to study properties such as saturability,

Table 1.1 Adrenergic agonists and antagonists used in this thesis and their relative selectivity for adrenergic receptors.

	Adrenoceptor selectivity			
	α_1	α_2	β_1	β_2
Agonists				
norepinephrine	++	(+)		+
epinephrine	+	(+)		++
isoproterenol	(+)		(+)	+++
salbutamol				+++
terbutaline				+++
dobutamine			+++	+
phenylephrine	+++			
B-HT920		+++		
Antagonists				
timolol			++	+++
betaxolol			+++	+
phenoxybenzamine	+++	++		

An empty space indicates little or no affinity; (+), +, ++ or +++ indicates moderate to high affinity.

specifity, and reversibility. This thesis deals with biological responses and refers to the literature on ligand binding. In vivo IOP, aqueous inflow, facility of outflow, and aqueous cAMP levels were studied together with in vitro AC responses in membrane fractions of trabecular meshwork.
Adrenergic drugs specificallly bind directly to adrenergic receptors and can be divided into agonists and antagonists. Agonists and antagonists are subdivided according to their selectivity for $\alpha(\alpha_1$ and/or $\alpha_2)$ and/or $\beta(\beta_1$ and/or $\beta_2)$ adrenergic receptors. The selectivity of the adrenergic agonists and antagonists used in this thesis is reviewed in Table 1.1.

Adenylyl cyclase

The effector component transduces the signal initiated by the ligand bound to a receptor to the cell via a second messenger system. A large variety of receptors are coupled to relatively few signal transduction systems. A few distinct systems have been described (Cooper et al, 1986). Schematic representations of the two major systems involved in adrenergic receptor responses are depicted in Figure 1.1.
The AC system is shown in the left-hand panel. The binding of an agonist switches the receptor to an activated state whereas antagonists stabilize the ground state. Binding of antagonists is slower but stronger and often irreversible. Once a blockade is achieved, it is long-lasting. Activation of the receptor leads to activation of the coupling G-proteins which have stimulating (G_s) or inhibitory (G_i) properties. The catalytic unit of AC is thus activated or inactivated, respectively, to modulate production of second messenger cyclic AMP using ATP as the substrate. Cyclic AMP activates phosphorylation of proteins but is metabolized intracellularly by the enzyme phosphodiesterase to inactive AMP. A variety of receptors couple to this AC system: β_1 and β_2- adrenoceptors but also non-adrenergic receptors, such as those for vasoactive intestinal peptide (VIP), prostaglandin E (PGE) and adenosine, stimulate AC via G_s; α_2-adrenoceptors inhibit AC via G_i. These (non-)adrenergic receptors have been demonstrated in ciliary processes and trabecular tissue; their presence, location and influence on IOP will be discussed below.
The second messenger system which leads to protein phosphorylation by phospholipase C is depicted in Figure 1.1 in the right-hand panel. Receptor binding activates phospholipase C, which in turn produces the second messenger molecules inositol triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 mobilizes intracellular Ca^{++}, a third second messenger molecule. IP_3, DAG and Ca^{++} activate protein kinase C and induce protein phosphorylation. Of the adrenergic receptors α_1-receptors activate this system.

Interactions between the cAMP and calcium/protein kinase C systems have been observed in a variety of tissues. Nishizuka (1986) has classified these interactions as monodirectional and bi-directional, indicating positive and negative feed-back of the response. In the eye several examples have been described. In the bovine iridic sphincter, cAMP may act as a regulator of responses to neurotransmitters that exert their action through the IP_3-Ca^{2+} system (Tachado et al, 1989). In rabbit ciliary processes protein kinase C activation has been linked to AC (Mittag et al, 1987c; Yoshimura et al, 1989), and a calcium/calmodulin-sensitive AC has been demonstrated in rabbit ciliary processes (Tormay et al, 1987).
How the second messenger molecules cAMP, Ca^{++}, IP_3 and DAG exert their intracellular and biological effects is not known in detail. Protein phosphorylation, a

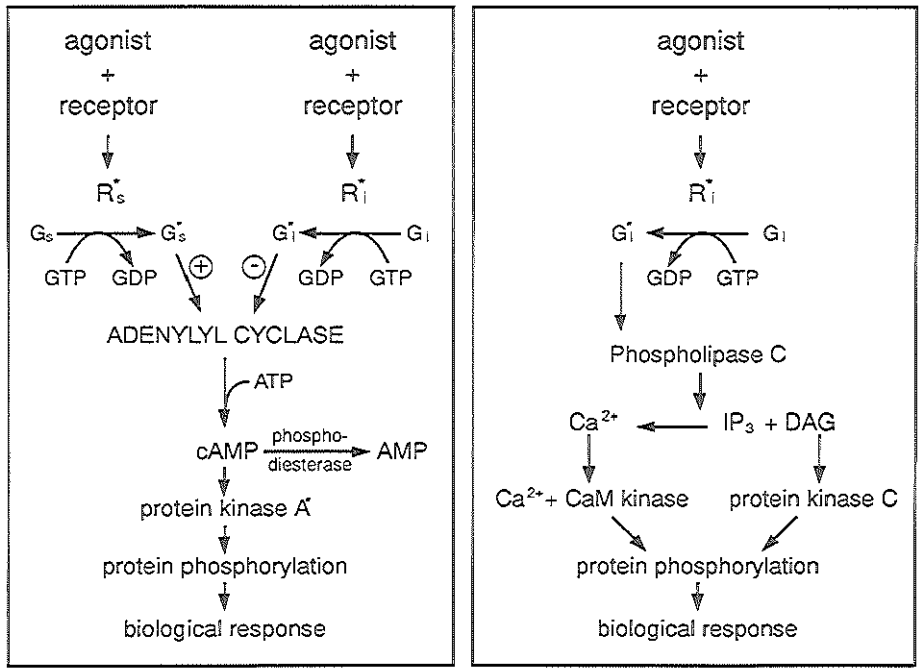

Figure 1.1 Schematic representation of the adenylyl cyclase/cAMP and calcium/protein kinase C second messenger systems. R^* = agonist bound to receptor; G^* = GTP bound to G-protein; G_s = stimulatory and G_i = inhibitory G-protein.

common phenomenon, may ultimately lead to

1. metabolic changes, enzyme activation or inactivation;
2. changes in receptor affinity, e.g. desensitization;
3. changes in ion conductance and
4. protein synthesis.

In conclusion, adrenergic agents exert their effect by binding to adrenergic receptors. Adrenergic receptors can be classified as α_1, α_2, $ß_1$ and $ß_1$-subtypes. A second messenger system is coupled to the receptor and transduces the receptor signal to the cell by formation of second messenger molecules. $Beta_2$(and $ß_1$)-adrenoceptors are coupled directly to AC. Activation of these $ß_2$-receptors stimulates AC via G_s, leading to synthesis of the second messenger molecule cAMP. $Alpha_2$-adrenoceptors are also coupled directly to AC but receptor activation inhibits AC via the inhibitory G-protein. In contrast, α_1-adrenoceptor activation initiates intracellular events directed by a different second messenger system, generating calcium, IP_3, and DAG as second messenger molecules.

Adrenergic agents, adenylyl cyclase and intraocular pressure

Adrenergic drug therapy is directed toward reducing IOP by manipulating adrenergic receptors pharmacologically. The gross non-visual innervation of the anterior eye of vertebrates consists of sensory, parasympathetic, and sympathetic fibres. The sympathetic nervous system is presumed to play an important role in maintaining IOP homeostasis and to be responsible for diurnal variations in intraocular pressure. Sympathetic innervation originates from thoracic root fibres and runs via the superior cervical ganglion and carotid plexus through the short and long ciliary nerves to the eye. Norepinephrine is the classical neurotransmitter of the effector organ. The trabecular meshwork contains nerve fibres in all regions, including both walls of Schlemm's canal (Holland et al, 1956); they consist of sympathetic (Nomura and Smelser, 1974) as well as parasympathetic and sensory fibres (Holland et al, 1957; Ruskell, 1976). The density of sympathetic fibres, however, is very low in comparison to the number of adrenergic receptors and adrenergic responses. Kaufman (1989) therefore suggested the presence of extrajunctional, non-innervated ß-receptors that respond to ambient free catecholamines, as demonstrated in various other tissues. Furthermore, paracrine cell clusters produce and release neurotransmitters or a local hormone that affects neighbouring cells, while the non-synaptic contact of nerves may

affect neighbouring cells by means of a neurosecretory mechanism (Stone et al, 1984; Mittag, 1989). The limbal vasculature, including the episcleral vascular system which receives aqueous humour from Schlemm's canal, contains adrenergic nerves but little is known about their function (Ehinger, 1964; Ehinger, 1966). In ciliary processes abundant adrenergic fibres innervate the uveal blood vessels and ciliary processes (Stone et al, 1989); intraepithelial nerve fibres have been demonstrated (Yamada, 1988; Yamada, 1989) in ciliary processes.
The presence of adrenergic receptors in ciliary processes and trabecular meshwork has been demonstrated by radiolabeled ligand binding techniques. These data will be discussed in the text below. The functional effects of adrenergic receptor stimulation and some non-adrenergic agents on AC activity in ciliary processes and trabecular meshwork in vitro are reviewed in Table 1.2. The physiological significance of the stimulation (or inhibition) of AC by adrenergic and some non-adrenergic agents for the regulation of IOP homeostasis is reviewed in Table 1.3. (Several authors have reviewed the pharmacology of the role of adrenergic agents in aqueous humour dynamics: Potter, 1981; Mishima, 1982; Sears, 1984; Mittag, 1989; Polansky, 1990).

Ciliary processes and aqueous flow

Ligand binding studies have provided ample evidence that ciliary processes contain ß-adrenoceptors. The rabbit iris and ciliary body (Neufeld et al, 1978) and ciliary processes (Bromberg et al, 1980) contain ß-receptors. About 20-25% of all adrenergic receptors in the iris and ciliary body are of the $ß_2$ subtype (Mittag and Tormay, 1985b). In human iris-ciliary body membrane preparations 90% of the ß-receptors were of the $ß_2$-subtype, as demonstrated by displacement studies of the high affinity binding of [^{125}I]iodopindolol (Wax and Molinoff, 1987). $Beta_1$-adrenoceptors were found only in small amounts around the blood vessels in sheep ciliary bodies (Trope and Clarke, 1982). In rabbit ciliary processes α_1 and α_2-receptors have been identified, the majority being of the α_2-subtype (Mittag and Tormay, 1985; Mittag et al, 1985; Mallorga et al, 1988; Jumblatt et al, 1987).
The discovery of AC in rabbit ciliary processes and its activation by catecholamines was the first indication that AC may play a role in aqueous humour formation (Waitzman and Woods, 1971) This was followed by many studies, in vitro as well in vivo, focussing on the role of AC (Tables 1.2 and 1.3, respectively). One consistent finding is that $ß_2$-receptors stimulate AC in ciliary processes, particularly in nonpigmented ciliary epithelial cells which secrete aqueous humour. AC stimulation was also obtained with non-adrenergic drugs, such as VIP, forskolin (FSK), fluoride

(F^-) and choleratoxin. It has been suggested that ß and VIP receptors are coupled to the same AC system in non-pigmented epithelial cells (Mittag, 1987b). However, the role of $ß_2$-receptor agonists and other cAMP stimulators in aqueous humour production is more complicated, as indicated by contradictory results. On the one hand $ß_2$-selective agonists and VIP increase the flow in primates (Nilsson et al, 1990); this ß-stimulated flow can be blocked by timolol. Comparable findings have been reported for rabbits. Timolol reduces aqueous flow during the night when the sympathetic tone is supposed to be high (Gregory, 1990; Yoshitomi and Gregory, 1991). The enhanced sympathetic tone was deduced from high aqueous cAMP (Rowland et al, 1986) and elevated endogenous norepinephrine levels (Liu, 1991a and b). In contrast to the $ß_2$-adrenergic-stimulated increase in flow, direct stimulators of AC, such as cholera toxin (Gregory et al, 1981b) and FSK (Caprioli, 1984a), have been reported to reduce aqueous flow in primates. Thus, opposite physiological effects have been reported for various stimulators of (the same?) AC in ciliary processes. Several explanations have been suggested. For example, Mittag and Tormay (1981) showed that the desensitization and uncoupling of ß-receptors could be attributed to high doses of ß-agonists; functionally, this implies that a ß-adrenergic agonist may have the same effect as a ß-blocker. The flow-decreasing effect of FSK has been questioned but may be due to a slight breakdown of the blood aqueous barrier (BAB) (Bartels et al, 1987), indicating that it can be a non-specific effect.

$Alpha_2$-receptors inhibit AC (see Table 1.3); they are located postjunctionally, as can be deduced from cervical sympathectomy experiments which show that denervation does not reduce the number of α_2 receptors in ciliary processes. This has led to the concept of dual control of AC by α_2 and $ß_2$-receptors (Mittag and Tormay, 1985a), whereby $ß_2$-stimulated AC is counterbalanced by inhibitory α_2-receptors. This is supported by the finding that both the $ß_2$ and VIP-receptor-stimulated formation of cAMP in ciliary bodies are both inhibited by the α_2-agonist clonidine (Bausher et al, 1989; Cepelic and Hynie, 1990a). Postsynaptic α_2-receptors and the negative coupling to AC might play an important role in aqueous production; for example, clonidine has been shown to decrease flow (Chiou, 1983). The significance of the large number of α_2-adrenoceptors in the ciliary body remains unclear (Mittag, personal communication).

Somewhat inconclusive is the role of phenylephrine/α_1 in ciliary processes; α_1-stimulation evokes primarily a calcium signal, which essentially is a different second messenger system from the AC/cAMP system. Physiologically, both stimulation and inhibition of aqueous inflow have been reported. The immediate effect may be an increase in flow accompanied by an initial hypertensive response, possibly due to BAB breakdown. The late effect is flow reduction and a drop in the IOP.

Among the non-adrenergic stimulators of AC in ciliary processes are PGE, calcitonin-

gene-related peptide (CGRP), and calcium calmodulin. PGE_2 might inhibit prejunctional norepinephrine release (Neufeld, 1976); the presence of calcium/ calmodulin-sensitive AC in rabbit ciliary processes provides evidence for an interaction between the α_1-adrenoceptor/calcium and the adenylyl cyclase/cAMP second messenger systems. Other non-adrenergic inhibitors of AC in ciliary processes are neuropeptide Y (NPY) (Bausher and Horio, 1990; Cepelic and Hynie, 1990b; Gooch et al, 1989), somatostatin in non-pigmented epithelial cells (NPE cells), and acetyl choline binding to $muscarinic_1$ receptors. Their physiological effects on IOP have not yet been clarified.
In conclusion, ciliary processes contain ß-adrenergic receptors predominantly of the $ß_2$-subtype, which are located in epithelial cells. They are directly coupled to AC; stimulation (terbutaline) seems to increase and inhibition (timolol) to decrease aqueous flow. $Alpha_2$-adrenoceptors are present in large quantities in ciliary processes, inhibit AC and may decrease flow. A dual control of AC by α_2 and $ß_2$-receptors has been proposed. $Alpha_1$-adrenoceptors are also present in ciliary processes, are not coupled to AC but to the calcium/IP_3/DAG second messenger system and may inhibit aqueous humour formation.

Trabecular meshwork and outflow facility

The $ß_2$-adrenergic receptors in human trabecular meshwork (Jampel et al, 1987; Wax et al, 1989; Elena et al, 1990) and in cultured human trabecular meshwork cells (Jampel et al, 1987; Wax et al, 1989) have been characterized by radioactive ligand binding techniques. In trabecular tissue, trabecular endothelium, and cultured human trabecular cells $ß_2$-receptor stimulation consistently activates AC (see table 1.2). Outflow facility increases after ß-adrenoceptor stimulation (see table 1.3); many authors report an increase in outflow facility after administration of epinephrine and isoproterenol, whereas timolol antagonizes this increase. Alpha-adrenoceptors in trabecular meshwork have not been studied by ligand binding techniques to our knowledge. A role for α_1-receptors, however, has long been suggested but is not yet firmly established. Neufeld and Sears (1974) reported that AC in rabbit trabecular tissue is activated by α-adrenergic stimulation since epinephrine stimulated AC activity could be antagonized by the α-adrenergic antagonist phenoxybenzamine.
Furthermore, PGE and VIP are non-adrenergic stimulators of AC in trabecular meshwork. PGs may be involved in the effect of epinephrine on outflow facility (Hoyng et al, 1982); in support of this is the finding that indomethacin partially inhibited the increase in outflow facility induced by topical epinephrine (Anderson and

Table 1.2 Adenylyl cyclase responses in vitro and in vivo

species	ocular tissue	stimulus	effect on adenylyl cyclase	references
Ciliary body				
rabbit	cil.body	ß	↑	Neufeld and Sears, 1974
rabbit	iris-cb	$ß_2$; α_2	↑;↓	Mittag and Tormay, 1985a
rabbit	iris-cb	PGE_2	↑	Battacherjee et al, 1991
rabbit	cil. proc	$ß_2$	↑	Cepelic and Cernohorsky, 1981
rabbit	cil. proc	α_2	↓	Kintz et al, 1988
rabbit	cil. proc	α_2 postsyn.	↓	Bausher et al, 1987
rabbit	cil. proc	VIP	↑	Mittag and Tormay, 1987b
rabbit	cil. proc	NPY; Som.st	↓;↓	Bausher and Horio,1990
rabbit	cil. proc	NPY	↓	Cepelic and Hynie, 1990b
rabbit	cil. proc	α_2;$ß_2$,VIP,PGE_2	↓;↑;↑;↓	Jumblatt et al, 1990
bovine	cil. proc	$ß_2$	↑	Elena et al, 1984
monkey	cil. proc	$ß_2$	↑	Crawford et al, 1991
human	cil.proc	$ß_2$	↑	Nathanson, 1981
human	npe-cells	CGRP	↑	Yabu et al, 1991
human	npe-cells	PGE_2	↑	Neltner et al, 1991
Trabecular meshwork				
rabbit	scl.trab.	ß; α; PGE_1	↑;↑;↑	Neufeld and Sears, 1974
bovine	trab.tissue	ß	0	Bartels, 1988b
monkey	scl.trab.	ß	↑	Neufeld and Sears, 1974
monkey	trab.cells	$ß_2$;VIP;PGE_1	↑;↑;↑	Koh and Yue, 1988
monkey	trab.membr	$ß_2$	↑	Crawford et al, 1991
human	scl.trab.	ß	↑	Neufeld and Sears, 1974
human	trab.cells	ß; (α)	↑	Tripathi and Tripathi, 1984
human	trab.cells	ß; (α)	↑	Polansky and Alvaredo, 1985
Aqueous humour				
rabbit	AH	ß	↑	Boas et al, 1981; Radius and Langham, 1973
rabbit	AH	ß; α_1	↑;↑	Rowland and Potter, 1979
rabbit	AH	$PGF_{2\alpha}$, PGI_2	↑;↑	Groeneboer et al, 1989

Effect on AC, i.e. stimulation (↑), inhibition (↓), or no effect (0) of adrenergic and some non-adrenergic agents in various species. AC activity was determined in vitro by the adenylyl cyclase assay and/or by measurement of cAMP synthesis in ocular tissue preparations by radioimmunoassay. Tissue preparations included from whole iris/ciliary body (iris-cb), ciliary body (cil.body), ciliary processes (cil.proc), non-pigmented epithelial cells (npe-cells), scleral-trabecular rings (scl.trab.), trabecular meshwork explants (trab.tissue), trabecular meshwork membrane preparations (trab.membr) and cultured trabecular endothelial cells (trab.cells). AC activity was determined in vivo by measuring cAMP levels in aqueous humour (AH) by radioimmunoassay. Adrenergic receptor stimuli (α, α_1, α_2, ß, $ß_1$, $ß_2$) were studied with adrenergic agonists and/or antagonists (see table 1.1), and non-adrenergic receptor stimuli with prostaglandin E (PGE), $PGF_{2\alpha}$, PGI_2, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and somatostatin (Som.st).

Table 1.3 Effect of adrenergic and some non-adrenergic agents on intraocular pressure and aqueous humour dynamics

drug	IOP	species	aqueous flow	outflow facility	references
Adrenergic agonists					
$ß_2$ (epinephrine,	↓	rabbit	↓/↑	↑	1-5
isoproterenol, salbutamol,	↓	primate	↑	↑	6-17
or terbutaline)					
α_1 (phenylephrine)	↑/↓	rabbit	↓	↑	2,4
	↑/↓	primate	↑/↓	?	18
α_2 (B-HT920)	↓	primate	↓		19
Adrenergic antagonists					
$ß_{2,1}$ (timolol)	0/↓	rabbit	0/↓	↓	4, 20
	↓	primate	↓	↓	21, 12
α_1 (corynanthine)	↓	primate	0	0	(uv.scl.flow↑) 2
α_2	↓	rabbit			
	↓	primate			
Non-adrenergic agents					
forskolin	↓	rabbit	↓	0	22
cholera toxin	↓	rabbit	↓		23
cAMP	↓	primate	?	↑	16,17,24,25

Effect of adrenergic and some non-adrenergic agents on intraocular pressure (IOP), aqueous humour production as measured by fluorophotometry and trabecular outflow facility as measured by tonography or two-level constant pressure perfusion, in rabbits and primates (including man).

References: (1) Eakins, 1963; (2) Sears and Sherk, 1964; (3) Lamble, 1977; (4) Araie, 1985; (5) Anderson and Williams, 1990; (6) Townsend and Brubaker, 1980; (7) Schenker et al, 1981; (8) Coakes and Siah, 1984; (9) Nilsson et al, 1990; (10) Bill, 1969; (11) Bill, 1970; (12) Miichi and Nagataki, 1983; (13) Gharagozloo et al, 1988; (14) Higgins and Brubaker, 1980; (15) Kaufman, 1985; (16) Neufeld et al, 1975; (17) Neufeld et al, 1978; (18) v Genderen, 1988; (19) Chiou, 1983; (20) Gregory, 1990; (21) Bartels, 1988a; (22) Serle et al, 1984; (22) Caprioli, 1984a; (23) Gregory, 1981b; (24) Kaufman, 1986; (25) Kaufman, 1987.

Williams, 1990).
A direct role for cAMP in increasing outflow facility has been suggested but has not yet been confirmed; perfusion of the anterior chamber with cyclic nucleotide analogues increased outflow facility (Neufeld, 1978; Kaufman, 1987), but a direct temporal relationship between aqueous cAMP levels and the drop in IOP could not be demonstrated (Boas et al, 1981; see summary of this thesis).
In conclusion, increasing evidence indicates that the trabecular endothelium contains $ß_2$-adrenergic receptors which are coupled to AC. $Beta_2$-adrenergic stimulation (e.g. epinephrine, isoproterenol) increases outflow facility. $Alpha_1$-adrenoceptor stimulation may increase outflow facility, although this is more likely to occur in rabbits than in primates.
An overview of the various stimuli of aqueous humour production and outflow facility is given in table 1.4.

Table 1.4: Schematic overview of the presence of adrenergic receptors and the effects of various stimuli on adenylyl cyclase activity

stimulus	presence of receptors in:		Effects on:		
	ciliary processes	trabecular tissue	second messenger	aqueous humour formation	outflow facility
$ß_2$	+++	+	AC ↑	↑	↑
VIP			AC ↑	↑	?
Forskolin			AC ↑	↓	0
Cholera toxin			AC ↑	↓	?
α_2	+++	?	AC ↓	↓	↑
α_1	+	?	IP_3 ↑,(AC↑)	↑ and ↓	↑

The presence of adrenergic receptors has been demonstrated by ligand binding techniques in ciliary processes and trabecular meshwork; the effects of selective adrenergic agonists and some non-adrenergic agents on the adenylyl cyclase (AC) or IP_3 second messenger system and on aqueous humour dynamics are shown.

Inhibition of the phosphodiesterase enzyme as pharmacological tool

In this thesis a phosphodiesterase-inhibitor was combined with adrenergic agents to

augment the AC/cAMP-mediated effects of these agents. The enzyme phosphodiesterase (see Figure 1.1) metabolizes active cAMP to inactive 5'AMP. For this purpose 3-isobutyl-1-methylxanthine (IBMX), a methylated xanthine derivative related structurally to caffein and theophylline, was used. Three basic cellular effects of the methylxanthines have been described (Rall, 1985):

1. increased accumulation of cyclic nucleotides, particularly cAMP, induced by non-specific inhibition of phosphodiesterases,
2. translocation of intracellular calcium; alterations in the cellular metabolism of calcium may occur in the presence of high levels of methylxanthines,
3. blockade of receptors for adenosine.

Other types of activity that have received relatively little attention include reduction of the re-uptake and/or metabolism of catecholamines in non-neural tissues (Kalsner, 1971; Kalsner et al, 1975).
The inhibition of cyclic nucleotide-phosphodiesterases and antagonism for adenosine receptors are the best known cellular activities. IBMX is a potent inhibitor of phosphodiesterases, 15 times stronger than theophylline (Beavo et al, 1970). Methylxanthines, especially IBMX and theophylline, have been shown to potentiate both the effects of neurotransmitters or hormones and the accumulation of cAMP or cGMP. In this thesis it was found that the potentiating effects of IBMX on IOP were accompanied by markedly elevated/potentiated levels of aqueous cAMP; this is considered indicative of the pronounced phosphodiesterase-inhibiting properties of IBMX.
The solubility of methylxanthines, which is low, can be greatly enhanced by the formation of either a salt or complexes with, for example, cyclodextrins. A well-known example is theophylline which is solubilized with ethylenediamine to form the salt aminophylline. In chapter 4 the ethylenediamine salt of IBMX and inclusion complexes of IBMX with cyclodextrin are investigated.

Aim of the thesis

This study was designed to gain more knowledge about the role of the AC/cyclic AMP second messenger system in the reduction of IOP, particularly in response to adrenergic agents.
The principle approach was to augment pharmacologically the AC/cAMP signals of adrenergic agonists in vivo by combination with the phosphodiesterase inhibitor

IBMX.

More specifically the aims were to:

Chapter 3	increase the ocular hypotensive effect of catecholamines by administering IBMX;
Chapter 4	test soluble forms of IBMX;
Chapter 5	study the effects on regional ocular blood flow of the administration of epinephrine and IBMX;
Chapter 6	study aqueous humour dynamics in the set-up of chapter 5;
Chapter 7	determine the adrenoceptor selectivity of catecholamines to obtain potentiation of the ocular hypotensive effect by IBMX;
Chapter 8	study the interaction between α_1-/calcium and β_2/cAMP signals, as observed in chapter 7, by measuring the parameters: IOP, outflow facility, and aqueous cAMP levels;
Chapter 9	study the direct effects of cyclic nucleotide analogs on IOP and
Chapter 10	study the basic characteristics of the AC enzyme in preparations of membranes of trabecular meshwork.

CHAPTER 2

MATERIALS AND METHODS

An introduction to the materials and methods used is given below. For a detailed description the reader is referred to the individual chapters and literature references.

Experimental animals and tissues

Measurement of intraocular pressure, analysis of aqueous humour composition and assessment of aqueous humour dynamics were performed in adult pigmented dutch rabbits of both sexes (chapter 3,4, 6-9). The IOP was also measured in normotensive beagle dogs (chapter 3). Effects on ocular blood flow were assessed in albino rabbits of either sex (chapter 5). Bovine eyes and human donor eyes, provided by the Cornea Bank of the Netherlands Ophthalmic Research Institute, were used to prepare trabecular meshwork membrane fractions to measure adenylyl cyclase activity (chapter 10).

Intraocular pressure

The relationship between factors determining the IOP may be described under steady state conditions as (Kaufman and Crawford, 1989):

$$\text{Inflow} = \text{Outflow} = C_{trab} \times (IOP - P_e) + \text{uveoscleral flow},$$

whereby C_{trab} is the trabecular outflow facility and P_e the episcleral venous pressure. Intraocular pressure was measured non-invasively by applanation tonography using an Alcon pneumatonograph. The pneumatonometer had been calibrated for the human eye at delivery. Figure 2.1 shows the calibration curve for rabbit eyes.

Artificial intraocular pressure levels were induced manometrically by a cannula connected to a water column and inserted into the anterior chamber. Pneumatonometer values were recorded in triplicate with the stopcock in closed position and then averaged. Step-wise 2.5 mmHg increments or drops in IOP levels were used to determine the pneumatonometric values which correspond to manometric IOP values in the range 7.5 to 40 mmHg. This procedure was repeated five times (3 times for increasing and twice for decreasing steps) in five eyes from different rabbits. The standard error of the mean of these five values for each pressure level in individual eyes ranged between 0 and 0.61 mmHg (mean 0.21 mmHg). This is indicative of a high reproducibility and low variation in the results of the pneumatonometer and/or the examiner. By interpolation conversion values were calculated for pneumatonometrically recorded IOP values (Figure 2.1). Near linearity was observed between 10 and 25 mmHg. An Alcon calibrator was used before and after each IOP experiment, and one person measured the IOP. The biological variability, as indicated

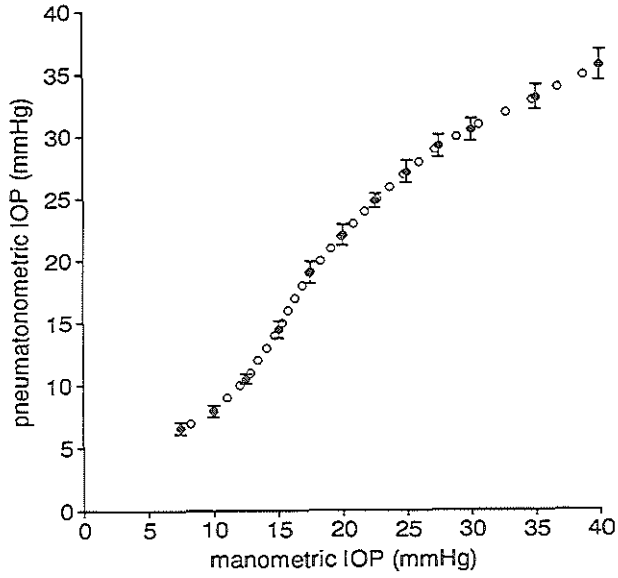

Figure 2.1 Calibration curve for the pneumatonometer for rabbit eyes. Closed circles indicate the mean pneumatonometric IOP values ± s.e.m., corresponding to the manometrically induced IOP levels. Open circles indicate the manometric IOP values corresponding to pneumatonometric IOP values calculated by interpolation. Five eyes from different rabbits were used.

in Figure 2.1 by error bars, is probably due to differences in corneal curvature and/or corneal elasticity. The effect of biological variation was minimized by using the contralateral eye as a control eye and/or by using the experimental eye as control eye on another day.
Local anaesthesia was achieved with 30μl 0.2% oxybuprocain.

Outflow facility (see chapters 6 and 8)

The outflow facility consist of trabecular outflow facility and uveoscleral outflow facility (chapter 1). The outflow facility was measured with a Berkely electronic Schiötz indentation tonograph.
A known weight was placed on the cornea; this raises the IOP. During the next 4 minutes aqueous is squeezed out of the anterior chamber and the IOP decreases. The rate of decrease in IOP is a measure of the pressure-dependent outflow facility. The flow across the trabecular meshwork is IOP-dependent, but uveoscleral flow is virtually independent of IOP (Bill, 1989; Kaufman and Crawford, 1989). Moreover, in rabbits uveoscleral flow is low (less than 2%). The ultrafiltration, which is the passive component of aqueous humour production, is also IOP-dependent; when the outflow facility is assessed with tonography this pressure sensitive decrease in aqueous flow will be falsely measured as outflow facility; this is termed pseudofacility. Pseudofacility is believed to account for 5-10% of the total facility (Kaufman, 1989). The contribution of this pseudofacility component,however, is approximately constant in all measurements. Pseudofacility may increase due to breakdown of the blood aqueous barrier (BAB), particularly in rabbits since they have a fragile BAB which is easily disrupted by drugs or trauma. In this thesis assessment of the aqueous protein content was always included as a control of the effect of drugs on the BAB and, as will be demonstrated, it did not increase. Furthermore, only one eye was used to exclude consensual effects and baseline C-values were assessed one or two days beforehand in the same experimental eye. Outflow facility determined after low dose drug therapy and baseline values from different days did not differ (Table 6.1). This indicates that variation of the baseline C-values on different days is small and neglectible.
The Friedenwald tables, modified for humans by Moses (1958), were used for conversion to C-values, in $\mu l.min^{-1}.mmHg^{-1}$. Data obtained should therefore be considered as qualitative changes in trabecular outflow facility rather than as quantitative changes. A major advantage of tonography is its non-invasiveness; e.g. indomethacin pretreatment, which is required for rabbits to prevent BAB breakdown

when invasive techniques are used, was not needed. Indomethacin may interfere with potential PG-mediated effects of catecholamines. Therefore, tonography may provide important qualitative information about changes in/effects on outflow facility.

Aqueous flow (see chapter 6)

Aqueous humour production was measured by fluorophotometry. Techniques for measuring the rate of aqueous humour formation were based on the timed dilution of exogenous tracer in the anterior chamber (Gaul and Brubaker, 1985). For the non-invasive approach, topical fluorescein is used as tracer while a fluorophotometer in front of the cornea measures the decrease in the fluorescence of an excitation light bundle. For the invasive technique, radioiodonated albumin is infused by cannulation into the anterior chamber and samples are withdrawn by a push-pull system to analyse activity (Bill, 1989). The non-invasive fluorophotometric technique employed in this thesis (v Genderen et al, 1988) was adapted for rabbits. Neither indomethacin nor sedatives were required.
Advantages and limitations of the techniques for measuring aqueous humour dynamics were reviewed by Kaufman and Crawford (1989).

Ocular blood flow (see chapter 5)

Regional ocular blood flow was measured by means of the radiolabeled microsphere technique (Buckberg et al, 1971; Alm and Bill, 1972; Stjernschantz et al, 1976) which is based on the following principle. Radioactive microspheres, 15μm in diameter, are injected into the circulation. The microspheres travel to the small peripheral vessels where they are trapped; next, the organ is removed and the radioactivity is measured. A known quantity of arterial blood, the reference sample, is withdrawn to measure its activity. The radioactivity of the tissue samples taken for assessment of the flow is divided by that of the reference sample and multiplied by the weight of the reference blood sample. The resulting blood flow is expressed as $mg.min^{-1}tissue^{-1}$.
Albino rabbits were anaesthetized with i.v. sodium pentobarbital and a catheter for microsphere injection was inserted into the left heart ventricle through the left brachial artery. The reference sample was taken from a cannulated femoral artery. The eyes were enucleated and dissected into the choroid, ciliary processes, iris and sclera to calculate blood flow as described above.

Aqueous cyclic AMP, protein and prostaglandins

Aqueous humour samples were aspirated with a tuberculin syringe and 27-gauge needle after topical anaesthesia was achieved with 30μl 2-4% tetracain. The needle was inserted into the anterior chamber near the limbus, avoiding the lens and iris; 100-200 μl were taken, divided into aliquots and stored at -80 °C. Samples were thawed only once. The protein content was assessed by the Bradford (1976) dye-binding method using human serum albumin as the reference protein. Cyclic AMP levels were determined in duplicate by radioimmunoassay (Rianen, Dupont, NEN) under non-acetylated assay conditions, using a 10 μl sample. PGE_2-levels were assessed in duplicate by radioimmunoassay (Rianen, Dupont, NEN), again using a 10 μl sample.

Adenylyl cyclase activity (see chapter 10)

Most of the AC enzyme is bound to plasma membranes. Trabecular tissue was prepared surgically from isolated trabecular meshwork from bovine (Anderson et al, 1980) and human (Tripathi and Tripathi, 1982) donor eyes. The human preparation was checked by histology (not shown). Membrane fractions were obtained by standardized procedures for homogenization, centrifugation and resuspension of the pellet in buffer solution.
Adenylyl cyclase catalyzes the conversion of ATP to 3'5'-cyclic AMP and pyrophosphate in the presence of GTP and magnesium ions. In the enzyme assay radiolabeled ATP was the substrate. The rate of conversion was determined by isolating and measuring the amount of ^{32}P-cyclic AMP formed from α^{32}P-ATP.

$$\underset{(\alpha^{32}\text{P-ATP})}{\text{Adenosine 5'-}^{32}\text{P-P-P}} \xrightarrow[\text{GTP, } Mg^{2+}]{} \underset{(^{32}\text{P-cyclic AMP})}{\text{Adenosine 3'5'-}^{32}\text{P}} + \text{P-P}$$

This was achieved by sequential chromatography on a Dowex 50 cation exchanger and on neutral alumina, as originally described by Salomon (1974). This AC assay has become a well-established method for measuring AC activity.
Interference due to degradation of ATP by various ATPases and nucleotidases was reduced by including an ATP-regenerating system (creatine kinase and creatine phosphate). Degradation of labeled cAMP by phosphodiesterases was reduced by including theophylline, a phosphodiesterase inhibitor, together with unlabeled cAMP. The activity of adenylyl cyclase was stimulated at various levels. A variety of

drugs/agents that stimulate receptors coupled to AC in various tissues was tested: for example, epinephrine, isoproterenol, PGE and VIP. Fluoride ions, which by-pass the receptor level, are known to stimulate G-proteins directly; the balance between G_i and G_s determines the final stimulatory or inhibitory response. Forskolin, an organic compound, was used to stimulate the catalytic unit of AC directly.

Statistics

All data are given as means $\pm$ SEM unless otherwise stated. Comparisons of means were based on the Student's t-test. Aqueous humour production data (chapter 6) are ananlyzed by the Wilcoxon rank sum test. Curve fitting of dose-response effects (chapter 3 and 10) was performed by means of non-linear regression analysis (proc. NLIN. SAS Institute Inc.)

CHAPTER 3

ISOBUTYLMETHYLXANTHINE ENHANCES ADRENERGIC-INDUCED OCULAR HYPOTENSION IN RABBITS AND BEAGLES

Philip FJ Hoyng, Carjoanne Groeneboer, and Michiel JWM Busch; published in Exp Eye Res 52, 511-517, 1991

Abstract

Isobutylmethylxanthine (IBMX), a strong phosphodiesterase/adenosine-inhibitor, was combined with norepinephrine (nE), epinephrine (Epi) and isoproterenol, respectively to evaluate their effect on intraocular pressure. Application of topical IBMX alone had no measurable effect on IOP. When IBMX was combined with nE or Epi the ocular hypotension in rabbits and beagles increased.
The E_{max} for nE alone was 2.9 $\pm$ 0.4 mmHg, for Epi alone 7.3 $\pm$ 0.5 mmHg and for isoproterenol alone 5.1 $\pm$ 0.3 mmHg. The EC_{50} was 0.2 $\pm$ 0.05% (nE), 0.05 $\pm$ 0.01% (Epi) and 0.003 $\pm$ 0.001% for isoproterenol. When given in combination with 1% IBMX the E_{max} for nE was 7.4 $\pm$ 1.7 mmHg, for Epi 9.0 $\pm$ 0.8 mmHg and for isoproterenol 6.1 $\pm$ 0.3 mmHg. The corresponding values for EC_{50} were 0.07 $\pm$ 0.03% (nE), 0.02 $\pm$ 0.006% (Epi) and 0.002 $\pm$ 0.001% for isoproterenol. Combining 1% IBMX with 0.1% Epi did increase the aqueous humour cyclic AMP-levels at 1, 3 and 5 hr in rabbits.
The results of this study demonstrate that a strong phosphodiesterase/ adenosine-inhibitor such as IBMX enhances the reduction in IOP induced by adrenergic agonists.

Introduction

Methylxanthines are phosphodiesterase-inhibitors and antagonists of the receptor-mediated activity of adenosine. Methylxanthines enhance both the contractions induced by epinephrine (Epi) in aortic strips (Kalsner, 1971) and the relaxation that follows ß-adrenoceptor stimulation in bovine coronary arteries (Kalsner et al, 1975).

At therapeutic plasma concentrations methylxanthines cause an increase in the level of circulating catecholamines (Robertson et al, 1978; Vestal et al, 1983). Of the three substituted xanthine derivatives 3-isobutyl-methylxanthine (IBMX) is one of the more potent phosphodiesterase-inhibitors (Beavo et al, 1970; Beavo et al, 1971). IBMX enhanced the increase in cyclic-AMP induced by biogenic amines and histamine in the guinea pig cerebral cortex in vitro (Schultz and Daly, 1973a, 1973b).
Topical ocular therapy with Epi or dipivalylepinephrine (DPE) is one of the pillars of glaucoma therapy at the present time. The reduction in IOP caused by these agents is attributable to both α- and $ß_2$-adrenoceptor stimulation in tissues of the anterior segment of the eye. The reduction in intraocular pressure (IOP) is accompanied by an increase in cyclic-AMP in the aqueous humour (Neufeld, Jampol and Sears, 1972a; Radius and Langham, 1973; Rowland and Potter, 1979; Boas et al, 1981). When applied intracamerally cyclic-AMP increases the outflow facility in both rabbit (Neufeld et al, 1975) and primate eyes (Neufeld and Sears, 1975). In two studies forskolin (Caprioli and Sears, 1983) and cholera toxin (Gregory et al, 1981), both stimulators of the adenylyl cyclase enzyme, were found to decrease IOP by inhibiting aqueous humour flow by approximately 50%; but neither of these studies provided evidence of increased cyclic-AMP levels in vivo. However, since the catecholamine-induced decrease in IOP seems to be at least in part cyclic-AMP-mediated, and since phosphodiesterase-inhibitors prevent the intracellular degradation of cyclic-AMP, we became interested in determining the effect of a combination of a phosphodiesterase-inhibitor with catecholamines on the reduction in IOP in normotensive rabbits and beagles.

Materials and methods

Young adult pigmented rabbits (2.5-4 kg, 6-12 months old) accustomed to IOP measurements were used. Six normotensive beagles (7.6-12.3 kg, 1-4 years old) were trained for at least 2 weeks to undergo tonometry while awake. The eyes of all animals were examined with the slit-lamp for ocular inflammation and cataracts before the experiments started.
After local anesthesia was induced with 30 μl of 0.2% (rabbits) or 0.4% (beagles) oxybuprocaine (Novesine, Chibret, Riom, France), the IOP was measured with an Alcon Pneumatonograph while the animals were awake. The pneumatonometer was manometrically calibrated for rabbit and beagle eyes. Once included in an experiment an animal was not used again for at least 3 weeks to allow the effect of the drug to wear off. One day before the experiments, baseline IOP diurnal curves were made.

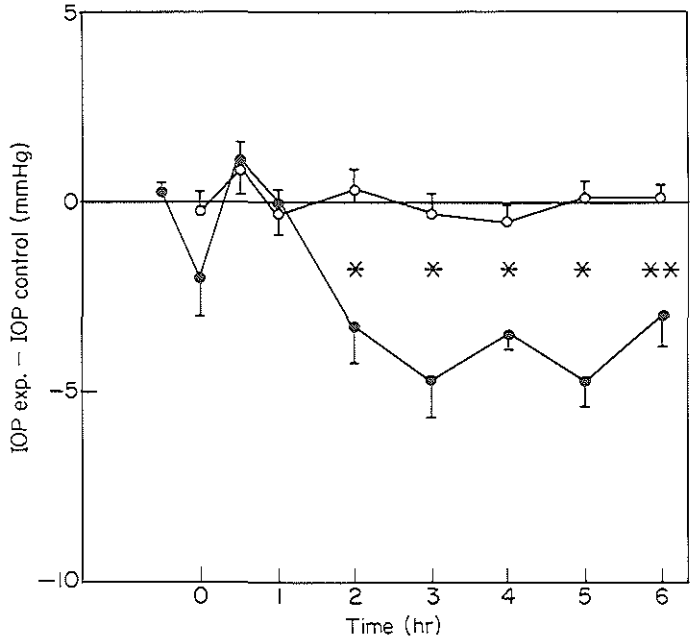

Figure 3.1 Effect of topical application of 30 μl 0.1% nE with (closed circle) and without (open circle) 30 μl 1% IBMX, both in artificial tears, on intraocular pressure in two groups of six rabbits. Points represent mean differences in IOP between treated and vehicle-treated contralateral eyes. Bars represent one SEM. P-values are calculated for corresponding data points (unpaired Student's t-test). IBMX was applied at -½ hr, nE at zero time. * $P<0.005$; ** $P<0.01$.

Epinephrine (Epi), norepinephrine (nE) and isoproterenol, all in tartrate form, were purchased from Sigma (St. Louis, MO) and 3-isobutyl-1-methylxanthine from Fluka (Buchs, Switzerland). Epi, nE and isoproterenol were dissolved in 0.5% hydroxypropylmethylcellulose in saline (AT) and IBMX was suspended in AT using an ultrasonic probe at high power. Each drug solution was applied topically in the same volumes: 30 μl for rabbits and 50 μl for beagles. The following concentrations of the catecholamines were given with and without 1% IBMX (IBMX was always applied 30 minutes prior to the catecholamine): rabbits: 0.00625 -4% nE, 0.0025 - 1% Epi and 0.001 - 2% isoproterenol; beagles: 0.1% nE and 0.1% Epi. In all experiments one eye was drug-treated and the contralateral vehicle-treated eye served as control. The IOP differences between treated and contralateral vehicle-treated eyes were determined every hour and between 2 and 6 hrs after application for each group of six rabbits (30 individual measurements) and than averaged. In all of these experiments

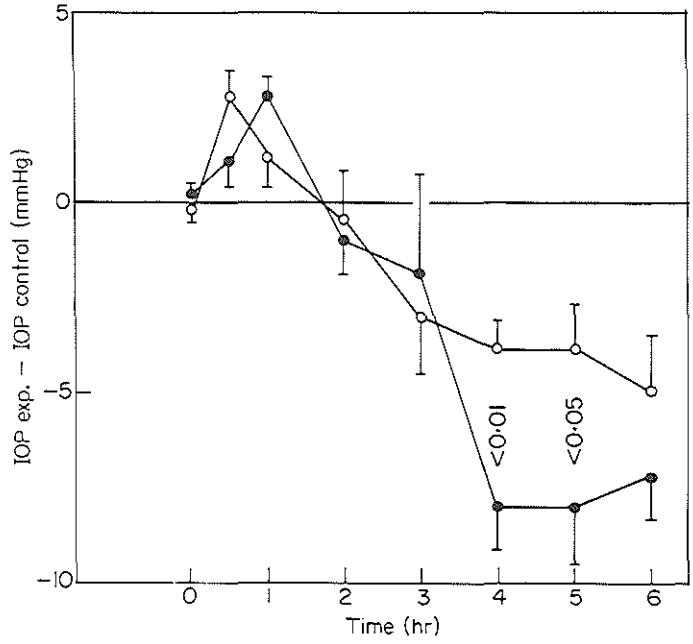

Figure 3.2 Effect of topical application of 30 μl 0.2% nE at zero time with (closed circle) and without (open) 30 μl 1% IBMX at 2 hr on intraocular pressure in two groups of six rabbits. Points represent mean values $\pm$ SEM, P-values calculated for corresponding data points.

no significant effect on IOP was observed in the contralateral vehicle-treated eyes compared to the baseline values made on the previous day.
The averaged IOP-differences (Δ IOP) found for the various concentrations of the catecholamines in the presence or absence of 1% IBMX were fitted to a log dose-response curve with the aid of a computer program. Non-linear regression analysis (proc. NLIN, SAS Institute Inc.) was applied, using the formula:

$$IOP = \frac{E_{max}}{1 + e^{A\,(\log EC_{50} - \log c)}}$$

where E_{max} is the maximum difference in IOP, EC50 the catecholamine concentration

that yields half of the maximum effect and A is a factor determining the slope of the curve. E_{max}, EC50 and A are calculated parameters.
In 9 groups of 8 rabbits aqueous humour protein and cyclic-AMP levels were determined 1, 3 and 5 hrs after topical application of 30 μl 0.1% Epi or 30 μl 1% IBMX or the two combined. Under local anesthesia, induced by two doses of 50 μl of 4% topical tetracaine, 200 μl of aqueous humour were carefully aspirated from restrained wrapped rabbits with a 27-gauge tuberculin needle, avoiding the lens and iris. The protein concentration of all aqueous humour samples was determined in duplicate in appropriate volumes according to Bradford (1976), using human serum albumin as a standard. Cyclic-AMP levels were determined in 10 μl of aqueous humour in duplicate by radioimmunoassay (New England Nuclear, Boston, MA). A non-acetylated assay condition was used. Since there was a considerable variation in aqueous humour cyclic-AMP in the individual vehicle treated control eyes and between the values of the vehicle treated control eyes of the different radioimmunoassay kits, aqueous humour cyclic-AMP was expressed as a ratio of the experimental to the control eye. (The values in untreated control eyes ranged from 2.5 p moles/ml to 41.0 p moles/ml.) The significances of the cyclic-AMP data were calculated from the means and all values are expressed $\pm$ SEM. Differences between IOP values were tested for significance with the two-tailed Student's t-test for unpaired data; aqueous humour protein and cyclic-AMP differences were tested with the two-tailed Student's t-test for paired data.

Results

Intraocular pressure

Rabbits. Baseline IOPs ranged between 20 and 25 mmHg. One percent IBMX (30 μl in AT) had no effect on IOP. Figure 3.1 shows the effect of 30 μl of 0.1% nE alone and in combination with 30 μl of 1% IBMX applied topically 30 min prior to the start of the experiment. It is clear that the effect of nE on IOP is enhanced by prior treatment with IBMX. Thirty microliters of 1% IBMX was also applied topically 2 hr after 0.2% nE administration (Figure 3.2). It can be seen that the hypotension induced in this case by 0.2% nE is also increased when IBMX is given after the topical nE. In Figure 3.3 a log dose-response curve for nE alone (open symbols) and nE in combination with 1% IBMX (closed symbols) is shown. The difference in IOP is obtained by averaging all differences between drug-treated and contralateral vehicle-treated eyes in the period between 2 and 6 hrs. This was done because the

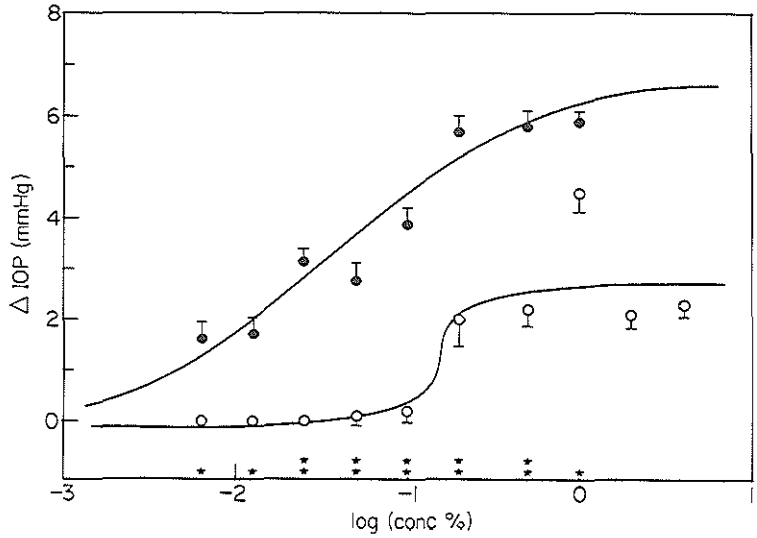

Figure 3.3 Effect of several concentrations of 30 μl nE with (closed circle) and without (open circle) 30 μl 1% IBMX, both in hydroxypropylmethylcellulose. The ordinate represents the mean of individual differences in IOP ± SEM between drug-treated and contralateral vehicle-treated eyes in the period between 2 and 6 hrs. The abcissa represents the log concentration of nE in per cent. Asterisks indicate P-value (* $P < 0.01$; ** $P < 0.005$; unpaired Student's t-test). Several groups of six rabbits were used. No effect was noted in the contralateral vehicle-treated eyes.

primary aim of glaucoma treatment is sustained ocular hypotension and not a maximum effect at a certain point in time. The E_{max} for nE was 2.9 ± 0.4 mmHg and EC_{50} was 0.2 ± 0.05%. The combination of nE with 1% IBMX gave an E_{max} of 7.4 ± 1.7 mmHg and an EC_{50} of 0.07 ± 0.03%.

Figure 3.4 shows the log dose-response curve for Epi with (closed symbols) and without (open symbols) 1% IBMX, obtained in the same way as that for nE. E_{max} was 7.3 ± 0.5 mmHg for Epi alone and 9.0 ± 0.8 mmHg for the combination. EC_{50} was 0.05 ± 0.01% for Epi alone and 0.02 ± 0.006% for Epi combined with 1% IBMX.

Figure 3.5 shows the effects of the concentrations of isoproterenol alone (open symbols) and in combination with 1% IBMX (closed symbols). The effect of isoproterenol is enhanced when the combination is given. The E_{max} was 5.1 ± 0.2 mmHg for isoproterenol alone and 6.1 ± 0.3 mmHg for the combination. EC_{50} was 0.0033 ± 0.001% for isoproterenol and 0.0022 ± 0.001% for the combination. It is interesting to observe that low doses of isoproterenol (0.01, 0.025 and 0.05%) in AT at pH 6.0 have a marked effect on IOP both when administered alone and in Combination with IBMX.

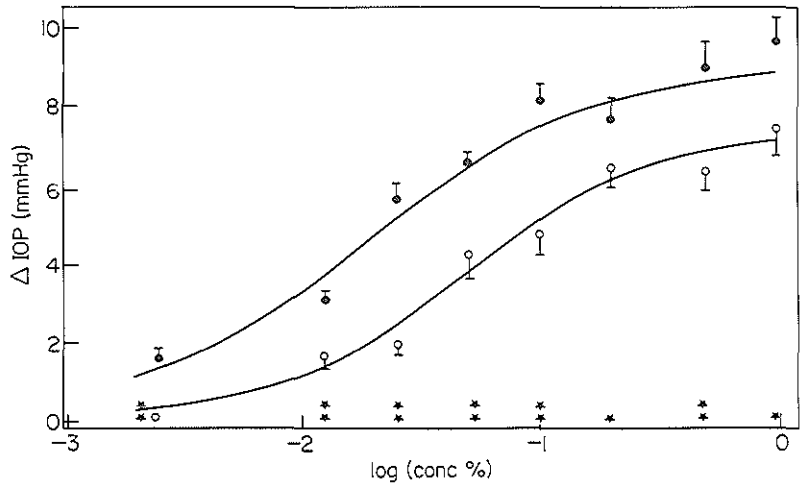

Figure 3.4 Effect of 30 μl of several concentrations of Epi with (closed circle) and without (open circle) 30 μl 1% IBMX both in 0.5% hydroxypropylmethylcellulose. The ordinate represents the mean of individual differences in IOP $\pm$ SEM between treated and contralateral vehicle-treated eyes in the period between 2 and 6 hrs. The abcissa represents the log concentration of Epi in percent. Asterisks indicate P-values (* $P < 0.05$; ** $P < 0.001$). No consensual effect was noted in the vehicle-treated eyes. Several groups of six rabbits were used.

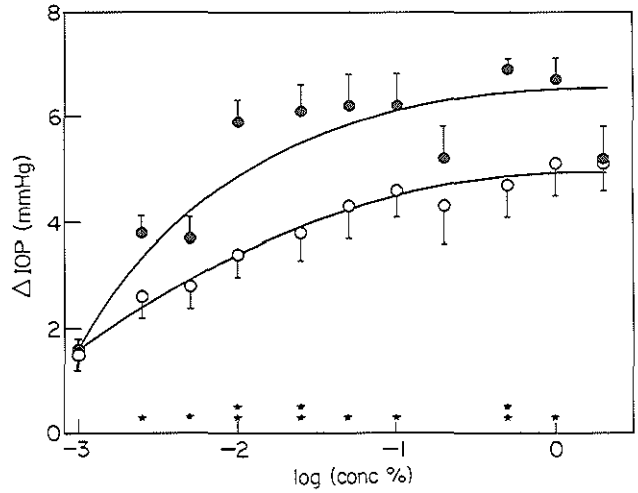

Figure 3.5 Effect of 30 μl of several concentrations of isoproterenol with (open) and without (closed circle) 30 μl 1% IBMX, both in 0.5% hydroxypropylmethylcellulose. The ordinate represents the mean of individual differences in IOP $\pm$ SEM between treated and contralateral vehicle-treated eyes in the period between 2 and 6 hrs. The abcissa represents the log concentration of isoproterenol in per cent. Significance: see Figure 3.4. No consensual effect on IOP was noted. Several groups of six rabbits were used in this experiment.

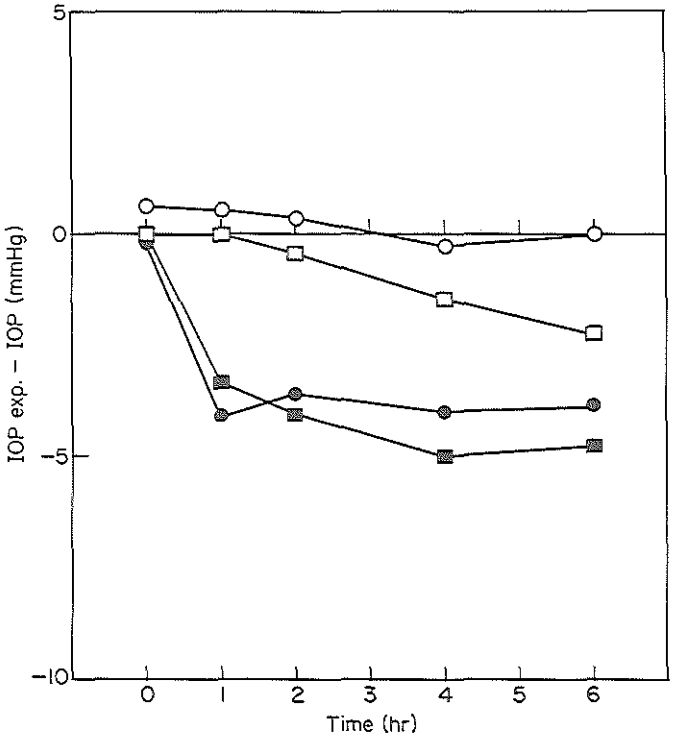

Figure 3.6 Effect of 30 μl 0.1% nE and 0.1% Epi with and without 1% IBMX on intraocular pressure in six normo-tensive beagles. The contralateral eye was vehicle-treated. Maximum SEM was 0.9 mmHg. Determination of significance see in Results. Open circles 0.1% nE; open squares 0.1% Epi; closed circles 0.1% nE in combination with 1% IBMX; closed squares 0.1% Epi with 1% IBMX.

Beagles. IBMX alone did not affect IOP. Figure 3.6 shows that the effects of 50 μl nE and Epi on IOP were significantly enhanced by 50 μl of 1% IBMX in normotensive beagles. The reduction in IOP in the period between 2 and 6 hrs was 0.1 $\pm$ 0.3 mmHg for 0.1% nE versus 3.9 $\pm$ 0.3 mmHg for the combination ($P < 0.005$) and 1.3 $\pm$ 0.3 mmHg for 0.1% Epi versus 4.6 $\pm$ 0.4 mmHg for the combination ($P < 0.005$).

Aqueous humour protein and cyclic-AMP

One, 3 and 5 hrs after 0.1% Epi, 1% IBMX or 0.1% Epi combined with 1% IBMX was applied, no significant effect on aqueous humour protein levels was observed (Table 3.1). At 1 hr the aqueous humour cyclic-AMP level was slightly elevated after administration of 1% IBMX and markedly elevated after the combination. At 3 and 5 hrs only the combination induced a marked increase in the cyclic-AMP content in the aqueous humour. The effect of 0.1% Epi alone at 5 hrs was significant but modest (Table 3.2).

Table 3.1 Aqueous humour protein

	Protein ($g.l^{-1}$)	
	Treated eyes	Contralateral control eyes
1 hr after 30 μl 0.1% Epi	0.3 ± 0.04	0.3 ± 0.03
1 hr after 30 μl 1% IBMX	0.2 ± 0.03	0.3 ± 0.04
1 hr after 30 μl 1% IBMX and 0.1% Epi	0.3 ± 0.08	0.3 ± 0.08
3 hrs after 30 μl 0.1% Epi	0.4 ± 0.08	0.4 ± 0.05
3 hrs after 30 μl 1% IBMX	0.4 ± 0.04	0.4 ± 0.04
3 hrs after 30 μl 1% IBMX and 0.1% Epi	0.5 ± 0.03	0.5 ± 0.03
5 hrs after 30 μl 0.1% Epi	0.3 ± 0.06	0.3 ± 0.06
5 hrs after 30 μl 1% IBMX	0.3 ± 0.03	0.3 ± 0.04
5 hrs after 30 μl 1% IBMX and 0.1% Epi	0.4 ± 0.1	0.4 ± 0.04

Aqueous humour protein in 9 groups of 8 rabbits. All data are mean values ± SEM.

Table 3.2 Aqueous humour cyclic AMP

	Time after treatment (hours)		
	1	3	5
With 30 μl 0.1% of Epi	1.3 ± 0.3	1.6 ± 0.4	1.3 ± 0.1*
With 30 μl of 1% IBMX	2.4 ± 0.4*	2.5 ± 1.2	1.0 ± 0.1
With the combination of 30 μl of 1 % IBMX and 0.1% Epi	24.5 ± 3.9**	4.9 ± 1.2**	2.0 ± 0.2**

Aqueous humour cAMP ratio exp/contr ± SEM in 9 groups of 8 rabbits. Asterisks indicate: * $P < 0.05$; ** $P < 0.005$.

Discussion

The results presented in this study provide the first evidence, to our knowledge, that a strong phosphodiesterase/adenosine-inhibitor enhances the pressure reduction

produced by catecholamines in rabbit and beagle eyes. IBMX alone has no effect on IOP. It is suggested that although catecholamines are continuously released from the sympathetic nerves in the iris-ciliary body, the level of intracellular cyclic-AMP is so low that IBMX is not able to increase it to the level needed for a reduction in IOP. Combining IBMX with nE results in an increase in the nE-induced reduction in IOP, and it was shown that this increase is independent of whether IBMX is given before or after nE.

The effect is most pronounced at concentrations of nE ranging from 0.025 to 0.5%. Similar results were found for Epi. The effects of Epi and IBMX are greatest when the concentrations of Epi range between 0.025 and 0.1%.

The log dose-response curves of Epi and isoproterenol reveal an enhanced maximal response after combination with IBMX. This effect of IBMX is consistent with it having an effect primarily by preventing metabolism of cAMP at the intracellular level, thereby increasing the maximal reponse. Low concentrations of isproterenol alone in AT induce a remarkable reduction in IOP, probably because the time of contact of the drug with the cornea is prolonged by hydroxypropylmethylcellulose and the pH is neutral. The maximal effect, i.e. efficacy, of nE is also enhanced if given in combination with IBMX, and it is more pronounced than that of Epi or isoproterenol. It was not expected that the effect of IBMX on nE-induced ocular hypotension would be more pronounced than that of isoproterenol. One would expect the reverse, if the effect of IBMX on catecholamine-induced hypotension was merely due to β_2-adrenoceptor-stimulated adenylyl cyclase.

However, methylxanthines are also competitive inhibitors of adenosine. In several studies it was shown that some of the effects of methylxanthines, which were attributed to adenylyl cyclase, are due to inhibition of adenosine (Fredholm, 1980; Evoniuk et al, 1986). Endogenous adenosine antagonizes some of the effects of catecholamines (e.g. by by inhibition of adenylyl cyclase), and adenosine receptor agonists have been shown to increase IOP in rabbits (Hirschfield et al, 1986).In the heart adenosine production is enhanced by exogenous catecholamines (Schrader et al, 1977). If IBMX also acts as an adenosine receptor antagonist, then the depressive effect of adenosine is inhibited, which could explain why the effect of IBMX and catecholamines on IOP is greater than with catecholamines alone. The flat shape of the nE response curve (0.01-0.1%) may reflect antagonism by adenosine which is relieved by IBMX, acting additionally as a competitive antagonist at adenosine receptors. The shift to the left which seems to be present after combination of nE with IBMX may indicate changes in drug-receptor interaction.

The catecholamine levels in the aqueous humour after combination with IBMX are not known. A major role for IBMX causing inhibition of catecholamine metabolism or increased corneal penetration is not likely since a recent study on effects on ocular

blood flow after Epi, IBMX and combined treatment revealed an increased blood flow after combined treatment, whereas Epi alone caused vasoconstriction (Busch et al, 1991c). An increased Epi content in the aqueous humour could not account for this change in ocular blood flow when the combination was used. In addition, in the present study changes primarily of the E_{max}, were observed while an increased corneal penetration or decreased catecholamine metabolism would more likely have affected the EC_{50}.

The role of cyclic-AMP in mediating the effect on IOP of catecholamines is debatable. The second messenger cyclic-AMP is known to mediate the effects of ß-adrenergic stimulation. Cyclic-AMP given intracamerally decreases IOP in rabbits (Neufeld et al, 1972a), and causes an increase in outflow facility (Neufeld and Sears, 1975; Neufeld et al, 1975). Furthermore aqueous humour inflow is reduced after stimulation of the adenylyl cyclase system of the ciliary body by ß-adrenergic agonists (Gregory et al, 1981a), cholera toxin (Gregory et al, 1981b) and forskolin (Caprioli et al, 1984a). These studies suggest an important role of cyclic-AMP in reducing IOP by either direct or indirect stimulation of adenylyl cyclase.

Several studies showed an increase in the aqueous humour cyclic-AMP level after nE (Radius and Langham, 1973) and Epi (Rowland et al, 1979; Boas et al, 1981). However, no direct time-relationship between the increase in aqueous humour cyclic-AMP and the reduction in IOP could be observed. It is known that Epi and nE, in the doses used in these studies, can produce an ocular hypertensive response (Norton and Vierstein, 1972; Lamble, 1973; Langham and Krieglstein, 1976; Rowland and Potter, 1980) which is α-adrenergic-induced (Rowland and Potter, 1980; Langham and Krieglstein, 1976). In rabbits the direct relation between aqueous humour cyclic-AMP and the response of IOP may be obscured by an initial increase in the IOP when higher doses of α-adrenergic agonists are given. This is not the case in our study. Administration of 0.1% Epi does not increase the cyclic-AMP level of the aqueous humour at 1 and 3 hrs, although there is a significant reduction in IOP at these points in time. Additionally, the combination of 0.1% Epi with IBMX results in a marked elevation of the aqueous humour cyclic-AMP level at 1 hr although there is not yet a reduction in IOP; the latter does occur, however, at 3 and 5 hrs. At those points in time there is still a five and two-fold increase, respectively, of aqueous humour cyclic-AMP levels. Although aqueous humour cyclic-AMP is suggested to be only a weak extracellular parameter of what is occurring at the intracellular level in the tissues surrounding the aqueous humour, our results show that during the ocular hypotension with the combination there is a sustained increase of aqueous humour cyclic-AMP.

In conclusion, the effect of IBMX on catecholamine-induced ocular hypotension may be due to enhancement of cyclic-AMP induced events by inhibition of the enzymatic

degradation, to antagonizing adenosine receptors, or to both acting together. Summarizing: this study shows that IBMX significantly enhances the reduction in IOP caused by catecholamines in rabbits and beagles. This finding may be of value in the treatment of glaucoma.

CHAPTER 4

SOLUBLE FORMS OF ISOBUTYLMETHYLXANTHINE ENHANCE THE OCULAR HYPOTENSION INDUCED BY CATECHOLAMINES

Michiel JWM Busch, and Philip FJ Hoyng; *published in Graefe's Arch Clin Exp Ophthalmol 229, 583-586, 1991*

Abstract

Isobutylmethylxanthine (IBMX) suspended in 0.5% hydroxypropyl methylcellulose has been shown to enhance the reduction in intraocular pressure (IOP) induced by epinephrine and norepinephrine in rabbits and beagles. In the present study, the effects of two soluble forms of IBMX, i.e. the ethylenediamine salt (IBMX-ED) and IBMX bound to cyclodextrin in saline (IBMX-CD), were studied in rabbits. When used alone, 1% IBMX-ED did not affect the IOP, but the hypotensive response to 0.1% epinephrine was enhanced dose-dependently by combination of the catecholamine with IBMX-ED. Although 1% IBMX-CD applied alone also failed to influence the IOP, the hypotensive response to epinephrine was enhanced by combination of these substances with IBMX-CD. Soluble IBMX-ED as well as soluble IBMX-CD and the IBMX suspension enhanced, to a similar extent and in a dos-dependent manner, the IOP reduction induced by catecholamines. IBMX-CD, which has a neutral pH, may be a suitable form of IBMX for future long-term experiments.

Introduction

Epinephrine and dipivalyl epinephrine are important in the management of ocular hypertension and primary open-angle glaucoma. The ability of epinephrine (Becker and Morton, 1966; Becker et al, 1961; Harris et al, 1970; and Obstbaum et al, 1974)

and dipivalyl epinephrine (Kass et al, 1979; Kohn et al, 1979; Krieglestein and Leydhecker, 1978) to lower IOP during long-term treatment has been demonstrated conclusively. In recent studies (Busch et al, 1991c; Hoyng et al, 1991/chapter 3) it was shown that isobutylmethylxanthine (IBMX), a phosphodieterase inhibitor/adenosine receptor antagonist (Daly, 1985; Fredholm, 1980; Rall, 1985), markedly enhanced the reduction of IOP induced by topical epinephrine, norepinephrine and isoproterenol in rabbits and beagles. IBMX alone did not affect the IOP. Thus, the results suggest that an interaction between IBMX and catecholamines reduces IOP, indicating that in single-drop experiments, lower doses of the catecholamines can induce marked ocular hypotension when combined with IBMX.
Long-term experiments are hampered by the instability of the IBMX suspension. Therefore, two soluble forms of IBMX, i.e. the ethylenediamine salt and IBMX bound to cyclodextrin in the form of inclusion complexes, were developed and their ability to enhance epinephrine- and norepinephrine-induced ocular hypotension in rabbits was tested and compared with the effect of the IBMX suspension. In addition, the effect of IBMX on the IOP reduction induced by dipivalyl epinephrine was studied.

Materials and methods

All studies were performed in the animal laboratory under standard light and atmospheric conditions using conscious adult pigmented rabbits (2.5-4 kg) restrained in cloth wrappers. The cornea was anaesthetized with 30 μl 0.2% oxybuprocaine (Novesine, Chibret, Riom, France), and the IOP was measured with an Alcon pneumatonometer. The pneumatonometer was manometrically calibrated for rabbit eyes and the digital readings were checked with an Alcon calibrator before and after each experiment.
Three different forms of methylxanthine were tested: 3-isobutyl-1-methylxanthine (Fluka, Buchs, Switzerland) suspended in 0.5% hydroxypropyl methylcellulose in saline (AT) using an ultrasonic probe at high power, pH 6 (IBMX-AT); 3-isobutyl-1-methylxanthine-ethylenediamine in saline, pH 9.7 (IBMX-ED); and 3-isobutyl-1-methylxanthine dissolved in saline containing 2% betacyclodextrin, pH 7 (IBMX-CD). The suspension of IBMX in AT was instilled in rabbit eyes within 15 min of its preparation. Epinephrine and norepinephrine (Sigma, St. Louis Mo.) were dissolved in AT and dipivalyl epinephrine eyedrops (Propine^R, Allergan, Irvine, Calif.) were diluted in saline; the catecholamine solutions were freschly prepared every day.
The drug was applied topically to one eye in a volume of 30 μl; the contralateral

control eye received the appropriate vehicle. IBMX-AT, IBMX-ED or IBMX-CD was applied at 30 min prior to catecholamine administration; a possible effect of cyclodextrin was tested by comparing the effect of 0.1% epinephrine dissolved in saline with that of 0.1% epinephrine dissolved in saline containing 2% beta-cyclodextrin. The same group of seven rabbits were used for an experiment carried out with and without an IBMX test substance; a 2-week interval between studies was established to ensure adequate washout.

The effect on IOP is expressed as the mean difference between treated and contralateral control eyes ± the standard error of the mean (ΔIOP ± SEM). The sustained ocular hypotension at 2-6 h after drug administration was calculated by averaging the differences in IOP ± the standard error of the mean (ΔIOP 2-6 h ± SEM). Statistical evaluation was done using Student's t-test for group means, whereby $P < 0.05$ was considered to indicate statistical significance.

Results

Baseline IOP values ranged between 20 and 25 mmHg; an effect on the contralateral control eye was not observed. When used alone, 1% IBMX-ED did not affect the IOP

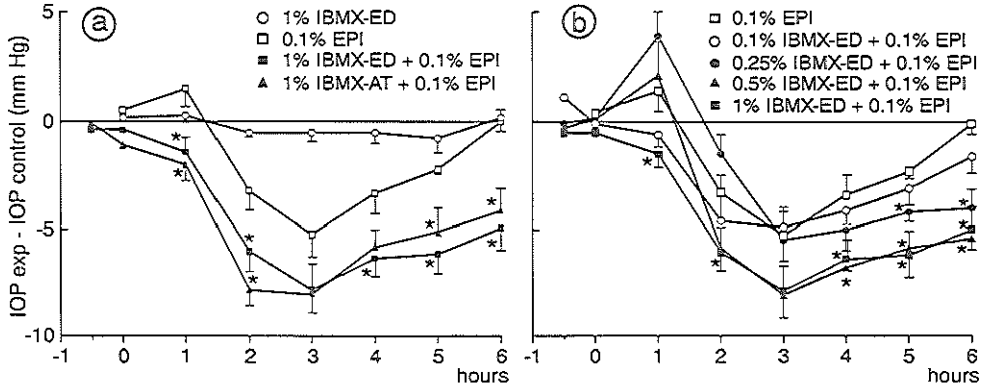

Fig. 4.1 A Effect of topical 1% IBMX-ED and 0.1% epinephrine (EPI) alone as well as topical 0.1% epinephrine in combination with either 1% IBMX-ED or 1% IBMX suspended in 0.5% hydroxypropylmethylcellulose (IBMX-AT) on the IOP in 7 rabbits. B Effect of topically applied 0.1% epinephrine alone (EPI) and 0.1% epinephrine in combination with 0.1%, 0.25% and 1% topical IBMX-ED on the IOP in 7 rabbits. The effect on IOP is expressed as the mean difference between treated and contralateral control eyes (IOP exp- IOP control, in mmHg); statistically significant differences between epinephrine alone and epinephrine in combination with IBMX-ED or IBMX-AT are indicated by asterisks. * $P < 0.05$

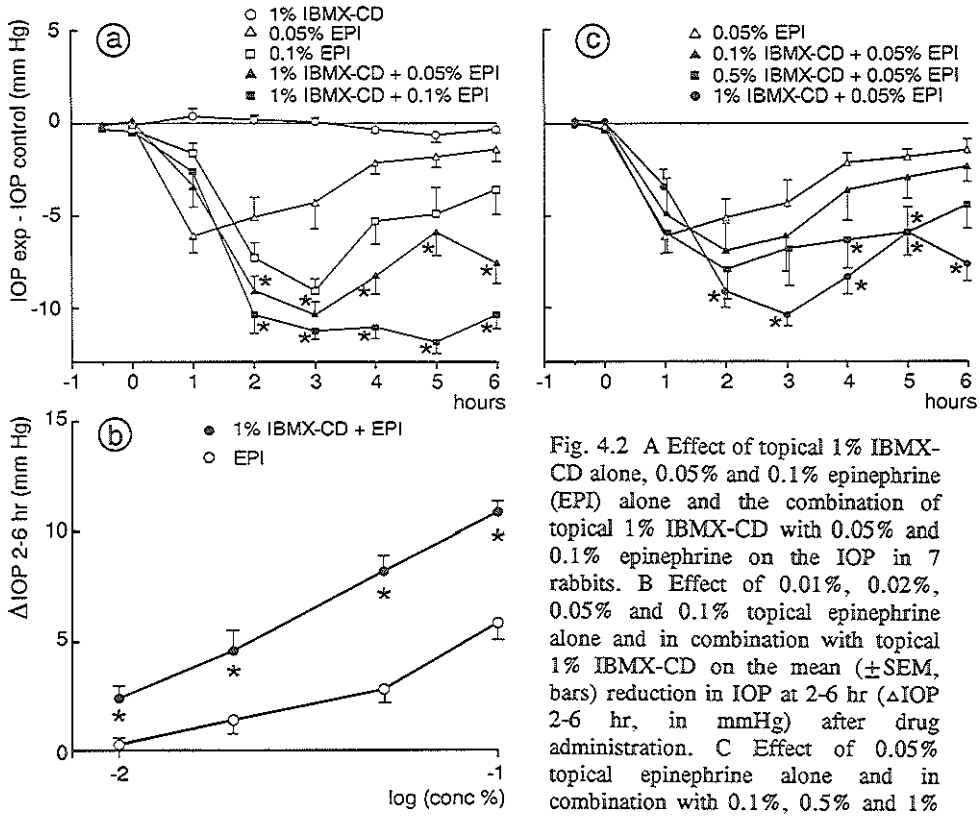

Fig. 4.2 A Effect of topical 1% IBMX-CD alone, 0.05% and 0.1% epinephrine (EPI) alone and the combination of topical 1% IBMX-CD with 0.05% and 0.1% epinephrine on the IOP in 7 rabbits. B Effect of 0.01%, 0.02%, 0.05% and 0.1% topical epinephrine alone and in combination with topical 1% IBMX-CD on the mean (±SEM, bars) reduction in IOP at 2-6 hr (ΔIOP 2-6 hr, in mmHg) after drug administration. C Effect of 0.05% topical epinephrine alone and in combination with 0.1%, 0.5% and 1% topical IBMX-CD on the IOP in 7 rabbits. * $P < 0.05$

(Fig. 4.1 A), but it enhanced the ocular hypotension induced by 0.1% epinephrine to the same extent as did 1% IBMX-AT (Fig. 4.1 A). Application of 1% and 0.5% IBMX-ED increased the mean IOP reduction observed at 2-6 hr after the administration of 0.1% epinephrine by 3.6 ± 0.6 and 3.4 ± 0.6 mmHg, respectively ($P < 0.05$). The effect of IBMX-ED on 0.1% epinephrine-induced ocular hypotension was dose-dependent (Fig. 4.1 B).

When applied alone, 1% IBMX-CD had no effect on the IOP (Fig. 4.2A); 0.1% epinephrine dissolved in saline reduced the IOP to the same extent as did 0.1% epinephrine dissolved in saline containing 2% beta-cyclodextrin (data not shown). IBMX-CD enhanced the ocular hypotension induced by 0.01%-0.1% epinephrine (Fig. 4.2 A, B), with the mean IOP reduction at 2-6 induced by 0.01%, 0.02%, 0.05% and

0.1% epinephrine being increased by 2.1 ± 0.4, 3.2 ± 0.8, 5.6 ± 0.6 and 5 ± 0.7 mmHg, respectively (all, $P<0.001$). The dose dependence of this effect is shown in Fig. 4.2C.
In addition, 1% IBMX-CD enhanced the IOP reduction iduced by 0.05% and 0.1% dipivalyl epinephrine (Fig. 4.3A) by 3.3. ± 0.6 and 3.9 ± 0.8 mmHg, respectively ($P<0.05$); when this test substance was combined with 0.1% norepinephrine, the mean enhancement of this effect at 2-6 h after catecholamine administration was 1.6 ± 0.5 mmHg ($P<0.05$, Fig. 4.3B).

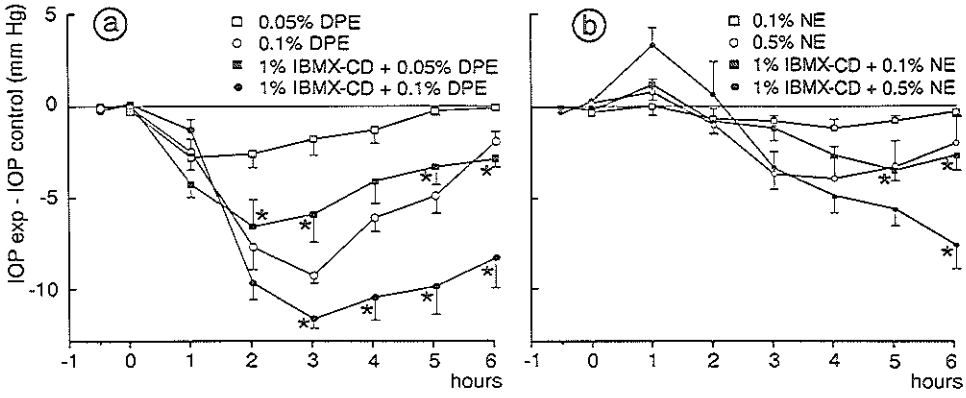

Fig. 4.3 A Effect of 0.05% and 0.1% topical dipivalyl epinephrine (DPE) alone and in combination with 1% topical IBMX CD on the IOP in 7 rabbits. B Effect of 0.1% and 0.5% topical norepinephrine (NE) alone and in combination with 1% topical IBMX-CD on the IOP in 7 rabbits. * $P<0.05$.

Discussion

In a previous study (Hoyng et al, 1991), it was demonstrated by means of dose-response curves that the ocular hypotension induced by 0.002%-1% epinephrine and 0.005%-1% norepinephrine was enhanced by combination of these catecholamines with a suspension of IBMX in 0.5% hydroxypropyl methylcellulose. The present investigation showed that IBMX-ED and IBMX-CD have a similar effect on epinephrine- and norepinephrine-induced ocular hypotension. The effect reached a maximum at 3 h after drug administration and the IOP reduction was sustained at a low level. Dose responsiveness was demonstrated for both IBMX-ED and IBMX-CD.

The formation of ethylenediamine salts of methylxanthines is a well-known method for improving the aqueous solubility of methylxantines (Rall, 1985). Cyclodextrins in general are used to enhance the solubility and/or stability of a variety of compounds through the formation of inclusion complexes (Cohen and Lach, 1963; Lach and Chin, 1964; Szejtli, 1982). Both soluble derivatives of IBMX seem to be suitable for long-term experiments to evaluate tachyphylaxis and tolerance, since IBMX-ED as well as IBMX in a cyclodextrin solution yield stable and soluble forms. In contrast, IBMX suspended in AT rapidly precipitates within 5-10 min.

It can be argued that IBMX and/or cyclodextrin may enhance corneal penetration or decrease the metabolism of catecholamines. However, the addition of cyclodextrin (2%) to the 0.1% epinephrine solution did not alter the pressure response induced by 0.1% epinephrine alone. Therefore, it is conceivable that the effect of IBMX-CD in combination with catecholamines is attributable to IBMX itself. Moreover, physiological experiments on regional ocular blood flow using the radioactively labelled microsphere technique (Busch et al, 1991c/chapter 5) showed that epinephrine alone induced slight vasodilation, whereas the combination of IBMX-CD and epinephrine induced a strong and long-lasting increase in ocular blood flow in the iris and ciliary processes. Increased corneal penetration or decreased metabolism of catecholamines cannot explain this qualitative change in response. Furthermore, dose-response curves illustrating the pressure response after the administration of epinephrine and norepinephrine showed an increased maximal effect (E_{max}) rather than a shift to the left following combination of these catecholamines with the 1% IBMX suspension (hoyng et al, 1991). Finally, a pronounced and consistent increase in the initial pressure response was not demonstrated in the present study, whereas high doses of epinephrine, dipivalyl epinephrine and norepinephrine are known to induce marked initial hypertensive responses.

The experiments carried out with and without IBMX always used the same group of rabbits, since variations occur in the response to catecholamines were noted among different groups of rabbits; a strong hypotensive response to 0.1% and 0.05% epinephrine (Fig. 4.2A) was found in the group treated with IBMX-CD as compared with the weaker response to 0.1% epinephrine seen in the group treated with IBMX-ED (Fig. 4.1A). However, when the effects of IBMX-ED and IBMX-CD were studied using equieffective doses of catecholamines, they proved to be very similar.

The effect of IBMX-CD in combination with norepinephrine was less pronounced. However, 0.5% norepinephrine induced an initial hypertensive response that was responsible for the late onset of the reduction in IOP, clearly present at 6 hr after catecholamine administration.

Dipivalyl epinephrine (DPE) is widely used for the treatment of glaucoma. This prodrug of epinephrine is known to facilitate the corneal penetration of this

catecholamine. It is interesting that IBMX-CD also increased the ocular hypotensive response to DPE.

IBMX-CD was studied more extensively than IBMX-ED; the pH of the solutions was 7 and 9.7, respectively; the IBMX-AT suspension had a pH of 6. Ophthalmic solutions with a buffered pH between 7 and 9 can generally be applied without causing pain in humans. For this reason, IBMX-CD should preferably be used in further experiments.

We conclude that a suspension of IBMX in 0.5% hydroxypropyl methylcellulose as well as IBMX-ED and IBMX-CD similarly enhance the reduction in IOP induced by catecholamines, the effect being maximal at 2-6 h after drug administration. In our opinion, IBMX-CD, which has a neutral pH and good solubility, is the preferred form of IBMX for further research on the mechanism(s) of its interaction with catecholamines in reducing IOP, and it may be of value in the treatment of glaucoma.

CHAPTER 5

INCREASE IN OCULAR BLOOD FLOW INDUCED BY ISOBUTYLMETHYLXANTHINE AND EPINEPHRINE

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Abstract

Alterations in regional blood flow of the eye were studied in rabbits using the radioactively labelled microsphere technique. The animals were topically treated with 1% IBMX, 0.1% epinephrine in combination with 1% IBMX or with 0.1% epinephrine only. After IBMX there was a tendency towards an increase in blood flow in the iris, the ciliary processes and the sclera whereas the choroidal blood flow tended to decrease. After epinephrine the iridial blood flow increased about 50% at 6 hours. After IBMX combined with epinephrine a biphasic response was obtained; an initial decrease in the blood flow of the iris, the ciliary processes and to some extent the choroid was followed by a marked and long lasting increase in blood flow from 2.5 up to 7.5 hours in the iris (up to 220%), the ciliary processes (123%) and the sclera (115%). The choroidal blood flow was not increased. The marked increase in blood flow of the iris and the ciliary processes indicates that the reduction of intraocular pressure seen after topical treatment with a combination of epinephrine and IBMX, is not based on a vascular mechanism. The increase of ocular blood flow after a combination of IBMX and epinephrine is probably based on increased cAMP secondary to inhibition of phosphodiesterases. This may indicate the presence of ß-adrenergic receptors in the ocular vasculature.

Introduction

Topical epinephrine is widely used to reduce intraocular pressure (IOP) in the treatment of glaucoma. A study of the combination of 3-isobutyl-1-methylxanthine (IBMX) and epinephrine in rabbits demonstrated that topically applied IBMX, a phosphodiesterase-inhibitor, enhanced the ocular hypotension induced by epinephrine (Hoyng and Groeneboer, 1987/chapter 3). IBMX alone did not affect IOP, but the ocular hypotensive effect of norepinephrine, dipivalylepinephrine and isoproterenol was also enhanced by prior treatment with IBMX. Thus, an interaction between catecholamines and IBMX to reduce intraocular pressure was shown.
The purpose of the present study was to investigate whether the epinephrine induced reduction of IOP, enhanced by IBMX, is based on a vascular mechanism. In addition, since very little is known about methylxanthines in the eye in general, it was thought to be of interest to study the vascular effect of IBMX in the eye.

Materials and methods

Albino rabbits of either sex, weighing 2.1-4.5 kg, were used. The animals were anaesthetized with sodium pentobarbital administered intravenously, 40-60 mg/kg b.w. Additional doses of the same anaesthetic were administered when necessary to maintain adequate anaesthesia. The animals were tracheotomized, and both the femoral arteries were cannulated, one for blood sampling and the other for blood pressure registration. The arterial blood pressure was continuously recorded during the entire experiment with a PDCR 75/1 pressure transducer (Druck Ltd) connected to a recorder (BBC, model SE 460). In two animals the blood pressure was sustained by giving 15-30 ml 3% macrodex (Pharmacia, Uppsala) intravenously. The pH, Pco_2 and Po_2 of arterial blood were analyzed in an ABL300 (Radiometer, Copenhagen) acid-base analyzer. When necessary the animal was ventilated artificially. Ventilation was adjusted to give normal values of arterial Po_2, Pco_2 and pH. If the animal suffered from marked metabolic acidosis 5-10 ml 5% sodium bicarbonate was administered intravenously. A heating pad was used to keep the animals at normal body temperature. A catheter, for the microsphere injections, was inserted into the left heart ventricle through the left brachial artery. The position of the tip of the catheter was controlled by recording the intraventricular blood pressure. All cannulas contained heparinized isotonic saline to prevent clotting.
Regional blood flow was determined with the reference flow method (Alm and Bill,

1972; Stjernschantz et al, 1976). Radioactively labelled microspheres with a size of 15 μm were obtained from New England Nuclear (Boston, Mass.). By using differently labelled microspheres it was possible to determine the blood flow at different stages of the experiment in each animal. The microspheres were labelled with 141-Ce, 113-Sn, 103-Ru or 95-Nb. Each injection contained 1-2.10^6 microspheres, suspended in 2 ml isotonic saline.

The microspheres were injected within 5-10 sec. into the left heart ventricle and from the start of the injection blood samples were collected from the femoral artery during the following 60 sec. In each animal three measurements were performed. The times of measurements were randomized between 0 and 7.5 hours after administration of the test substance. The time interval between the first and last injection was usually less than three hours. After the third sphere injection the animal was sacrificed with an overdose of pentobarbital, the eyes were enucleated and dissected and lung samples were collected as a control of microsphere recirculation. The eye was opened from the posterior pole, and the different ocular tissues were collected. The ciliary and iridial processes were scraped from the back of the iris-ciliary body preparation. The iris preparation thus contained some of the ciliary body and remnants of the ciliary and iridial processes.

The radioactivity of the blood and tissue samples was determined in a multi-channel gamma spectrometer (1282 Compugamma Wallac LKB-Pharmacia). The blood flow in the different tissues of the eye was calculated by dividing the radioactivity of the tissue sample with that of the reference blood sample obtained during a one minute period, multiplied with the weight of the blood sample. The resulting blood flow was expressed as mg/min/tissue.

Three different drug regimens were tested; in group 1 consisting of 13 animals only 1% IBMX was tested; in group 2 consisting of 14 animals the combination of 1% IBMX and 0.1% epinephrine was tested. IBMX was administered 30 min prior to epinephrine and the time was set to zero at the IBMX administration; in group 3 consisting of 6 animals 0.1% epinephrine was tested. One eye, randomly left or right, was treated and the contralateral eye received only the vehicle. In group 3 with administration of epinephrine only, the IBMX vehicle was administrated 30 min prior to epinephrine in both eyes. IBMX (Fluka, Buchs, Switzerland) was dissolved in a phosphate buffered solution containing 2% betacyclodextrin (Avebe, Groningen, the Netherlands). Epinephrine eye drops (1%) (Eppy^R, Pharmacia, Uppsala, Sweden) were freshly diluted with isotonic saline to 0.1%. All test substances were applied topically in a volume of 30 μl.

The results are expressed as the arithmetical mean value $\pm$ standard error of mean (Mean $\pm$ SEM). The change in blood flow i.e. differences between the experimental eye and contralateral control eye was expressed as percentage of the blood flow in the

control eye. Statistical evaluation of the results between treated and contralateral control eyes was performed using the matched pair t-test. Significances between different groups were calculated using the t-test between group means. $P < 0.05$ was taken as statistically significant.

Table 5.1 Blood flow in control eyes after IBMX

Time (h)	N	MABP (mmHg)	Choroid ($mg.min^{-1}$)	Sclera ($mg.min^{-1}$)	Ciliary processes ($mg.min^{-1}$)	Iris ($mg.min^{-1}$)
0	6	85 ± 3	1190 ± 97	43 ± 13	146 ± 10	125 ± 19
0.75	5	88 ± 3	1198 ± 117	43 ± 8	173 ± 22	155 ± 19
1.5	6	87 ± 4	1016 ± 101	32 ± 7	178 ± 33	135 ± 19
2.5	4	81 ± 5	1042 ± 89	49 ± 10	106 ± 8	147 ± 12
3.5	5	85 ± 5	1002 ± 61	45 ± 9	107 ± 13	142 ± 28
4.5	4	83 ± 5	949 ± 97	49 ± 11	105 ± 15	136 ± 35
5.5	4	89 ± 3	1015 ± 143	68 ± 16	69 ± 3	58 ± 11
6.5	3	86 ± 4	911 ± 186	45 ± 10	64 ± 5	59 ± 15
7.5	1	80	730	64	56	58

Blood flow in different tissues of the contralateral control eyes of animals treated with 1% IBMX (mean ± SEM). Time in hours after drug application. MABP, mean arterial blood pressure.

Table 5.2 Blood flow in control eyes after IBMX + epinephrine

Time (h)	N	MABP (mmHg)	Choroid ($mg.min^{-1}$)	Sclera ($mg.min^{-1}$)	Ciliary processes ($mg.min^{-1}$)	Iris ($mg.min^{-1}$)
1.0	3	88 ± 4	1155 ± 98	61 ± 14	126 ± 10	134 ± 30
1.5	4	86 ± 3	1012 ± 95	55 ± 11	121 ± 10	144 ± 36
2.0	4	88 ± 5	1038 ± 187	41 ± 10	116 ± 6	133 ± 19
2.5	5	79 ± 6	1261 ± 328	74 ± 30	68 ± 13	145 ± 72
3.5	6	85 ± 7	1058 ± 129	54 ± 16	79 ± 22	127 ± 30
4.5	7	78 ± 5	900 ± 128	43 ± 9	66 ± 12	97 ± 18
5.5	5	96 ± 6	1038 ± 79	79 ± 28	101 ± 9	154 ± 34
6.5	5	93 ± 7	1043 ± 93	80 ± 22	98 ± 8	162 ± 35
7.5	2	88 ± 8	1117 ± 36	133 ± 4	89 ± 16	264 ± 57

Blood flow in contralateral control eyes of animals treated with 1% IBMX and 0.1% epinephrine (mean ± SEM). Time in hours after drug application. MABP = mean arterial blood pressure.

Results

The blood flow values in the different tissues of the contralateral control eyes in the IBMX, IBMX-epinephrine and epinephrine groups are presented in Tables 5.1, 5.2 and 5.3, respectively. The percentual changes in blood flow of the iris, the ciliary processes, the sclera and the choroid in the experimental eyes compared with the contralateral control eyes are presented in the Figures 5.1, 5.2, 5.3 and 5.4.
IBMX alone showed a tendency towards increased blood flow in the ciliary processes, the iris and the sclera. Pooled data from the increased level at 1.5-4.5 hours showed a significant change of 63 $\pm$ 19% ($P < 0.005$) in the iris, 24 $\pm$ 8% ($P < 0.01$) in the ciliary processes and 50 $\pm$ 18% ($P < 0.05$) in the sclera. After 4.5 hours no effect was seen. On the contrary the choroidal blood flow tended to decrease slightly.
Combined treatment with IBMX and epinephrine induced an initial decrease in blood flow, followed by a marked increase ranging from 3.5 to 7.5 hours in the iris, the ciliary processes and the sclera. The maximal increase was 220 $\pm$ 50% ($P < 0.05$), 123 $\pm$ 32% ($P < 0.01$) and 115 $\pm$ 29% ($P < 0.01$), respectively. The choroidal blood flow was only slightly affected with a small, but statistically significant, decrease at 1.5 hour.
The blood flow after epinephrine alone was studied at 4.5, 5.5 and 6.5 hours after administration of the drug. During this time period there was only an increase in the iridial blood flow which was 50 $\pm$ 13% ($P < 0.05$) 6.5 hours after administration of epinephrine.

Table 5.3 Blood flow in control eyes after epinephrine

Time (h)	N	MABP (mmHg)	Choroid ($mg.min^{-1}$)	Sclera ($mg.min^{-1}$)	Ciliary processes ($mg.min^{-1}$)	Iris ($mg.min^{-1}$)
4	6	93 $\pm$ 2	1048 $\pm$ 140	61 $\pm$ 13	92 $\pm$ 9	97 $\pm$ 20
5	6	91 $\pm$ 2	885 $\pm$ 106	52 $\pm$ 12	92 $\pm$ 5	82 $\pm$ 18
6	6	88 $\pm$ 2	842 $\pm$ 131	43 $\pm$ 10	93 $\pm$ 10	91 $\pm$ 21

Blood flow in contralateral control eyes of animals treated with 0.1% epinephrine (mean $\pm$ SEM). Time in hours after drug application. MABP, mean arterial blood pressure.

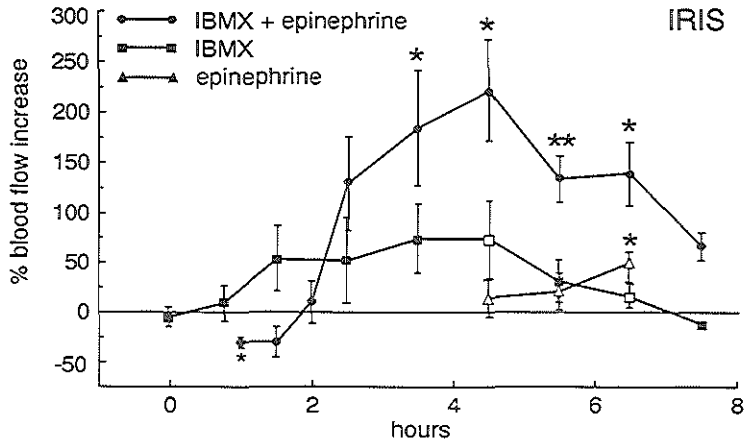

Figure 5.1 Effect of 1% IBMX, 0.1% epinephrine, and the combination of 1% IBMX and 0.1% epinephrine on blood flow in the iris. The change in blood flow is expressed as percentage of the blood flow in the contralateral control eye (mean ± SEM) * $P < 0.05$, ** $P < 0.01$. Significantly different effects between 0.1% epinephrine or 1% IBMX and the combination of 1% IBMX and 0.1% epinephrine are indicated in the former curves with open symbols ($P < 0.05$). The increase in blood flow after combination of IBMX and epinephrine was statistically significantly different from that of IBMX alone at various points in time as seen in Figures 1-3 ; the difference compared to epinephrine alone was also statistically significant in the iris, the ciliary processes and the sclera (Figures 1-3).

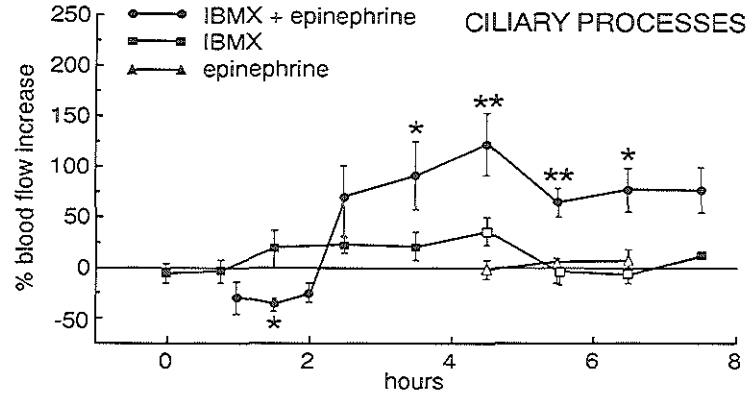

Figure 5.2 Effect on blood flow in the ciliary processes of 1% IBMX, 0.1% epinephrine and the combination of both. For legends see Figure 5.1.

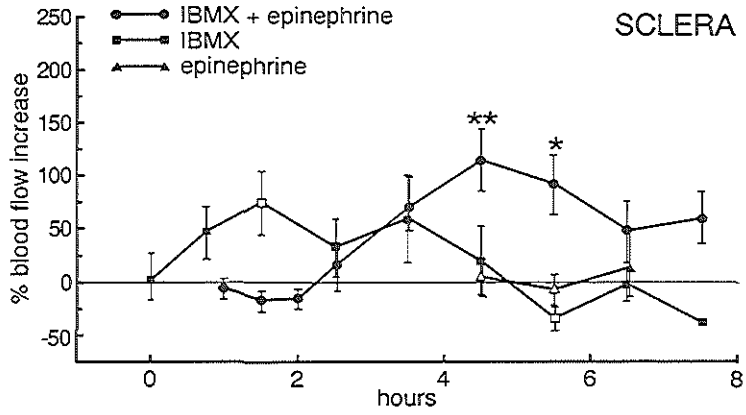

Figure 5.3 Effect on blood flow in the sclera of 1% IBMX, 0.1% epinephrine and the combination of both. For legends see Figure 5.1.

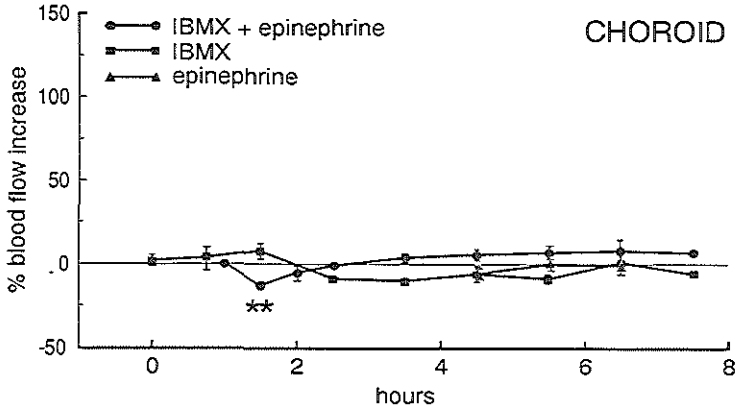

Figure 5.4 Effect on blood flow in the choroid of 1% IBMX, 0.1% epinephrine and the combination of both. For legends see Figure 5.1.

Discussion

The radioactively labelled microsphere technique is a well established method for measurement of regional blood flow. If the interval between the first microsphere

injection and the third microsphere injection is long, a recirculation of microspheres from the peripheral tissues could theoretically occur. Therefore, lung samples were obtained from each animal and analysis of these confirmed that practically no recirculation had occurred, when comparing the values obtained with the first and the last microsphere injection. This indicates that a three hour time interval between the first and last microsphere injection was acceptable. Moreover, any recirculation can be expected to affect both eyes to a similar extent and should therefore not influence the results with respect to differences in blood flow between the eyes. In the rabbit the retinal circulation is very poorly developed. Therefore, the blood flow of the retina was not determined.

The main objective of this study was to determine whether changes in the haemodynamics could explain the IBMX-enhanced reduction of intraocular pressure compared to epinephrine alone. Epinephrine is known to induce marked vasoconstriction in the anterior uvea of monkeys (Alm, 1980; Caprioli et al, 1984) and rabbits (Morgon et al, 1981), and a marked vasoconstriction would tend to lower the IOP. Vasodilation, however, cannot reduce the IOP. Indeed, 1 % IBMX alone induces a moderate increase in blood flow but is not capable of lowering the IOP (Hoyng and Groeneboer, 1987). The moderate decrease and following increase in blood flow of the anterior uvea after IBMX in combination with epinephrine indicate that changes in haemodynamics do not play a causative role in the IBMX enhanced reduction of the IOP to epinephrine. Control experiments with indomethacin pretreated rabbits (not included in Results) showed that the vasodilatory response of epinephrine and IBMX could not be prevented by cyclooxygenase inhibition, thus making an involvement of prostaglandins in this response unlikely. Increased uveal blood flow concomitant with a reduction in the IOP may be explained on the basis of an increased perfusion pressure (Bill, 1975), but cannot account for the marked increase in blood flow seen in the iris and the ciliary processes since similar and significant choroidal vasodilation is not observed.

Epinephrine has been shown to induce vasodilation in the anterior uvea of rabbits after an initial vasoconstriction (Morgon et al, 1981). A 60 times higher dose of epinephrine was used however compared with the present study, and a 2.5 fold increase in blood flow was measured only in the iris, 3 hours after administration. In the group treated with 0.1 % epinephrine alone in this study a moderate vasodilation was seen at 6 hrs in the iris only. Early measurements were not performed in the epinephrine group since the vasoconstrictive response has been well documented. It is most likely that the initial decrease in blood flow after IBMX combined with epinephrine was due to the effect of epinephrine. Analysis of the results shows that the vasodilation after IBMX alone was not statistically significant at any individual time point, due to a fairly large variation in results and the small number of animals tested. Some animals

showed a marked vasodilation, some responded less. The tendency, however, of IBMX to induce vasodilation in the eye is demonstrated. In contrast, all animals treated with IBMX combined with epinephrine responded clearly with vasodilation, 3.5 up to 7.5 hours after treatment. Thus, it appears to be a very constant phenomenon. The marked increase in blood flow after IBMX with epinephrine cannot be explained on the basis of an additive effect of IBMX to epinephrine because the individual treatment responses to IBMX alone and epinephrine alone are too weak and the onset of the vasodilation after the combination is too early.

Except for the cerebral circulation, the effect of methylxanthines on the peripheral vasculature generally is vasodilation (Rall, 1982; Rutherford et al, 1981). Thus, methylxanthines seem to have a similar effect in the anterior uvea of the eye, although relatively weak. Methylxanthines are known to have two major cellular effects. They inhibit intracellular phosphodiesterases and they antagonize adenosine receptors (Rall, 1982; Rall, 1985). The effect of adenosine on the peripheral vasculature is vasodilation (Rall, 1982; Daly, 1985) and data supporting a similar effect in the eye have recently been presented (Braunagel et al, 1988; Campochiaro and Sen, 1989). 8-Phenyltheophylline, an adenosine receptor antagonist, blocked vasodilation in the anterior uvea after combined treatment with adenosine and dipyridamol applied intravitreally. Dipyrimadol is an adenosine-re-uptake inhibitor (Braunagel et al, 1988). Thus, it seems less likely that the mechanism responsible for the increased blood flow in the anterior uvea after IBMX and IBMX in combination with epinephrine is based on antagonism of adenosine receptors. One would therefore be inclined to interpret the mechanism of IBMX-epinephrine induced blood flow increase as being based on an adenylate cyclase mediated process. Furthermore, we have found that the cAMP content in the aqueous humor after topical treatment with IBMX and epinephrine is markedly increased compared to IBMX and epinephrine alone (unpublished data). Our data suggest potentiation of a beta adrenergic vasodilation by IBMX. So far, however, evidence for beta adrenergic receptors in the intraocular vasculature is very sparse (Bill, 1975; Nathanson, 1981; Trope, 1982; Ferrari-Dileo, 1988; Martin and Rabineau, 1989; Denis, 1989) and a systematic study to investigate this aspect seems warranted.

The slight increase in the choroidal blood flow seen after combination of IBMX and epinephrine may be explained by a reduction in the IOP resulting in an increased perfusion pressure. A reduction in the IOP of 3-4 mmHg can be anticipated to increase the choroidal blood flow with 5-10% since autoregulation of blood flow is essentially lacking in the choroid (Bill, 1975). The cause of the tendency towards a slight decrease in the choroidal blood flow after IBMX alone remains unclear. One may speculate that a contributing factor could be shunting of blood from the anterior part of the choroid to the anterior uvea. In fact, a 30-60% increase in the anterior uveal

blood flow could be anticipated to reduce total choroidal blood flow with around 5-10%.

In conclusion, the results of the present study indicate that the epinephrine induced reduction of the IOP, enhanced by IBMX, is not based on a vascular mechanism. The methylxanthine IBMX in combination with epinephrine induces a strong and long lasting increase in blood flow of the iris, the ciliary processes and the sclera, a phenomenon that best can be characterized as a potentiation probably based on increased cAMP due to inhibition of phosphodiesterases.

CHAPTER 6

AQUEOUS HUMOUR DYNAMICS AFTER TOPICAL ISOBUTYLMETHYLXANTHINE AND EPINEPHRINE IN RABBITS

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Abstract

Combination drug treatment of topical isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, with topical epinephrine has demonstrated that IBMX enhances the ocular hypotensive response induced by epinephrine in rabbits. The present study investigates the site of action for this effect of IBMX by measuring outflow facility by indentation tonography and aqueous humour production by fluorophotometry.

It was observed that topical 1% IBMX, 1% epinephrine, and 0.05% dipivalyl epinephrine alone did not individually affect outflow facility; however, combined treatment of 1% IBMX with 1% epinephrine, and 1% IBMX with 0.05% dipivalyl epinephrine increased outflow facility by 43% ($P < 0.01$) and 32% ($P < 0.05$), respectively.

Topical 0.05% dipivalyl epinephrine alone induced a reduction of flow between 3 and 6 hours after medication, from 3.0 ± 0.3 to 2.6 ± 0.4 $\mu l.min^{-1}$ ($P < 0.05$). Flow after 1% IBMX in combination with 0.05% dipivalyl epinephrine was 3.2 ± 0.1 $\mu l.min^{-1}$. This was not different from baseline flow but higher than after dipivalyl epinephrine alone ($P < 0.05$).

It is concluded that IBMX potentiates the ocular hypotensive effect of epinephrine by increasing the effect of epinephrine on outflow facility and not by decreasing aqueous humour flow. These in vivo findings lend support to a role for adenylyl cyclase in controlling outflow facility in rabbits, particularly in response to epinephrine.

Introduction

Topical epinephrine is widely used to reduce intraocular pressure (IOP) in the treatment of glaucoma. Combination drug studies with topical 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, and topical epinephrine in rabbits have shown that IBMX enhances the ocular hypotensive response elicited by epinephrine (Hoyng et al, 1991; Busch and Hoyng, 1991b). IBMX itself did not affect IOP. This effect of IBMX was also found for combination of IBMX with dipivalyl epinephrine, norepinephrine, and isoproterenol. Thus, an interaction between catecholamines and IBMX to reduce IOP was demonstrated.
Adenylyl cyclase/cyclic AMP is thought to play an important role as second messenger system in the regulation of aqueous humour secretion by ciliary processes (Waitzman and Woods, 1971; Sears et al, 1981; Neufeld et al, 1972a; and Cepelic and Cernohorsky, 1981). Increasing evidence accumulates that cAMP is also important in controlling outflow mechanisms in the trabecular meshwork, particularly in response to adrenergic drugs such as epinephrine (Neufeld et al, 1972a, Neufeld et al, 1975). IBMX prevents metabolism of cAMP (Rall,1982), and may conceivably increase effects of epinephrine in trabecular meshwork as well as in ciliary processes.
The purpose of the present study was to investigate whether IBMX potentiates the ocular hypotensive response of epinephrine by increasing outflow facility and/or by reducing aqueous humour formation.

Materials and methods

Drug preparation

3-Isobutyl-1-methylxanthine (IBMX) (Fluka, Buchs, Switzerland) was dissolved in saline containing 2% beta-cyclodextrin. A 1% IBMX solution was thus prepared. L-epinephrine bitartrate (Sigma) and dipivalyl epinephrine (Propine^{R}, Allergan) were dissolved or diluted in 0.5% hydroxypropyl methylcellulose. Drugs were topically

administered in a 30μl volume. IBMX was applied 30 minutes prior to (dipivalyl) epinephrine. The contralateral eyes received the appropriate vehicle.

Measurement of outflow facility by indentation tonography

Outflow facility was non-invasively measured by indentation Schiötz tonography using an electronic Schiötz tonograph (Berkeley, CA). Animals were anaesthetized with 15mg.ml^{-1} thiopental sodium, slowly infused i.v. into a cannulated marginal ear vein. Additionally, one drop 0.2% oxybuprocaine was administered topically to the eye. The tonograph was placed on the corneal surface during a 4 minute period. The tonograph was calibrated before and after each individual measurement. The C-value was estimated using the 1955 Friedenwald Tables, modified by Moses. C-values were determined in three groups of 8 rabbits, in one eye, 4 hours after topical administration of 0.2%, 1% epinephrine or 0.05% dipivalyl epinephrine. One or several days prior to the experiment baseline C-values were determined in the same eyes. After a 2 week wash-out period the experiments were repeated applying topically 1% IBMX 30 minutes prior to 0.2%, 1% epinephrine or 0.05% dipivalyl epinephrine. The same experimental eyes were used. In 8 rabbits the effect of 1% IBMX alone was measured.

Measurement of aqueous flow by fluorophotometry

Aqueous flow was determined with a computer fluorophotometer (Fluorotron Master, Coherent Radiation Inc; Palo Alto, CA), equipped with an anterior segment adaptor for detailed scanning of the anterior segment of the eye. The fluorescence excitation wavelength was 430nm to 490nm, and the fluorescence range was 510nm.
Ten drops of a 2% fluorescein solution in saline were instilled into the lower conjunctival sac of both eyes at a rate of 1 drop every two minutes. Ten minutes after instillation of the last drop both eyes were flushed 5 times with saline. Flow measurements were performed starting 4 hours after fluorescein instillation to obtain homogenous fluorescein distributution in the cornea. Four consecutive measurements of the fluorescein concentration in the cornea and anterior chamber were performed at half hour intervals during a 3 hours period. Measurements were corrected for autofluorescence and spatial resolution. The corneal thickness of each rabbit was measured by pachymetry and the corneal volume was calculated using pachymetric measurements at the limbus and at the center of the cornea. The anterior chamber volume was calculated using the anterior chamber depth and the inner corneal radius

(v Genderen et al, 1988). Both values were estimated from histologic sections of a rabbit eye.
The average aqueous flow values between 4 and 5.5 hours and between 5.5 and 7 hours after fluorescein instillation were calculated from the decay of fluorescein concentration in the cornea and the anterior chamber according to a method described previously (v Genderen et al, 1988). This method is based on the assumption that all fluorescein in the corneal stroma diffuses homogeneously into the anterior chamber, from which it vanishes by aqueous flow only (Gaul and Brubaker, 1985). It was further assumed that a steady state of fluorescein transfer did occur 4 hours after fluorescein instillation, resulting in an identical slope of fluorescein concentration decay in the cornea and anterior chamber.
A group of 7 rabbits was used for the flow experiments. One eye received topically 30μl 0.05% dipivalyl epinephrine or 30μl 1% IBMX combined with 30μl 0.05% dipivalyl epinephrine. The contralateral eye received the vehicle(s). This medication was administered 1 hour after fluorescein instillation, i.e. 3 hours before starting with flow measurements. Flow values were assessed in both eyes. Additionally, baseline flow values were determined in the same eyes that were used for testing drug effects. An interval of 14 days was allowed to permit adequate washout between experiments.

Statistics

Tonographic data were subjected to a two-tailed Student's t-test for paired observations; the non-parametric Wilcoxon paired test was used for flow data. A P-value less than 0.05 was considered statistically significant.
The investigations adhered to the the ARVO resolution on the Use of Animals in Research.

Results

Tonography

Values for tonographic outflow facility are presented in Table 6.1. Topical 1% IBMX alone, topical 0.2 and 1% epinephrine alone, and topical 0.1% dipivalyl epinephrine alone did not affect the C-value individually, relative to their baseline values. However, combination treatment of 1% epinephrine with 1% IBMX, and 0.05% dipivalyl epinephrine with 1% IBMX increased the C-value by 43% ($P < 0.01$) and

32% ($P < 0.05$), respectively. This effect can be characterized as potentiation since these drugs had no effect individually at the doses used. The effect of 0.2% epinephrine on the C-value was not significantly affected by prior treatment with 1% IBMX. Only a tendency was observed reflected by the relative large s.e.m. value; some animals responded with an increase while others had no effect.

Table 6.1 Tonographic facility of outflow

	Facility of outflow in $\mu l.min^{-1}.mmHg^{-1}$		
	baseline	treated eye	difference
1% IBMX	0.21 ± 0.01	0.22 ± 0.02	0.01 ± 0.02
0.2% epinephrine	0.20 ± 0.02	0.22 ± 0.03	0.02 ± 0.03
1% IBMX + 0.2% epinephrine	0.20 ± 0.02	0.23 ± 0.03	0.03 ± 0.04
1% epinephrine	0.21 ± 0.01	0.20 ± 0.01	-0.02 ± 0.01
1% IBMX + 1% epinephrine	0.21 ± 0.01	0.30 ± 0.02	0.09** ± 0.02
0.05% dipivalyl epinephrine	0.21 ± 0.01	0.22 ± 0.01	0.01 ± 0.01
1% IBMX + 0.05% dipivalylepinephrine	0.21 ± 0.01	0.28 ± 0.03	0.07* ± 0.03

Mean facility of outflow ± s.e.m., measured by indentation tonography 4 hour after topical drug treatment in three groups of eight rabbits (n=8). The same rabbits were used for measurements in the presence and absence of 1% IBMX. * $P < 0.05$, ** $P < 0.001$; two tailed t-test for paired observations.

Fluorophotometry

On examination with blue light no corneal lesions were observed. The mean corneal thickness measured in all rabbits by pachymetry was 0.348 mm. The anterior chamber depth estimated from histological sections of one rabbit eye amounted to 3.42 mm. The anterior chamber volume was calculated to be 210μl (v Genderen et al, 1988). Values for aqueous flow are presented in Table 6.2. Aqueous flow slightly decreased after topical 0.05% dipivalyl epinephrine administration as compared to the contralateral vehicle treated eye; the mean decrease was 11 ± 2.7 % ($P<0.05$). Combined treatment of dipivalyl epinephrine and IBMX had no net effect on flow; in fact, the flow reducing effect of dipivalyl epinephrine was abolished. Flow in the contralateral control eyes was not different from baseline flow values, indicating that no significant contralateral drug induced effects were present.

Table 6.2 Aqueous flow assessed by fluorophotometry

		average aqueous flow in $\mu l.min^{-1}$		
		experimental eye	contralateral eye	difference
	vehicle (n=7)	3.0 ± 0.3	-	-
0.05%	dipivalyl epinephrine (n=6)	2.6 ± 0.4	3.0 ± 0.4	0.4* ± 0.1
1%	IBMX + 0.05% dipivalyl epinephrine (n=6)	3.2 ± 0.1	3.2 ± 0.2	0.02 ± 0.2

Average aqueous flow ± s.e.m. between 3 and 6 hours after topical drug treatment to one eye in $\mu l.min^{-1}$, measured by fluorophotometry in one group of 7 rabbits. A two week wash-out period was allowed between experiments. * $P < 0.05$, Wilcoxon test for paired measurements.

Discussion

The present results demonstrate that IBMX enhances the ocular hypotensive effect of epinephrine by increasing the effect of epinephrine on outflow facility and not by decreasing aqueous humour production.

The increase of tonographic outflow facility may conceivably be interpreted as a qualitative increase of trabecular outflow facility. Tonography is known to measure pressure dependent changes of outflow while uveoscleral flow is probably not pressure sensitive. Moreover, uveoscleral flow in rabbits has been reported to be less then 2% under physiologic conditions (Bill, 1989). An increase of outflow facility is therefore not likely due to an enhanced uveoscleral outflow. Major changes due to pseudofacility are also not likely, since neither epinephrine, IBMX, nor the combination of both disrupted the blood-aqueous barrier as reflected by normal aqueous protein levels, observed in a previous study (Hoyng et al, 1991).

Epinephrine has been demonstrated to reduce IOP in rabbits mainly by increasing outflow facility (Eakins, 1963; Lamble, 1974). Evidence from various sources indicates that cAMP plays an important role as a second messenger mediating the increase of outflow facility (Neufeld et al, 1972a, 1975; Potter and Rowland, 1978). The major cellular action of IBMX is the inhibition of phosphodiesterases, which leads to an inhibition of cAMP degradation. Moreover, a variety of $ß_2$-adrenoceptor/cAMP mediated physiological responses has been reported to be increased by methylxanthines

(Rall, 1982). The current results lend support to the proferred physiological role of adenylyl cyclase in regulating outflow facility, particularly in response to epinephrine. IBMX alone had no effect on intraocular pressure (Hoyng et al, 1991) and also did not affect outflow facility in the present experiments. Comparable findings have also been observed for forskolin, a direct stimulator of adenylyl cyclase by activating the catalytic unit. Forskolin had no effect on outflow facility by itself, but potentiated the outflow increasing effect of isoproterenol (Caprioli, 1984a).

Aqueous humour cAMP levels have been considered to play a role in affecting outflow facility (Neufeld et al, 1975). Kaufman (1987) demonstrated in monkeys that infusion of exogenous cAMP into the anterior chamber increased outflow facility. On the other hand, a close time relationship between aqueous cAMP levels and the intraocular pressure response could not be demonstrated (Boas et al, 1981). Previous observations (Hoyng et al, 1991) showed slightly raised aqueous cAMP levels after epinephrine and IBMX alone. However, combination treatment markedly enhanced the aqueous cAMP levels one hour after medication whereas after 3 hours this effect was levelled off. At that time point the IOP effect was maximal. This suggests that a close time-relationship between aqueous cAMP levels and the outflow facility induced intraocular hypotensive response is not likely.

In rabbit ciliary processes AC stimulation by epinephrine has been well documented (Waitzman and Woods, 1971; Neufeld et al, 1972a; Cepelic and Cernohorsky, 1981). However, contradictory effects on aqueous flow have been reported for epinephrine, and also for other ß-selective adrenergic agonists and non-adrenergic stimulators of adenylyl cyclase. Employing an invasive inuline diluting method (Lamble, 1977) an initial decrease of flow was observed after topical epinephrine. Some studies using fluorophotometric techniques report an increase, others a decrease of aqueous flow due to stimulation of ß-adrenergic receptors. For example, ß-blockade with timolol reduces intraocular pressure and aqueous flow only during the dark (Gregory, 1990; Yoshitomi and Gregory, 1991) when aqueous levels of cAMP (Rowland et al, 1986) and endogenous norepinephrine are high (Liu and Dacus, 1991, Liu et al 1991). From these findings it was concluded that ß-adrenoceptor stimulation increases aqueous flow. On the other hand, Araie (1985) found that salbutamol reduced aqueous flow 1 to 4 hours after drug instillation. In humans and primates ß-adrenergic stimulation increases flow (Townsend and Brubaker, 1980; Schenker et al, 1981; Coakes and Siah, 1984; Nilsson et al, 1990); but direct activators of AC such as cholera toxin (Gregory et al, 1981b) and forskolin (Caprioli, 1984a) can reduce flow.

The present experiments show that IBMX abolished an 11% flow reduction after epinephrine. This tendency to increase flow cannot account for the potentiation of the ocular hypotensive effect of epinephrine by IBMX. Epinephrine is predominantly a ß-selective adrenergic agonist. Theoretically, IBMX can be expected to increase this

flow reducing effect, but an adverse response was observed. Several explanations may be profferred. Desensitization of ß-receptors has been described in rabbit ciliary processes when using high doses of epinephrine (Neufeld et al, 1978; Mittag and Tormay, 1981). IBMX could conceivebly accelerate this process of desensitization. Another factor may be found in adverse effects of epinephrine alone and in combination with IBMX on local haemodynamics. A 2-fold increase of basal blood flow in ciliary processes was observed after combination of epinephrine with IBMX; epinephrine alone however, induced vasoconstriction. This was previously found using the radioactively labelled microsphere technique (Busch et al, 1991c). The marked increase of blood flow in ciliary processes, probably due to beta-mediated relaxation of the vascular bed, could well increase the ciliary perfusion rate which may in turn increase the aqueous humour formation rate. This would oppose the slight flow reducing effect of epinephrine alone.
Summarizing, IBMX potentiates the ocular hypotensive effect of epinephrine by increasing the effect of epinephrine on outflow facility and not by further inhibition of aqueous humour formation. The present results provide in vivo evidence for a role for AC in controlling outflow facility, particularly in response to epinephrine.

CHAPTER 7

POTENTIATION OF OCULAR HYPOTENSIVE RESPONSE OF CATECHOLAMINES BY ISOBUTYLMETHYLXANTHINE: INTERACTION BETWEEN α_1 AND β_2-ADRENERGIC STIMULATION

Michiel JWM Busch, and Philip FJ Hoyng; submitted

Abstract

Combination drug treatment of IBMX, a phosphodiesterase inhibitor, with catecholamines has demonstrated that IBMX potentiates the ocular hypotensive response of catecholamines. Potentiation by IBMX, however, was observed to be better for less selective or non-selective agonists nor- and epinephrine as compared to the ß-selective agonist isoproterenol. The present study investigates in rabbits the role of the α-signal of catecholamines for potentiation by IBMX. Combination drug experiments were performed with phenylephrine (α_1), salbutamol and IBMX (β_2/cAMP).

Topical 1% IBMX increased the ocular hypotension after topical salbutamol; IBMX alone does not affect IOP. In contrast, IBMX had no effect on topical phenylephrine; however, additional treatment of a sub-threshold dose (0.1%) phenylephrine further potentiated the ocular hypotensive response to IBMX with salbutamol.

The results indicate that phosphodiesterase inhibition with IBMX potentiates the ocular hypotensive effect induced by catecholamines not only due to β_2-adrenoceptor/cAMP stimulation but also to α_1-adrenergic receptor stimulation.

Introduction

In a recent study topically applied 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, has been shown to enhance the ocular hypotensive response induced by norepinephrine, epinephrine, and isoproterenol in rabbits (Hoyng et al, 1991; Busch and Hoyng, 1991b). Topical IBMX alone had no effect on IOP. Thus an interaction between catecholamines and IBMX to reduce intraocular pressure was shown. The site of action for this effect of IBMX has been localized in the outflow pathway since IBMX increased the effect of epinephrine on outflow facility, whereas a slight flow reduction induced by epinephrine was abolished by combination with IBMX (Hoyng and Busch, 1992).

Potentiation of the intraocular pressure response by topical IBMX was more pronounced for epinephrine and norepinephrine as compared to isoproterenol. This was an unexpected finding since IBMX could be expected to increase primarily responses mediated by adenylyl cyclase/cAMP pathway. IBMX is a non-specific inhibitor of phosphodiesterases which prevents metabolism of cAMP (Rall, 1982) and isoproterenol is a highly selective ß-agonist which mediates its effect by activating adenylyl cyclase (AC). Norepinephrine, however, is a non-selective adrenergic agonist with high affinity for α_1-adrenoceptor and moderate affinity for ß-adrenoceptors; and α_1-adrenoceptors primarily evoke a calcium-signal which is generally not directly related to AC/cAMP.

The purpose of the present study was to investigate systematically the adrenoceptor selectivity of catecholamines for potentiation of the intraocular pressure response by IBMX, particularly regarding the role of, additional, α_1-stimulation. Combination-drug experiments were performed with a selective α_1-or α_2-adrenergic agonist (phenylephrine/B-HT920) in combination with a selective $ß_2$-or $ß_1$-agonist (salbutamol/dobutamine) in the presence and absence of IBMX.

Materials and methods

Drug preparation

3-Isobutyl-1-methylxanthine (IBMX) (Fluka, Buchs, Switzerland) was dissolved in saline containing 2% beta-cyclodextrin to prepare a 1% solution. The following adrenergic agonists were used: phenylephrine HCL (Sigma), B-HT920 (Thomae), dobutamine HCL (Lilly), terbutaline sulphate (Astra), salbutamol sulphate (Sigma),

L-epinephrine bitartrate (Sigma), L-norepinephrine bitartrate (Sigma). Adrenergic antagonists: phenoxybenzamine (Smith, Kline and French), timolol maleate (Timolol^{R}, Chibret). Drug solutions were daily prepared in 0.5% hydroxypropyl methylcellulose and administered topically in a 30μl volume. Phenoxybenzamine was dissolved in saline. The contralateral eyes received the appropriate vehicle.

Measurement of intraocular pressure

The experiments were performed under standard light and atmospheric conditions on conscious 2.5-4 kg pigmented rabbits of either sex. The rabbits had free acces to water and food. Intraocular pressure (IOP) was measured tonometrically using an Alcon applanation pneumatonograph, calibrated for rabbit eyes. Digital readings were standardized with an Alcon Calibrator before and after each experiment. Local anaesthesia was obtained with topical administration of 30μl 0.2% oxybuprocain (Novesine^{R}, Chibret).
One eye was drug treated unless indicated otherwise; the contralateral control eye received the appropriate vehicle. Adrenergic drugs were topically applied at zero-time. 1% IBMX was topically administered at t=-0.5 hr. Groups of 6 to 10 rabbits were used. The same group of rabbits was used for treatment with adrenergic drugs alone and in combination with IBMX to minimize variability due to different responsiveness to catecholamines. A 7-10 day interval was allowed to permit adequate washout and 14 days if adrenergic antagonists were used. IOP was measured prior to drug administration followed by hourly measurements up to 6 or 8 hours. Results were expressed as the mean of the difference between treated and control eye $\pm$ S.E.M. determined every hour and then averaged between 3 and 6 or 8 hrs. The control eye was the contralateral, vehicle treated eye. Since B-HT920 is known to reduce IOP in the contralateral eye, the IOP values were recalculated to a baseline curve made the day before if treatment included B-HT920.

Statistics

Data were subjected to two-tailed Student's t-test for paired or unpaired observations. A p-value less than 0.05 was considered statistically significant.

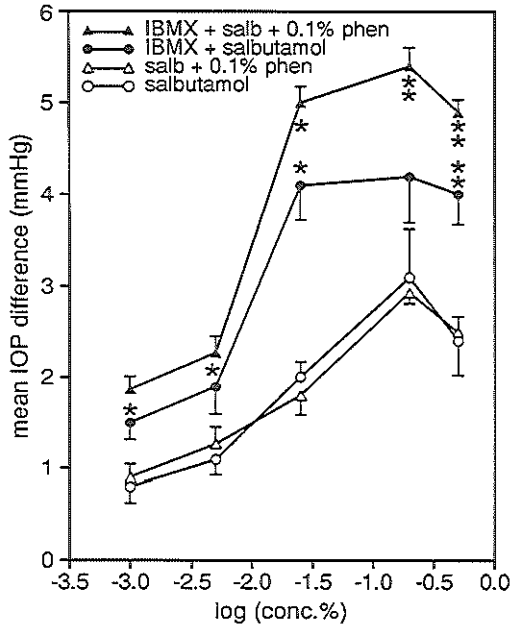

Figure 7.1 Dose-response curves for the ocular hypotensive effect of salbutamol with and without 1% IBMX (one eye drug-treated); and for the effect of adding a subthreshold dose of 0.1% phenylephrine to salbutamol or to salbutamol with 1% IBMX (both eyes treated respectively with salbutamol or salbutamol with IBMX, and one eye additionally with 0.1% phenylephrine). The latter differences between contralateral eyes are superimposed on the curves for salbutamol and IBMX + salbutamol, respectively; $^{*}P < 0.05$, $^{**}P < 0.01$ paired t-test. The ordinate represents the mean difference ± s.e.m. from 3-8 hours in groups of 8-10 rabbits, and the abcissa the log concentration of salbutamol in %.

Results

A dose-response curve for the hypotensive effect of the ß-selective adrenergic agonist salbutamol is demonstrated in Figure 7.1. 1% IBMX alone does not affect IOP (Hoyng et al, 1991). 1% IBMX pretreatment significantly potentiated the IOP-responses of salbutamol; the upward shift of the dose-response curve indicates that IBMX increased the efficacy of salbutamol. Topical 1% IBMX had no effect on moderate and high effect doses of phenylephrine (0.1, 1 and 2%; Table 7.1). Topical 0.1% phenylephrine alone did not reveal a detectible effect on IOP; this sub-threshold

dose was used for combination experiments with either salbutamol alone or with salbutamol in combination with IBMX (Figure 7.1). Phenylephrine, 0.1%, did not alter the hypotensive response of salbutamol alone. However, addition of this sub-threshold dose phenylephrine to 0.03, 0.2 and 0.5% salbutamol in 1% IBMX pretreated eyes elicited significantly increased hypotensive responses; this effect of phenylephrine can be characterized as synergism, and indicates that the α_1-signal potentiates the β_2/cAMP stimulated ocular hypotensive response of salbutamol with IBMX.

Similar combination experiments were performed with terbutaline, another β_2-selective agonist, in stead of salbutamol. IBMX enhanced the response of high but not low dose terbutaline alone (Table 7.1); the combined effect of low dose terbutaline with phenylephrine was potentiated by IBMX (Figure 7.2a), and confirms the observations

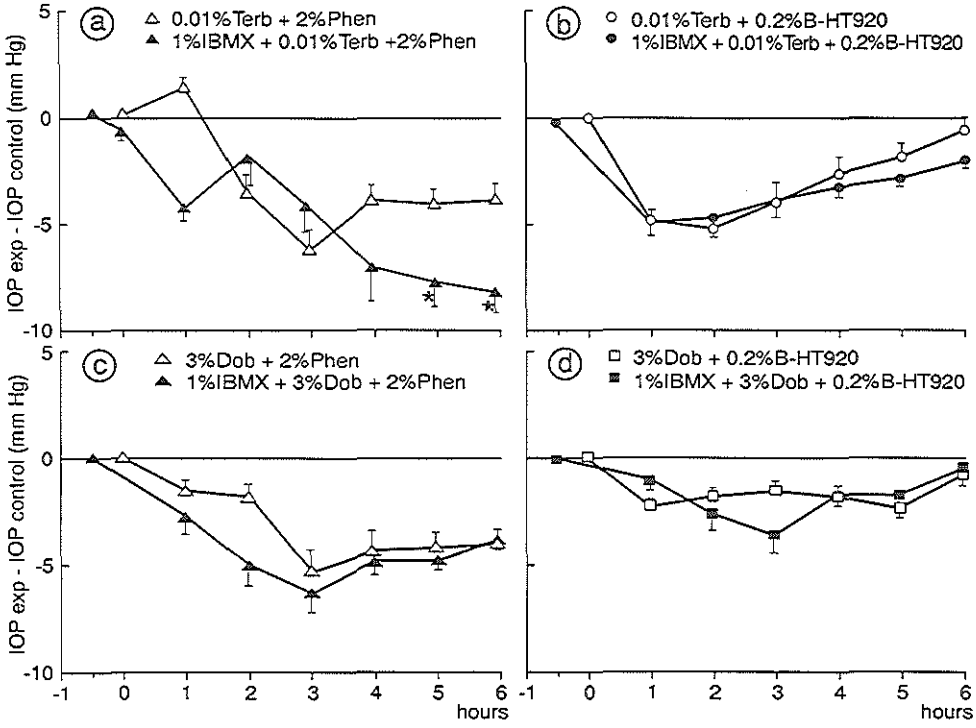

Figure 7.2 Effect of topical 1% IBMX pretreatment on the ocular hypotensive response to combination treatment of (a) terbutaline with phenylephrine ($\beta_2+\alpha_1$), (b)terbutaline with B-HT920 ($\beta_2+\alpha_2$), (c)dobutamine with phenylephrine ($\beta_1+\alpha_1$), and (d) dobutamine with B-HT920 ($\beta_1+\alpha_2$)(d) in 4 groups of 8 rabbits. * $P<0.05$.

found with salbutamol.
Control experiments were performed to evaluate involvement of selectivity for α_2- and $ß_1$-adrenoceptors for potentiation by IBMX. Threshold doses of 3% dobutamine ($ß_1$) and 0.2% B-HT920 (α_2) were used, and IBMX did not affect the hypotensive responses to B-HT920 alone and dobutamine alone (Table 7.1). The moderate hypotensive responses to combination treatment of 0.01% terbutaline and 0.2% B-HT920 ($\beta_2 + \alpha_2$), 3% dobutamine and 2% phenylephrine ($\beta_1 + \alpha_1$), 3% dobutamine and 0.2% B-HT920 and ($\alpha_2 + \beta_1$), were not enhanced by IBMX (Figure 7.2b, c, and d respectively). This indicates that phosphodiesterase inhibition with IBMX enhances the ocular hypotensive responses after combined $ß_2$ and α_1 adrenoceptor stimulation. Finally, two sets of experiments (Figure 7.3) show that both α- and ß-adrenoceptors are involved in the potentiation of the ocular hypotensive response of epinephrine and norepinephrine by IBMX. Beta-blockade with topical timolol, but also α-blockade with topical phenoxybenzamine, inhibited the markedly increased IOP responses to IBMX with either norepinephrine or epinephrine. Timolol 0.5% and 1% phenoxybenzamine, alone and in combination with 1% IBMX, did not affect IOP (not shown).

Table 7.1 Selective adrenergic agonists with and without IBMX

	Mean ocular hypotensive response between 3 and 6 hours in mmHg	
	without IBMX	with IBMX
0.1% phenylephrine	0.1 ± 0.2	0.2 ± 0.2
1% phenylephrine	1.4 ± 0.2	0.8 ± 0.2
2% phenylephrine	1.9 ± 0.4	1.4 ± 0.7
0.2% B-HT920	2.9 ± 0.4	3.2 ± 0.5
3% dobutamine	2.5 ± 0.4	2.9 ± 0.5
10% dobutamine	4.9 ± 0.5	4.7 ± 0.3
0.01% terbutaline	2.1 ± 0.4	2.6 ± 0.4
1% terbutaline	3.0 ± 0.3	4.7* ± 0.3

Ocular hypotensive effect of topical selective adrenergic agonists with and without topical 1% IBMX ± s.e.m.. Significant enhancement induced by pretreatment with IBMX is indicated by * $P < 0.05$, two tailed t-test for paired observations.

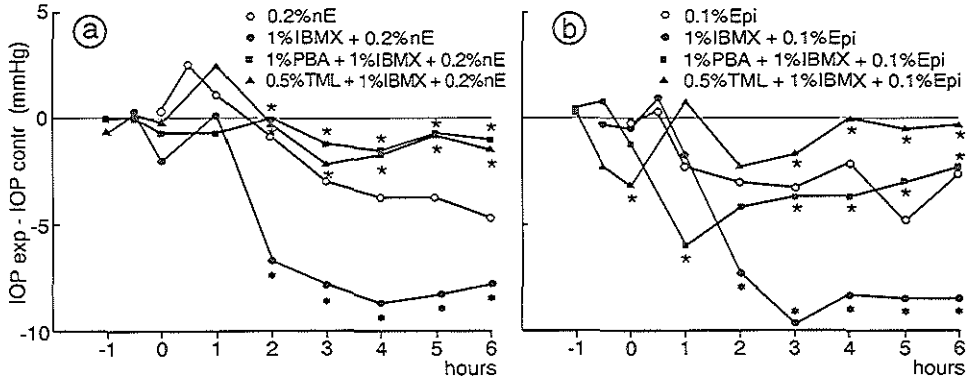

Figure 7.3 Effect of topical 0.5% timolol (TML) and 1% phenoxybenzamine (PBA) pretreatment on the 1% IBMX-enhanced ocular hypotensive response to (a) 0.2% norepinephrine (nE) and (b) 0.1% epinephrine (Epi) in two groups of 7 rabbits. Significant enhancement of the effect of nE/Epi by IBMX is indicated by a bold *; significant inhibition of these IBMX-enhanced responses of nE/Epi by TML or PBA are indicated by a small *. * $P < 0.05$.

Discussion

The present study demonstrates that phosphodiesterase inhibition with IBMX enhances the ocular hypotensive response of catecholamines which are selective for $ß_2$-adrenoceptors. However, additional subthreshold α_1-adrenoceptor stimulation further potentiates this effect of IBMX. These results support and explain our previous observation that the ocular hypotensive response of the ß-selective agonists isoproterenol was less potentiated by combination with IBMX as compared to combination treatment with non-selective agonists epinephrine and norepinephrine (Hoyng et al, 1991). The order of responsiveness of catecholamines for potentiation by IBMX can be summarized as:

norepinephrine = epinephrine > isoproterenol = salbutamol > terbutaline.

Recent experiments on aqueous humour dynamics showed that potentiation of the ocular hypotensive response to epinephrine by IBMX was due to an increase of outflow facility since IBMX increased epinephrine's effect on outflow facility (Hoyng and Busch, 1992; chapter 6 this thesis) whereas a slight flow reduction after

epinephrine was abolished by a combination with IBMX which could thus not account for an IOP fall. It may therefore be hypothesized that in this study the IBMX-effects on IOP are mainly due to affecting outflow mechanisms and that combination treatment of salbutamol and phenylephrine mimics the properties of epinephrine. Both α- and β-adrenoceptors have been reported to be involved in decreasing IOP and increasing outflow facility in rabbits (Sears and Barany, 1960; Eakins, 1963; Langham and Diggs, 1974; Potter and Rowland, 1978).

Adenylyl cyclase has been postulated to play an important role in controlling outflow mechanisms, particularly after ß-receptor stimulation (Neufeld et al, 1972a; Neufeld et al, 1973). Biochemically, the question arises how α_1-receptor stimulation may increase the β_2-adrenergic/and cAMP mediated ocular hypotensive response, since phenylephrine evokes a calcium-calmodulin signal whereas IBMX/$ß_2$-selective agonists generate increased cAMP responses. Neufeld and Sears (1974), however, demonstrated in scleral trabecular tissue that α-antagonism with phenoxybenzamine could inhibit the epinephrine stimulated production of cAMP, suggesting also α-mediated stimulation of AC. Other studies suggested and showed involvement of prostaglandins in the IOP decreasing and outflow increasing effect of epinephrine (Hoyng et al, 1982; Abdel Latiff, 1989; Camras, 1989; Anderson and Williams, 1990), and prostaglandins in turn may activate AC.

It is concluded that IBMX enhances $ß_2$- adrenoceptor/cAMP mediated ocular hypotensive responses; additional α_1-adrenergic receptor stimulation significantly potentiates this effect. For this reason potentiation of the ocular hypotensive response of catecholamines by IBMX is better for non-selective agonists (nor)epinephrine than for selective $ß_2$-agonists terbutaline and salbutamol. Since the site of action for the IBMX effect is on trabecular outflow facility, these results may indicate interaction between the α_1- and $ß_2$/cAMP signalling systems in the outflow pathway.

CHAPTER 8

INTERACTION BETWEEN SELECTIVE α_1 AND β_2-ADRENERGIC AGONISTS: REDUCTION OF INTRAOCULAR PRESSURE AND INCREASE IN OUTFLOW FACILITY

Michiel JWM Busch, and Philip FJ Hoyng; submitted

Abstract

Combination drug studies of phenylephrine with either salbutamol or terbutaline were performed, measuring intraocular pressure (IOP), outflow facility, and correlations with aqueous cAMP levels in rabbits.

Additive ocular hypotensive responses were observed after combining topical phenylephrine, a selective α_1-agonist, with either salbutamol or terbutaline, selective β_2-agonists. In contrast no additivity was found after combining terbutaline with B-HT920 (α_2), or after combining dobutamine (β_1) with either phenylephrine or B-HT920.

Tonographic outflow facility was neither affected by 4% phenylephrine nor by 1% salbutamol alone; combination treatment, however, increased C-value by 32%.

Aqueous humour cAMP levels increased after salbutamol. A high dose of phenylephrine increased aqueous cAMP only if administered in combination with a phosphodiesterase inhibitor. The effects on aqueous cAMP levels after combination treatment of salbutamol with phenylephrine were not additive.

It is concluded that there is an interaction between selective α_1- and selective β_2-adrenoceptor stimulation which mediates a reduction of IOP and an increase of outflow facility. The results suggest that the IOP reduction and increase of outflow facility after non-selective agonists (nor)epinephrine depends on α_1- and β_2-adrenergic stimulation, probably by interaction and not merely addition. Adenylyl cyclase may be involved after both the β_2- and α_1-signal.

Introduction

There is convincing evidence that ß-adrenergic stimulation by topical application of catecholamines reduces intraocular pressure by increasing outflow facility in rabbits (Eakins, 1963; Sears and Sherk, 1964; Lamble, 1977; Potter, 1981). The role of α-adrenergic stimulation in regulating outflow facility is less clear but various studies performed in rabbits have indicated a role for α-adrenergic receptors in increasing outflow facility, particularly in response to non-selective drugs such as (nor)epinephrine (Potter, 1981). For example, the increase in outflow facility after intravitreous norepinephrine, a non-selective agonist with strong α-adrenergic properties, was substantially inhibited by prior administration of the α-adrenergic antagonist phentolamine (Eakins and Ryan, 1964). Also α-adrenergic antagonists have been shown to antagonize the increase of outflow facility during denervation (Sears and Barany, 1960), which was supposed to be induced by endogenous norepinephrine release.

Recently, it has been shown that a topical phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), potentiated the outflow facility increase after epinephrine (Hoyng and Busch, 1992). This was probably due to both α_1- and $ß_2$-adrenoceptor stimulation since the enhancement of the ocular hypotensive response by IBMX (Hoyng et al, 1991; Busch and Hoyng, 1991b) was mediated by selective $ß_2$- as well as selective α_1-adrenoceptor stimulation (Busch and Hoyng, 1991a). Furthermore, some studies suggest that the reduction of IOP and increase of outflow facility after (nor)epinephrine may be, partially, mediated by endogenous prostaglandin synthesis in response to α-adrenergic stimulation (Anderson and Williams, 1990; Camras, 1989).

The present study investigates interactions between α_1- and $ß_2$-adrenergic stimulation in reducing IOP and in increasing outflow facility. Combination drug experiments were performed with a selective α1- (phenylephrine) and a selective β2- (salbutamol or terbutaline) adrenergic agonist. Additionally, correlations with aqueous cAMP levels were evaluated since cyclic AMP is considered to play an important role as second messenger in controlling outflow facility (Neufeld et al, 1975; Potter, 1981; Caprioli et al, 1984a; and Kaufman, 1987).

Materials and methods

The experiments were performed in the animal facilities under standard light and

atmosphere conditions on restrained, conscious 2.5- 4 kg pigmented rabbits of either sex. The rabbits had free access to water and food.
The following selective adrenergic drugs were used: phenylephrine HCL (Sigma), B-HT920 (Thomae), salbutamolsulphate (Sigma), terbutalinesulphate (Sigma), and dobutamine HCL (Lilly); 3-isobutyl- 1-methylxanthine (IBMX) was from Fluka (Buchs, Switzerland). All drugs were daily freshly prepared in 0.5% hydroxypropylmethyl-cellulose. Each drug was administered topically in a volume of 30μl. One eye was drug-treated and the contralateral control eye received the appropriate vehicle. Adrenergic agonists were applied at zero-time. The experiments were performed in groups consisting of 6-8 rabbits, and the same group of rabbits was used for each set of combination experiments. Between drug treated experiments a period of at least 14 days was allowed to permit adequate washout.

Intraocular pressure

Intraocular pressure (IOP) was measured tonometrically using an Alcon applanation pneumatonograph, calibrated for rabbit eyes (Chapter 2 of this thesis). Digital readings were standardized with an Alcon Calibrator before and after each experiment. Local anaesthesia was obtained with topical 30μl 0.2% oxybuprocain (Novesine, Chibret, RIOM, France). IOP was measured before drug administration and afterwards hourly up to 8 hours. Results are expressed as mean differences between treated and control eye $\pm$ S.E.M. at every hour, and were averaged between 3 and 6 or 8hrs as indicated. The control eye was the contralateral, vehicle treated eye. When using B-HT920 a baseline curve was made the day before because of effects of B-HT920 on the contralateral eye; IOP values were compared with these baseline values.

Outflow facility

Outflow facility was measured by indentation tonography, using an electronic Schiötz tonograph (Berkely, CA). General anaesthesia was induced with i.v. thiopental sodium, 40-60 mg.kg bodyweight^{-1}, via a cannulated marginal ear vein. Tonographic measurements were made 4 hrs after topical drug administration. Baseline control C-values were determined one day in advance in the same, experimental, eyes. Three groups of 6-8 rabbits were used, and only one eye was drug treated. Measurements were made during a 4 minute period and the C-value was approximated from the 1955 Friedenwald Tables, modified by Moses (1958).

Aqueous humour analysis

Effects of selective adrenergic agonists on aqueous humour cAMP levels were determined in the presence and absence of 1% IBMX pretreatment. IBMX was given topically at t=-0.5 hr. Aqueous humour, 100-200 μl, was carefully aspirated from restrained wrapped rabbits with a 27-gauge tuberculin needle after local anaesthesia (50μl 4% tetracaine), aliquotted, and immediately stored at 80°C. Cyclic AMP levels were determined in duplicate in 10μl of aqueous humour by radioimmunoassay (New England Nuclear, Boston, MA) under non-acetylated assay conditions.
Aqueous humour protein was determined in duplicate by the Bradford (1976) Dye-binding method using human serum albumin as the reference protein.

Statistical analysis

Statistical significance was determined based on conventional two-tailed Student t-test for unpaired, or paired if appropriate, observations. A P-value less than 0.05 was considered statistically significant.

Table 8.1 Intraocular pressure

		mean IOP fall between 3 and 6 hours in mmHg
0.01%	terbutaline	2.2 ± 0.5
2%	phenylephrine	1.9 ± 0.6
0.01%	terbutaline + 2% phenylephrine	4.5 ± 0.5
0.01%	terbutalin	2.7 ± 0.6
0.2%	B-HT920	0.9 ± 0.2
0.01%	terbutaline + 0.2% B-HT920	2.3 ± 0.5
3%	dobutamine	1.2 ± 0.1
2%	phenylephrine	2.9 ± 0.3
3%	dobutamine+ 2% phenylephrine	3.4 ± 0.5
3%	dobutamine	1.2 ± 0.1
0.2%	B-HT920	1.0 ± 0.3
3%	dobutamine+ 0.2% B-HT920	1.6 ± 0.3

Ocular hypotensive effect after topical combination treatment of two selective adrenergic agonists. Drugs were applied in a 30μl volume to one eye in groups of 6-8 rabbits.

Results

Intraocular pressure

Baseline IOP values ranged between 20-25mmHg. Fig. 8.1 shows the ocular hypotensive responses induced by salbutamol, phenylephrine, and by combined treatment of salbutamol and phenylephrine. Weak dose-response characteristics were

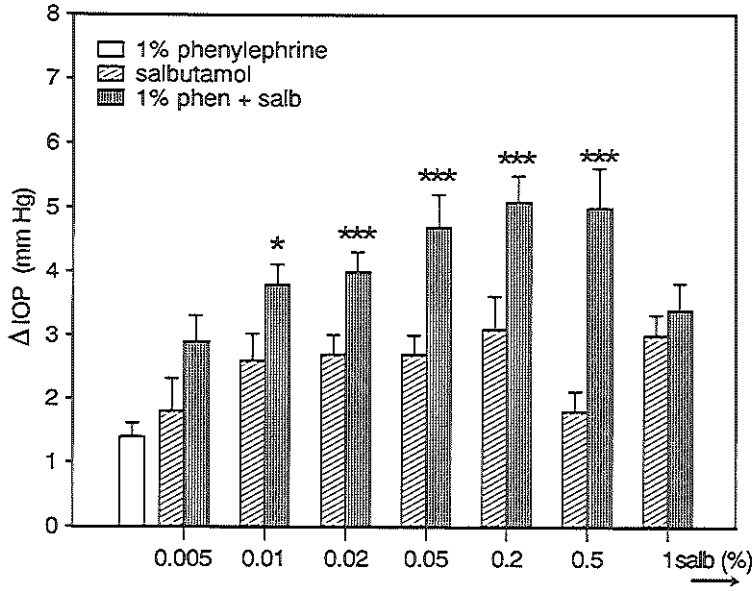

Figure 8.1 Mean ocular hypotensive effect between 3 and 8 hours after topical treatment of one eye with salbutamol alone, 1% phenylephrine alone, and after combination treatment of salbutamol with 1% phenylephrine. The concentration salbutamol in a group with and without phenylephrine remained the same. Significant difference between the combination treatment versus salbutamol alone is indicated by asterisk. $^{*}P < 0.05$, $^{***}P < 0.005$, unpaired t-test.

present for salbutamol, a selective β_2-agonist, alone. A near maximal response was noted at low doses. The selective α_1-agonist phenylephrine (1%) alone yielded a 1.3 mmHg IOP fall. Combination treatment of salbutamol with this threshold dose phenylephrine induced additive responses. This additive effect was more pronounced if 1% phenylephrine was combined with relatively high doses of salbutamol.

Figure 8.2 demonstrates that a 1 to 4 ratio between the doses salbutamol and phenylephrine was more effective in showing additivity; e.g. treatment of 0.05% salbutamol in combination with 0.2% phenylephrine (Figure 8.2) induced a higher response compared to combination of 0.05% salbutamol with 1% phenylephrine (Figure 8.1). The former response may be indicative for potentiation.
Table 8.1 shows that combination of phenylephrine with another β_2-selective adrenergic agonist, terbutaline, also induced an additive response. This is consistent with the data obtained with salbutamol. Control combination experiments were performed using small effect doses of dobutamine (mainly β_1) and B-HT920 (α_2). No additivity was found for combination treatment of either terbutaline with B-HT920, or

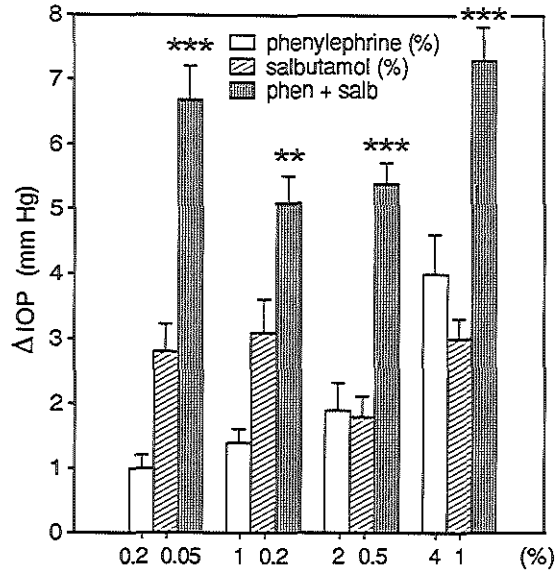

Figure 8.2 Mean ocular hypotensive effect between 3 and 8 hour after topical treatment of one eye with salbutamol alone, phenylephrine alone, or combination of salbutamol with phenylephrine. The ratio between [salbutamol]:[phenylephrine] = 1:4. Significance for the smaller difference between combination treatment versus salbutamol alone or phenylephrine alone is indicated by asterisks. ** $P < 0.01$, *** $P < 0.005$, unpaired t-test.

for dobutamine in combination with either phenylephrine or B-HT920. Thus, additivity of the ocular hypotensive responses was observed only when combining a selective β_2- and α_1-adrenergic agonist.

Outflow facility

Outflow facility was measured 4 hours after topical treatment with either 1% salbutamol or 4% phenylephrine alone and after a combination of both. Phenylephrine 4% alone and 1% salbutamol alone did not significantly alter C-value as compared to the respective baseline values performed the day before in the same eyes. However, combination treatment of 4% phenylephrine and 1% salbutamol significantly increased C-value by 32%, ie from 0.22 $\pm$ 0.02 to 0.29 $\pm$ 0.03 ($P < 0.05$) (Table 8.2). This effect is indicative for potentiation.

Table 8.2 Tonographic facility of outflow

		Facility of outflow in $\mu l.min^{-1}.mmHg^{-1}$		
		control eye	treated eye	difference
4%	phenylephrine(n=7)	0.22 $\pm$ 0.03	0.21 $\pm$ 0.01	-0.01 $\pm$ 0.03
1%	salbutamol(n=6)	0.22 $\pm$ 0.02	0.19 $\pm$ 0.01	-0.03 $\pm$ 0.03
4%	phenylephrine + 1% salbutamol (n=8)	0.22 $\pm$ 0.02	0.29 $\pm$ 0.03	0.07* $\pm$ 0.03

Outflow facility (μl/min/mmHg), measured by indentation tonography, in three groups of rabbits after topical application of 4% phenylephrine,1% salbutamol and combined treatment of 4% phenylephrine and 1% salbutamol. Measurements are performed 4 hours after drug administration and control baseline facility measurements were performed one day prior in the same, experimental, eye. Data are mean values $\pm$ SEM. * $P<0.05$, paired t-test.

Aqueous humour parameters

Aqueous humour cyclic AMP levels (Figure 8.3) were assessed one hour after topical adrenergic drug treatment. IBMX, a non specific phosphodiesterase inhibitor which prevents intracellular metabolism of cyclic AMP to AMP (Rall, 1982), was used to increase effects on aqueous humour cAMP levels. Baseline cAMP in the aqueous humour were 19.5 $\pm$ 1.3 $pg.ml^{-1}$ without IBMX, and 29.4 $\pm$ 2.6 $pg.ml^{-1}$ in the presence of IBMX. Low and high doses salbutamol moderately increased aqueous cAMP levels. These responses were markedly increased by IBMX pretreatment. Phenylephrine alone, 0.2 and 4%, did not reveal detectible effects on aqueous cAMP. However, in combination with IBXM a significant effect was found with 4%

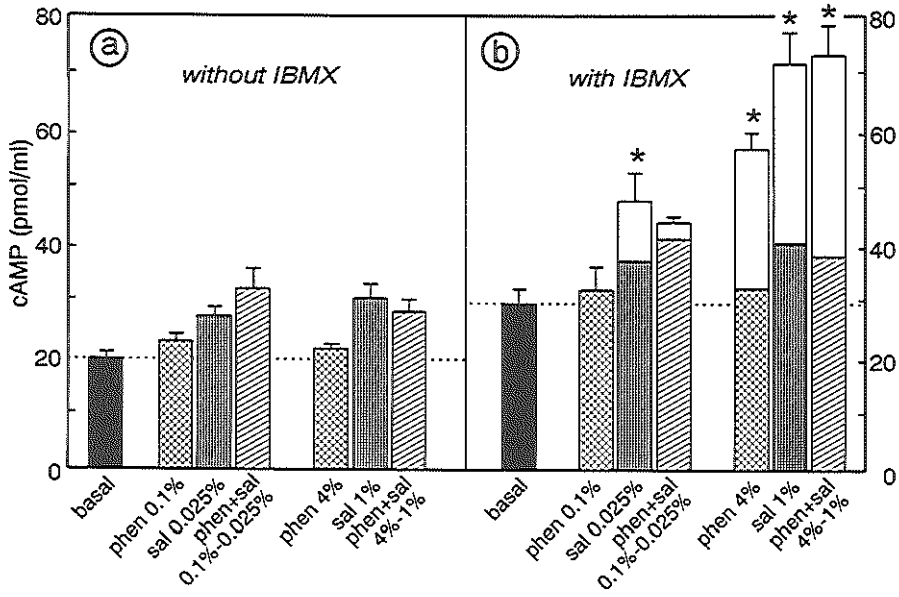

Figure 8.3 Aqueous cyclic AMP levels, in pmol.ml^{-1}, one hour after topical adrenergic drug treatment. These experiments were done in the absence (a) and in the presence (b) of topical 1% IBMX, administered at t=-0.5 hr, in groups of 7-9 rabbits. Potentiation by IBMX in the right panel is indicated by blank parts of the bars, * $P < 0.05$, paired t-test.

Table 8.3 Aqueous humour protein

	Protein in g.l^{-1}
basal	0.61 ± 0.11
0.1% phenylephrine	0.73 ± 0.17
0.025% salbutamol	0.34 ± 0.03
0.1% phen + 0.025% salb	0.60 ± 0.22
4% phenylephrine	0.64 ± 0.05
1% salbutamol	0.54 ± 0.05
4% phen + 1% salb	0.56 ± 0.05

Aqueous humour protein in mg.ml^{-1}, determined one hour after topical drug treatment in groups of 8 rabbits.

phenylephrine. Combining salbutamol with phenylephrine did not induce a higher aqueous cAMP levels than with salbutamol alone; pretreatment with IBMX did not change this response, indicating that additivity of aqueous cAMP responses was not present.
Aqueous humour protein (table 8.3) was determined one hour after topical drug treatment. No effects on aqueous humour protein were present. This indicates that neither high and low doses of salbutamol and phenylephrine, nor combinations of both, affected the blood aqueous barrier.

DISCUSSION

This study was performed to analyze interactions between selective α- and selective ß-adrenergic stimulation in reducing IOP and increasing outflow facility. Additivity of hypotensive responses can only be obtained using phenylephrine in combination with either terbutaline or salbutamol. Tonographic measurements indicate that the mechanism for this fall in IOP is, at least partially, by increasing outflow facility. This effect can be characterized as potentiation since neither phenylephrine nor salbutamol alone affect outflow facility and provides direct evidence for an interaction between α_1- and $ß_2$-adrenergic stimulation in mediating an increase in outflow facility. Aqueous humour protein levels were not affected and this excludes major bias due to increased pseudofacility because of blood aqueous barrier breakdown.
Adenylyl cyclase (AC) is thought to play an important role in controlling outflow facility. Many in vitro studies indicate the presence of $ß_2$-adrenoceptor stimulated AC in trabecular tissue in rabbits (Neufeld and Sears, 1974) and other species (Wax et al, 1989; Crawford et al, 1991; Busch et al, 1992). The effect of salbutamol in the present study is likely mediated by $ß_2$-adrenoceptors coupled to AC. $Alpha_1$-adrenoceptor stimulation is known to generate primarily a calcium-calmodulin signal but a high dose phenylephrine in the present study markedly increased the level of aqueous cAMP, if combined with IBMX. This is suggestive for interaction of the Ca-signalling system with AC/cAMP. Other studies have also indicated that α_1-adrenoceptor stimulation may lead to increases of cAMP. Neufeld (1974) has demonstrated in rabbit corneoscleral rings that the epinephrine-stimulated cAMP synthesis could be partially blocked by the α-adrenergic antagonist phenoxybenzamine. Rowland and Potter (1979) have observed previously similar findings with phenylephrine on aqueous cAMP levels since topical 100μl 2% phenylephrine alone raised the aqueous cAMP level.
Various biochemical mechanisms may be responsible for the rise of aqueous cAMP

after phenylephrine. For example, it is observed that rabbit ciliary processes contain AC that could be activated by Ca-calmodulin (Lind et al, 1989). Alpha-stimulation has also been demonstrated to induce prostaglandin synthesis in iris and ciliary body and to increase PG release in the aqueous humour (Hoyng et al, 1982; Abdel-Latif, 1989; Camras, 1989); and PGs in turn may stimulate AC. In fact, this mechanism may be present in trabecular meshwork as well, since the increase of outflow facility by epinephrine could be blocked in rabbits by indomethacin and piroxicam, both inhibitors of PG synthesis (Anderson and Williams, 1990). Furthermore, AC in rabbit corneoscleral rings could be stimulated by PGE (Neufeld, 1974); and in trabecular tissue membrane preparation of bovine and human trabecular meshwork, PGE_1 and PGE_2 have been shown to stimulate cAMP synthesis mediated by EP-receptors linked to AC (Busch et al, 1992).

Additivity of raised aqueous cAMP responses after combining high doses phenylephrine and salbutamol was not present. AC seemed to be maximally stimulated by phenylephrine as well as by salbutamol. It can be speculated that both α_1- and $ß_2$-adrenergic stimulation have the same AC as target for activation. On the other hand, one would expect an additive response using low doses but this was neither present. The use of IBMX, however, raises in our opinion the value of aqueous humour cAMP levels since metabolism of cAMP is inhibited, resulting in increased aqueous humour responses.

It is concluded that an interaction between α_1- and $ß_2$-adrenoceptor stimulation mediates an increase of outflow facility and reduction of IOP. The mechanisms by which phenylephrine and salbutamol interact and increase outflow facility likely involve the AC pathway. The results suggests that the IOP reduction and increase of outflow facility seen after non-selective agonists (nor)epinephrine depends on α_1- and $ß_2$-adrenergic stimulation, probably by interaction and not merely addition. Adenylyl cyclase may be involved after both the $ß_2$- and α_1-adrenergic stimulation.

CHAPTER 9

EFFECTS OF CYCLIC NUCLEOTIDE ANALOGS ON INTRAOCULAR PRESSURE AND TRAUMA-INDUCED INFLAMMATION IN THE RABBIT EYE

Michiel JWM Busch, Eric JGM van Oosterhout, and Philip FJ Hoyng; *published in Current Eye Research 11, 5-13, 1992*

Abstract

In this study the effects of cell-permeable 8-bromo-cAMP and 8-bromo-cGMP on intraocular pressure (IOP) and puncture-induced inflammatory response were investigated. Both 8-bromo-cAMP and 8-Bromo-cGMP reduced IOP when given subconjunctivally, but not topically. Subconjunctival administration of 8-bromo-cAMP induced a moderate disruption of the blood-aqueous barrier (BAB); in addition, subconjunctival 8-bromo-cAMP, but not topical 8-bromo-cAMP or subconjunctival 8-bromo-cGMP, reduced the disruption of the BAB and elevation of the aqueous PGE_2 level after puncture trauma.

It is concluded that the effects of 8-bromo-cAMP depend on the mode of administration, since this determines the concentration of 8-bromo-cAMP reached in the aqueous humour. It is suggested that 8-bromo-cAMP can partially suppress a slight inflammatory response by interference with the release of arachidonic acid from the tissues surrounding the aqueous humour.

Introduction

Corneal puncture has been shown to be a traumatic procedure which provokes a mild

inflammation in the rabbit eye (Hoyng et al, 1986). The mechanism for this model of inflammation is, amongst others, presumed to be that puncture liberates endogenous prostaglandins (PGs) into the aqueous humour (Neufeld et al, 1972b, Neufeld and Sears, 1973). The released PGs are to a large extent responsible for the breakdown of the blood aqueous barrier (BAB), slight cellular infiltration of the aqueous humour (AH), conjunctival hyperemia and the biphasic intraocular pressure (IOP) response (Hoyng et al, 1986).
Exogenous, topically administered, PGs can partially inhibit the inflammatory response after trauma (Hoyng et al, 1986) and after bacterial endotoxin (Wong and Howes, 1983) in the rabbit eye. The underlying mechanism for this this effect of exogenous PGs is not clear. Weissmann et al (Weissman et al, 1980) suggested that a secondary rise in intracellular cAMP mediates anti-inflammatory effects of PGs. Other studies have shown that topical isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, raises cAMP levels in the aqueous humour (Hoyng et al, 1991) and protects the BAB in human serum albumin (HSA)-induced uveitis (Verbey et al, 1987). Thus, topical exogenous PGs as well as topical IBMX modulate endogenous PG formation after corneal puncture and HSA-induced uveitis, respectively. These and other studies (Weissman et al, 1980; Bourne et al, 1974; Deporter, et al, 1976, Deporter, 1977; and Seo and Saeki, 1980) suggest involvement of intracellular cAMP in modulation of the inflammatory response.
However, it is not clear whether this inhibitory effect can be specifically related to cAMP. Therefore, this study investigates the direct effect of a permeable c-AMP analog, i.e. 8-bromo-cAMP, on trauma-induced inflammation in the rabbit eye and a comparison was made with the effects after 8-bromo-cGMP. In addition, effects of 8-bromo-cAMP and 8-bromo-cGMP on IOP were evaluated.

Materials and methods

Young adult pigmented rabbits (2.0-3.5 kg), aged 6-12 months, were used. They received food and water ad libitum. Before entering an experiment the eyes were examined for the absence of inflammatory signs and cataract with a Zeiss hand slit lamp.
IOP was measured in conscious animals after local anesthesia (30 μl 0.2% oxybuprocaine; Novesine, Chibret, RIOM, France) using a pneumotonograph (Alcon, Fort Worth, Texas), standardized with an Alcon calibrator. The day before testing drug effects, a baseline IOP-curve was obtained over a 6-hour period. On the experimental day, the drug was applied after the first pressure reading, and the IOP

was measured during the subsequent 6 hours. Contralateral eyes received the vehicle and served as control. After an IOP experiment the animal was not used for three weeks to allow adequate wash-out of the drug.

IOP-experiments were performed after topical application of 8-bromo-cAMP (0.1, 0.5 or 1%) and topical 8-bromo-cGMP (1%). Twice 50 μl was topically administered with a 15 min interval. The IOP effect after subconjunctival administration was studied for saline, 1% 8-bromo-cAMP and 1% 8-bromo-cGMP; once a 100 μl volume was applied subconjunctivally. Thus, the same dose was administered either topically or as a subconjunctival depot. The contralateral control eye was treated with the appropriate vehicle, administered topically or subconjunctivally.

Effects on aqueous humour protein and PGE_2 were assessed at 1hr after subconjunctival 100 μl saline, and after subconjunctival 100 μl of 1% 8-bromo-cAMP in 2 groups of 8 rabbits. Aqueous humour protein was determined in duplicate according to Bradford (1976), using human serum albumin as standard. Aqueous PGE_2 was determined in duplicate in 10 μl of aqueous humour with a RIA (NEN, Boston, MA).

Penetration, i.e. the concentration of 8-bromo-cAMP in the aqueous humour and effects on aqueous protein were measured 1, 2, 3, 4, 5 and 6hr after topically and subconjunctually administered 100 μl 1% of 8-bromo-cAMP. For each time point a group of 7 or 8 rabbits was used. The contralateral control eyes were not treated.

8-bromo-cAMP and 8-bromo-cGMP were purchased from Sigma (St. Louis, M.O.) and dissolved in saline.

The cAMP levels were determined in duplicate by radioimmunoassay (RIA) (NEN,Boston, MA) using 10 μl of aqueous humour. A non-acetylated assay condition was used. Standard curves were made for cAMP as well as for 8-bromo-cAMP to determine cross-reactivity for the RIA. The results for the vehicle-treated contralateral eyes were plotted on the cAMP standard curve and those for 8-bromo-cAMP treated eyes on the 8-bromo-cAMP standard curve. Baseline aqueous c-AMP levels were obtained from a group of 8 rabbits that received the vehicle only. Slight ocular inflammation was induced by puncturing the anterior chamber with a 27G needle after local anesthesia with 2% tetracaine. The eye was immobilized with a conjunctival forceps and the needle was inserted 3 mm through the corneal stroma before entering the anterior chamber. Care was taken that neither the iris nor the lens was touched; the needle was carefully withdrawn to prevent leakage of aqueous humour. Eight rabbits underwent corneal puncture of one eye; aqueous protein and PGE_2 levels were determined one hour later in both eyes providing baseline values and values for the effect of corneal puncture. Effect of drug pretreatment on this puncture induced inflammatory response is studied for topical 1% 8-bromo-cAMP (twice 50 μl); for subconjunctival 1% 8-bromo-cAMP (once 100 μl) and for subconjunctival 1% 8-

bromo-cGMP (once 100 μl). The drug was administered to the experimental eye and the vehicle to the contralateral control eye one hr before corneal puncture of both eyes. Groups of 7-8 rabbits were used. Samples of 100 μl of aqueous humour were obtained at 1, 2, 3 or 5 hr after corneal puncture and stored at -70°C to determine aqueous humour protein and PGE_2 levels.
IOP values and concentrations are presented as means $\pm$ SEM. Differences between values were tested for significance with Student's t-test.
These experiments adhered to the ARVO resolution on the use of animals in experimental research.

RESULTS

Intraocular pressure was not affected by topical 0.1%, 0.5% and 1% 8-bromo-cAMP or 1% 8-bromo-cGMP (data not shown). Subconjunctival application 1% 8-bromo-cAMP (Figure 9.1) or 8-bromo-cGMP (Figure 9.2), however, decreased

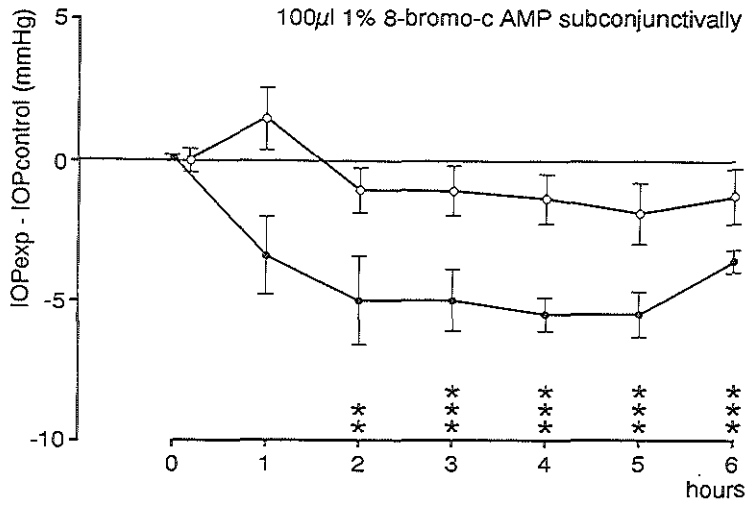

Figure 9.1 Effect on intraocular pressure of subconjunctival administration of 100 μl of saline (open circles), the contralateral eyes untreated, and of subconjunctival administration of 100 μl 1% 8-bromo-cAMP (closed circles), the contralateral eyes received the vehicle subconjunctivally (both n=7). The points represent means $\pm$ 1 SEM. Asterisks in the figure represent p-values for the difference IOPexp - IOPcontr (** P (0.001; *** P (0.005, two-tailed paired t-test).

IOP. No effect in the contralateral vehicle-treated eyes was observed. 8-bromo-cAMP reduced the IOP between 1 and 6 hours after application, with a maximum of 5.5 $\pm$ 0.8 mmHg ($P < 0.005$) at 4 and 5 hrs; 8-bromo-cGMP reduced IOP between 2 to 6 hrs after application, the maximum being 3.4 $\pm$ 0.8 mmHg at 4 hrs. ($P < 0.005$). Subconjunctivally administered 100 μl saline alone did not affect IOP (Figure 9.1).

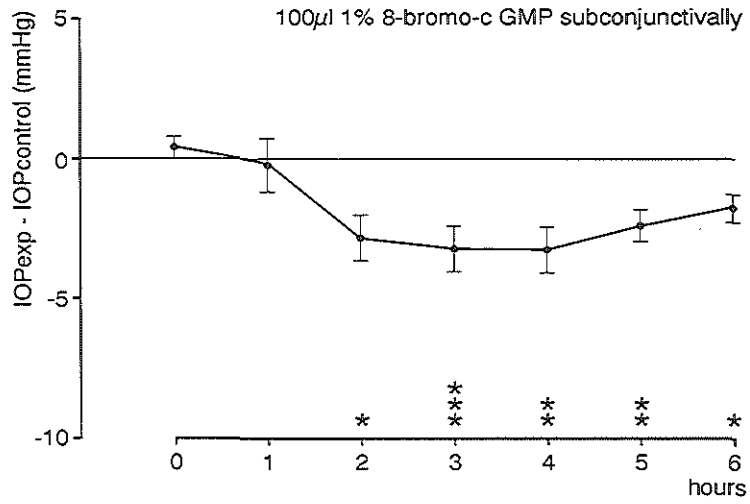

Figure 9.2 Effect of subconjunctival application of 100 μl 1% 8-bromo-cGMP (contralateral eyes received the vehicle subconjunctivally) on intraocular pressure (n=7). The points represent means $\pm$ 1 SEM. Asterisks indicate: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, two-tailed paired t-test.

Effect on aqueous humour protein and PGE_2 of subconjunctival injection of 100 μl saline and 100 μl 1% 8-bromo-cAMP is illustrated in Table 9.1. A slight increase of aqueous protein and aqueous PGE_2 was observed both with saline and 8-bromo-cAMP; the increased aqueous PGE_2 level after subconjunctivally injected 8-bromo-cAMP tended to be lower than after saline, the difference being 52.3 $\pm$ 27.6 pg/ml ($P < 0.1$, unpaired two-tailed t-test).

The baseline c-AMP level in aqueous humour was 19.8 $\pm$ 1.4 p moles/ml. Table 9.2 demonstrates the cross-reactivity of 8-bromo-cAMP for the adenosine 3',5'cyclic monophosphate radioimmuno assay.

Table 9.1 Aqueous humour protein and PGE_2 levels

	Protein in $g.l^{-1}$	PGE_2 in $pg.ml^{-1}$
contralateral untreated eye	0.4 ± 0.05	32.8 ± 5.7
saline subconjunctival	1.4 ± 0.4*	119.6 ± 24.5*
1% 8-bromo-cAMP subconjunctival	2.8 ± 0.7*	67.3 ± 12.6*

A volume of 100 μl saline or 8-bromo-cAMP was administered subconjunctivally.* indicates: significantly different from values in the contralateral untreated eye, $P < 0.05$ two-tailed paired t-test. Two groups of 8 rabbits were used.

Table 9.2 Cross-reactivity of 8-bromo-cAMP for the cAMP radioimmunoassay

Standard concentration cyclic AMP and 8-bromo-cAMP in $pmol.ml^{-1}$	Percentage bound labeled tracer to antiserum	
	cyclic AMP	8-bromo-cyclic AMP
0	100	100
0.5	86.3	82.4
1.0	82.2	75.2
2.5	75	59.5
5.0	69.2	47.7
10.0	52.4	33.5
25.0	32.6	19.9
50.0	20.4	11.8

Percent bound labeled tracer to antiserum for standard concentrations cAMP and 8-bromo-cAMP, calculated by dividing the average net counts of the standard with the average net counts of the zero standard * 100%. The cross-reactivity for 8-bromo-cAMP, calculated at the 50% point by interpolation, is 2.6.

Aqueous 8-bromo-cAMP levels after topical and subconjunctival administration are presented in Table 9.3 and 9.4, respectively. Topical 100 μl 1% 8-bromo-cAMP induced a maximum concentration of 290 ± 82 p moles/ml ($P < 0.01$) at 1 hour; subsequently, the level gradually decreased. No effect on aqueous humour protein levels in either treated or vehicle-treated contralateral eyes was present (Table 9.3).

Subconjunctival administration of 100 μl 1% 8-bromo-cAMP induced high aqueous humour concentrations of 8-bromo-cAMP over a period of 6 hours, with a maximum of 2789 $\pm$ 752 p moles/ml ($P<0.001$) at 2 hrs (Table 9.4). This is about 10-fold

Table 9.3 Aqueous protein, aqueous cAMP and aqueous 8-bromo-cAMP levels after *topical* application of 100 μl 1% 8-bromo-cAMP solution.

Time (hrs)	8-bromo-cAMP in pmoles.ml^{-1}	cAMP in pmoles.ml^{-1}	Protein in g/l^{-1}	
	Treated	Control	Treated	Control
1	290 $\pm$ 82**	29 $\pm$ 8	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1
2	148 $\pm$ 18***	36 $\pm$ 8	0.4 $\pm$ 0.1	0.4 $\pm$ 0.04
3	166 $\pm$ 53*	48 $\pm$ 7	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1
4	122 $\pm$ 54	26 $\pm$ 2	0.3 $\pm$ 0.04	0.2 $\pm$ 0.03
6	32 $\pm$ 8	19 $\pm$ 6	0.3 $\pm$ 0.04	0.3 $\pm$ 0.1

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-tailed paired t-test. For each time point a group of 7-8 rabbits was used.

Table 9.4 Aqueous protein, aqueous cAMP and aqueous 8-bromo-cAMP levels after *subconjunctival* application of 100 μl 1% 8-bromo-cAMP solution.

Time (hrs)	8-bromo-cAMP in pmoles.ml^{-1}	cAMP in pmoles.ml^{-1}	Protein in g/l^{-1}	
	Treated	Control	Treated	Control
1	874 $\pm$ 218***	66 $\pm$ 13	3.4 $\pm$ 2.0	0.2 $\pm$ 0.04
2	2789 $\pm$ 752***	29 $\pm$ 4	5.4 $\pm$ 1.5**	0.9 $\pm$ 0.03**
3	2610 $\pm$ 619***	34 $\pm$ 4	2.0 $\pm$ 1.3	0.3 $\pm$ 0.03
4	1142 $\pm$ 183***	23 $\pm$ 2	1.2 $\pm$ 0.5	0.3 $\pm$ 0.03
5	704 $\pm$ 152***	36 $\pm$ 9	0.5 $\pm$ 0.1	0.3 $\pm$ 0.05
6	501 $\pm$ 134*	26 $\pm$ 7	0.7 $\pm$ 0.2	0.4 $\pm$ 0.1

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-tailed paired t-test. For each time point a group of 7-8 rabbits was used.

higher compared to the maximal concentration after topical application. In addition, subconjunctival 100 μl 1% 8-bromo-cAMP induced higher aqueous humour protein levels, the maximum being 5.4 $\pm$ 1.5 g/l ($P < 0.01$) at 2 hrs. A slight increase in aqueous humour protein was also measured in the contralateral untreated eyes at 2 hrs, i.e. 0.9 $\pm$ 0.03 g/l ($P < 0.01$).

Table 9.5 Aqueous protein and aqueous PGE_2 levels after *topical* application of 100 μl 1% 8-bromo-cAMP solution to one eye (at -1 hr) and puncture of both eyes (at 0 hr)

Time (hrs)	PGE_2 in $pg.ml^{-1}$		Protein in $g.ml^{-1}$	
	Treated	Control	Treated	Control
1	1030 $\pm$ 337	806 $\pm$ 263	33.0 $\pm$ 6.8	31.0 $\pm$ 5.9
5	137 $\pm$ 23	148 $\pm$ 25	11.1 $\pm$ 3.8	10.2 $\pm$ 4.1

For each time point a group of 7-8 rabbits was used.

Table 9.6 Aqueous protein and aqueous PGE_2 levels after *subconjunctival* application of 100 μl 1% 8-bromo-cAMP solution to one eye (at -1 hr) and puncture of both eyes (at 0 hr)

Time (hrs)	PGE_2 in $pg.ml^{-1}$		Protein in $g.ml^{-1}$	
	Treated	Control	Treated	Control
1	138 $\pm$ 45*	861 $\pm$ 282	4.9 $\pm$ 1.5**	29.1 $\pm$ 6.2
2	105 $\pm$ 41*	518 $\pm$ 190	4.1 $\pm$ 2.6**	13.7 $\pm$ 2.9
3	119 $\pm$ 77	277 $\pm$ 136	5.4 $\pm$ 1.9	10.8 $\pm$ 2.2
5	130 $\pm$ 11	200 $\pm$ 49	8.0 $\pm$ 1.6	12.9 $\pm$ 2.1

For each time point a group of 7-8 rabbits was used. * $P < 0.05$; ** $P < 0.01$; two-tailed paired t-test.

Corneal puncture of the rabbit eye increased aqueous humour protein and PGE_2 levels to 33.5 ± 5.6 g/l and 1600 ± 400 pg/ml, respectively, at 1 hr. In the contralateral non-punctured eyes protein was 0.6 ± 0.1 g/l and PGE_2 was 30 ± 10 pg/ml.
Topical application of 100 µl 1% 8-bromo-cAMP had no effect on the increased aqueous humour PGE_2 and protein levels in the eyes, 1 and 5 hrs after puncture (table 9.5). Subconjunctival administration of 100 µl of 1% 8-bromo-cAMP, however, significantly inhibited the increased aqueous PGE_2 and protein levels 1 and 2 hr after puncture; a time course is presented in table 9.6.

Table 9.7 Aqueous protein and aqueous PGE_2 levels after subconjunctival administration of 100 µl 1% 8-bromo-cGMP to one eye (at -1 hr) and puncture of both eyes (t=0 hr)

Time (hrs)	PGE_2 in $pg.ml^{-1}$		Protein in $g.ml^{-1}$	
	Treated	Control	Treated	Control
1	931.2 ± 265	1390 ± 298	31.4 ± 5.5	28.8 ± 5.6
5	162 ± 23.4	227 ± 47.2	6.9 ± 2.0	10.8 ± 3.5

For each time point a group of 7-8 rabbits was used.

Subconjunctival administration of 100 µl 1% 8-bromo-cGMP showed to have no inhibitory effect on aqueous humour protein and PGE_2 levels after corneal puncture (Table 9.7).

DISCUSSION

This study shows that 8-bromo-cAMP and 8-bromo-cGMP reduce IOP and that 8-bromo-cAMP but not 8-bromo-cGMP inhibit trauma-induced ocular inflammation. These effects of exogenously administered cyclic nucleotide analogs depend on the mode of administration.
Differences in pharmacokinetics for subconjunctival and topical administration (Maurice and Mishima, 1989) are clearly demonstrated. Subconjunctival application of 8-bromo-cAMP induced 3 to 15-fold higher 8-bromo-cAMP concentrations in the aqueous humour compared to topical administration of the same dose. A plateau

maximal concentration at 2 and 3 hr shows the long duration achieved after giving a subconjunctival depot. Topical treatment rapidly reached a 10-fold lower maximal concentration at one hour. These results are in agreement with literature on ocular pharmacokinetics (Maurice and Mishima, 1989).

In our opinion the different penetration into the anterior chamber is the reason why subconjunctival but not topical administration of 8-bromo-cAMP induced a sustained reduction of IOP. High levels of 8-bromo-cAMP are needed to achieve an IOP reduction; these levels correlated well in time with the hypotensive effect. Intracamerally administered cAMP has been shown to induce a two-fold increase in outflow facility in rabbits (Neufeld et al, 1975). In another study (Wong and Howes, 1983), intravitreal injections of cAMP did not cause elevation of the intracellular cAMP levels. In the current study, 8-bromo-cAMP was used instead of cAMP for mainly two reasons. Firstly, cyclic AMP is rapidly degraded by cAMP dependent phosphodiesterases, while 8-bromo-cAMP is a poor substrate for this enzyme (Beebe et al, 1988). Secondly, 8-bromo-cAMP is cell-permeable whereas cAMP itself does not easily pass cell membranes.

Many studies have indicated that endogenous cAMP is involved in the reduction of IOP achieved with various stimulators of adenylate cyclase such as catecholamines (Hoyng et al, 1991; Neufeld et al, 1972a, 1973; Radius and Langham, 1973; Rowland and Potter, 1979; and Boas et al, 1981), cholera toxin (Gregory, 1981b; Sears et al, 1981) or forskolin (Caprioli et al, 1983; Caprioli et al, 1984a). In this study we provide evidence that also the cAMP analog 8-bromo-cAMP can lower IOP.

It was not expected that subconjunctival administration of only saline induced a slight increase of aqueous humour PGE_2 and protein; the PGE_2 probably derives from conjunctival tissue. However, the raised level of aqueous humour PGE_2 was insufficient to have an effect on IOP. Moreover, it should be noted that, when treating one eye subconjunctivally, the contralateral control eye received the vehicle subconjunctivally too, so that the effect on IOP can only be attributed to 8-bromo-cAMP itself.

Like 8-bromo-cAMP, 8-bromo-cGMP also induced a reduction of IOP when given subconjunctivally but not when given topically. Recently, it has been reported that atrial natriuretic factor (ANF) (Sugrue and Viader, 1986; Mittag et al, 1987a; Nathanson, 1988; Becker, 1990; and Korenfeld and Becker, 1989) reduced IOP in rabbits and that a simultaneous elevation of cGMP in the ciliary processes was observed (Mittag, 1987a; Becker, 1990; Korenfeld, 1989; and Nathanson, 1987). The 1% topical application of 8-bromo-cGMP in our study had no hypotensive effect. This is slightly different from Becker (Becker, 1990) who demonstrated a shortlasting effect with this topical dose in NZW rabbits. Our finding that 8-bromo-cGMP reduces IOP when given subconjunctivally may support the hypothesis that the peptide ANF

reduces IOP via a cGMP regulating mechanism in pigmented rabbits.
There is evidence that drugs that activate intracellular adenylate cyclase can modulate the inflammatory response after bacterial endotoxin (Wong and Howes, 1983) or immunogenic provocation (Verbey et al, 1987; Deporter et al, 1976, Deporter, 1977; and Seo and Saeki, 1980). Therefore we were interested in the effect of the cell-permeable c-AMP analog 8-bromo-cAMP on protein and PG levels in aqueous humour after a slight trauma such as induced by corneal puncture. Subconjunctival but not topical 8-bromo-cAMP partially protected the breakdown of the BAB. It reduced the endogenous formation of PGs as indicated by the PGE_2 content in aqueous humour 1, 2 and 3 hr after corneal puncture. In the contralateral eyes saline was given subconjunctivally. It can be argued, that the effect of 8-bromo-cAMP on aqueous humour PGE_2 after corneal puncture is due to the effect of subconjunctival injection of saline inducing PGE_2-formation. However, the eyes pre-treated with subconjunctival saline followed by puncture showed an increase in aqueous humour PGE_2-level not different from that after corneal puncture only. The breakdown of the BAB after puncture with subconjunctival administration of 8-bromo-cAMP did not exceed the breakdown of the BAB after 8-bromo-cAMP alone. Similar to effects of 8-bromo-cAMP on IOP, the puncture experiments also reveal that higher levels of 8-bromo-cAMP, up to 3 nmoles/ml, are needed in the aqueous humour to be effective, i.e. inhibit endogenous PGE-production. It has been reported that intracellular c-AMP elevation reduces phosphoinositide breakdown in platelets (Nishizuka, 1984) and inhibits the formation of inositol 1,4,5,-triphosphate in bovine iris sphincter muscle (Tachado et al, 1989). It is suggested that in this study 8-bromo-cAMP may suppress the pathways by which arachidonic acid is released from phosphoinositides after a slight trauma. The protective effect on the BAB seems to be a secondary effect due to inhibition of PG release.
In control experiments, neither topical or subconjunctival application of 8-bromo-cGMP had influence on the breakdown of the BAB or PG formation in aqueous humour after puncture. This is supportive for a specific anti-inflammatory effect of 8-bromo-cAMP.
In conclusion: subconjunctival administration of 8-bromo-cAMP as well as 8-bromo-cGMP reduces IOP, although probably by different mechanisms. Subconjunctival application of 8-bromo-cAMP but not 8-bromo-cGMP reduces the breakdown of the BAB and formation of PGE_2 after puncture trauma of the cornea. A role for c-AMP in modulation of the arachidonic acid cascade during inflammation is suggested.

CHAPTER 10

ADENYLYL CYCLASE IN HUMAN AND BOVINE TRABECULAR MESHWORK

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Abstract

Adenylyl cyclase activity was compared in bovine and human trabecular meshwork membrane fractions and in whole tissue homogenate (bovine only). Basic characteristics and responses to various drugs/agents were tested including forskolin (FSK), manganese (Mn^{2+}), fluoroaluminate (AlF_4^-), isoproterenol (ISO), prostaglandins (PGE_1, PGE_2, $PGF_{2\alpha}$) and vasoactive intestinal peptide (VIP).

In bovine trabecular meshwork particulate fraction was stimulated 3.3- and 2.6-fold (x) basal by 60 and 2μM FSK respectively, 2.2 x by ALF_4^-, 1.5 x by PGE_1 and PGE_2, while no or very weak response was obtained with $PGF_{2\alpha}$, ISO, norepinephrine, phenylephrine, p-amino clonidine, adenosine and VIP. The PGE_1 stimulation was dose- and G-protein dependent, evidence for prostaglandin E receptor (EP-receptor) mediated activation.

In human trabecular meshwork membrane fractions AC stimulation was more pronounced, being 12.4 and 5.5 x for 60 and 2μM FSK, and 3 x for PGE_1 and PGE_2. $PGF_{2\alpha}$ had no effect. Significant AC stimulation was found with ISO, 2.8 x, and with VIP, 1.8 x.

The results indicate EP-receptor mediated activation of adenylyl cyclase in bovine and human trabecular meshwork. Bovine trabecular meshwork may lack or have few $ß_2$-adrenoceptors, or if present they are not coupled to adenylyl cyclase, and had no receptor-mediated responses for the drugs tested except for PGE_1 and PGE_2. The findings indicate significant species differences in receptors coupled to AC in TM tissue. The results also suggest that AC may have a regulatory role in human trabecular meshwork controlling outflow facility acting via $ß_2$-adrenoceptors, PGE_2 and VIP, all of which could have a local physiologic function in this tissue.

Introduction

Adenylyl cyclase/cyclic AMP (cAMP) is thought to play a role as second messenger in the regulation of aqueous humour secretion by ciliary processes. Increasing evidence accumulates that cAMP is also important in regulating outflow mechanisms in the trabecular meshwork. Several drugs or agents that act on receptors coupled to adenylyl cyclase in various tissues affect outflow facility; particularly adrenergic drugs such as epinephrine (Neufeld et al, 1972a, Neufeld et al, 1973). The importance of cAMP as second messenger in response to in vivo treatment with catecholamines is further demonstrated by combination drug studies with IBMX, a phosphodiesterase inhibitor. IBMX potentiated the hypotensive effect of epinephrine (Hoyng et al, 1991; Busch and Hoyng, 1991b) by increasing its effect on outflow facility (Hoyng and Busch, 1992) in rabbits. Other agents like prostaglandins have been suggested, but also questioned (Kaufman, 1989), to play a role in controlling outflow facility if topically applied (Camras et al, 1977), or as endogenous intermediate in response to epinephrine (Anderson and Williams, 1990).
In vitro studies report the presence of adenylyl cyclase activity and some drug responses in trabecular meshwork explants in bovine (Bartels, 1988b), in monkey (Crawford et al, 1991) and human (Wax et al, 1989) trabecular meshwork membrane fractions and in cultured human trabecular meshwork cells (Wax et al, 1989; Polansky and Alvaredo, 1985). $ß_2$-Adrenoceptors have been localized and characterized in human trabecular meshwork (Wax et al, 1989; Jampel et al, 1987; Elena, 1990). In the present study trabecular meshwork membrane fractions were prepared from bovine (also tissue homogenate) and from donor bank human eyes. Basic AC characteristics and stimulatory responses to various drugs/agents were investigated and demonstrated for catecholamines, forskolin, prostaglandins, and the peptide VIP.

Materials and methods

Chemicals and reagents

Biochemical reagents were purchased from Sigma Chemical Company (St.Louis, MO). ^{3}H-c-AMP was obtained from New England Nuclear Corp. (Boston, MA) and α-^{32}P-ATP from New England Nuclear or from Amersham (Arlington Heights,IL). Synthetic porcine VIP was from Cambridge Research Biochemials (Atlantic Beach NY11509). All other reagents and drugs were obtained from Sigma or from Fisher Scientific (Pittsburg, PA). Forskolin (FSK) was made up as 10 mM stock in dimethyl sulfoxide. The assay kit for protein determination by dye-binding was purchased from BioRad (Richmond,CA 94804).

Preparation of the tissues

Fresh bovine eyes were delivered on ice from a nearby slaughterhouse and, upon arrival, immediately dissected on ice. Trabecular meshwork explants were prepared according to a method described by Anderson, Wang and Epstein (1980). The anterior segment of the eye was cut off between the limbus and the equator. Choroidal tissue was severed from the sclera together with the lens and anterior vitreous. The iris-ciliary body was gently lifted from the sclera. The meshwork could be identified as a grey band encircling the cornea; posteriorly adjacent nontrabecular grey tissue was removed. Trabecular tissue was lifted from the sclera by using a small blunt gouge and immediately stored at -80^{0}C.

Thawed tissues were hand homogenized in isotonic divalent ion chelating buffer (0.3 M sucrose, 20 mM Tris pH 7.6, 5 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol) in a teflon/glass homogenizer using 25 strokes of the pestle. The centrifuged particulate fraction (15 min, 27000 g) was washed once in the homogenizing buffer (6ml), recentrifuged (15 min, 27000 g), decanted and resuspended in the same buffer (3ml), containing 0.1 mM indomethacin and 10μg/ml leupeptin, and passed through a 0.5mm nylon mesh. If whole tissue homogenate was used then the thawed tissue was homogenized in 3 ml of the homogenizing buffer containing 0.1 mM indomethacin and 10μg/ml leupeptin with 25 strokes of the pestle and passed through the mesh. The protein content of the preparations usually ranged between 1-1.5 mg/ml.

Human donor eyes were provided by the donor eye bank of the Netherlands Ophthalmic Research Institute. In the procedure for preserving corneas for transplantation purposes the eyes were enucleated as soon as possible and delivered

on ice by courier. The donors' ages ranged between 20-80 years. The anterior part of the eyes was cut off at the ora serrata, quickly frozen at -80°C and stored up to 4 weeks before use. The post-mortem time before freezing the tissues was usually less than 8 hours. The tissue was thawed to 4°C and the trabecular meshwork was surgically prepared on ice under an operating microscope according to a procedure described by Tripathi and Tripathi (1982). The lens was removed after being severed from the zonular attachment. The iris-ciliary body was pulled away from the sclera; the remaining corneo-scleral ring was dissected in 8-10 equal radial segments; the scleral spur was identified by its ridge-like configuration. In each segment a partial thickness cut was made anteriorly in front of the scleral spur and a second parallel cut posterior to the line of Schwalbe. Trabecular meshwork tissue was lifted away with a sharp forceps. Histological sections confirmed the presence of predominantly trabecular tissue (not shown). Trabecular tissue from 4 human eyes was prepared for one experiment, hand homogenized in 1.2 ml of the same isotonic divalent ion chelating buffer as described above containing 0.1 mM indomethacin and 10μg/ml leupeptin in a small Dounce glass/glass homogenizer using 35 strokes of the pestle, passed through a mesh and centrifuged (15 min, 27000 g). The supernatant was removed by decanting and the particulate fraction resuspended in 1.2ml homogenizing buffer containing 0.1 mM indomethacin and 10μg/ml leupeptin. The resuspended particulate fraction had a protein content of approximately 0.10- 0.30 mg/ml. Only once frozen and thawed tissue was used.

Adenylyl cyclase assay (AC)

Enzyme activity was determined in glass test tubes in a total volume of 250 μl for bovine tissue and 125μl for human tissue, containing 60 mM sucrose, 80 mM Tris buffer pH 7.6, 3 mM $MgCl_2$ (or $MnCl_2$ as indicated), 1 mM EDTA, 0.2 mM EGTA, 5 mM creatinine phosphate (CP), 125 μg creatinine phosphokinase, 20 μM GTP (or 0.1 mM GDPßS as indicated), 4 mM theophylline, 20 μM indomethacin, 0.2 mM ATP, α-^{32}P-ATP (1-2 * 10^6 cpm for bovine and 4-6 * 10^6 for human tissue), 1 mM cAMP, and ^{3}H-cAMP (1-3 * 10^4 cpm). The tubes were preincubated at 30°C to equilibrate. To start the assay fifty μl aliquots of the membrane or full tissue suspension were added to triplicate (bovine) or quadruplicate/quintuplicate (human) tubes containing a premix of all the other incubation ingredients and drugs as indicated. The assay was terminated after 5 (bovine) or 10 (human) minutes by addition of SDS, followed by placing the tubes in boiling water (3 min) and isolation of the ^{32}P-cAMP with added ^{3}H-cAMP tracer by the double column method of Salomon et al (1974). When FSK was used, the control assay tubes contained

DMSO(< 1 %) equivalent to the amount used to dissolve FSK. GDPßS, if used, was added to the bovine cell homogenate prior to the assay to allow 10 min pre-equilibration with the tissue.

Data analysis

Adenylyl cyclase stimulation was expressed as the ratio of corrected ^{32}P-cAMP cpm for a drug effect relative to the corrected cpm for the control (baseline) assay tubes. Correction was based on recovery of the ^{3}H-cAMP tracer, which was in the range of 55 to 80%. This yielded a relative value, i.e. stimulation index. Mean basal AC specific activities were calculated for bovine and human tissue preparations in tri-/quadruplicates and were based on membrane protein solubilized with NaOH determined by the Bradford Dye-binding method with bovine gamma globulin as the reference protein. Stimulation indices multiplied by the basal specific activity approximated specific activities for drug effects. Experiments illustrated by bar graphs were done at least three (up to 8) times using different membrane/full tissue preparations. Error bars represent S.E.M. values, and significance (discriminating at P value 0.05) were determined by paired t-test. The AC activation dose-response curve depicted in figure 10.2 was fitted with nonlinear regression analysis using the formula:

$$\text{Effect} = \frac{E_{max} \times c^{\eta}}{EC_{50}^{\ \eta} + c^{\eta}} .$$

E_{max} is maximal response, EC_{50} is drug concentration which yields 50% of the maximal effect, and η is Hill slope coefficient, all being calculated values.

Results

Basal AC specific activity in bovine trabecular meshwork preparations was calculated to be 31.1 ± 10.7 pmol cAMP/min/mg protein (n=4).
Routine assay conditions included Mg^{2+} as divalent ion regulator required for catalytic activity. The 3mM concentration used yielded approximately 1.8 mM free Mg^{2+} ion concentration, calculated by correcting for the presence of total EDTA + EGTA chelators of divalent ions. Stimulation of AC in bovine trabecular meshwork .

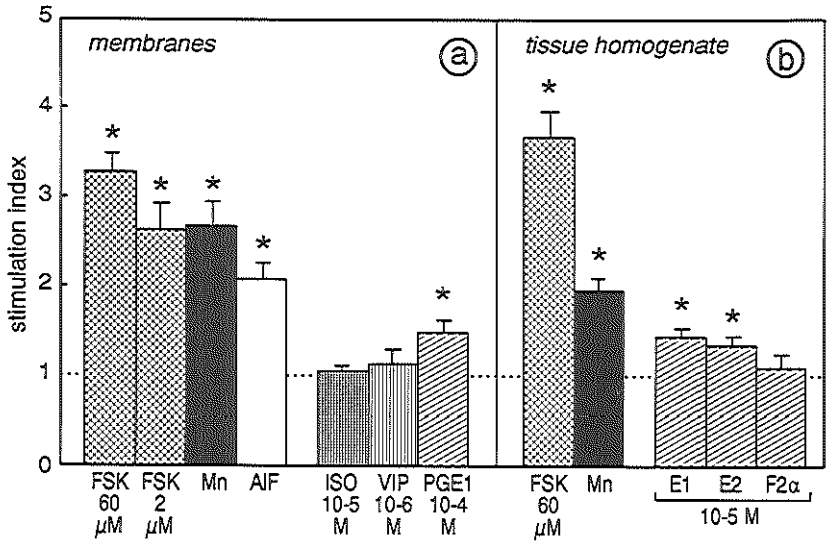

Figure 10.1 Adenylyl cyclase stimulation in membrane and tissue homogenate preparations of bovine trabecular meshwork in response to 60 and 2 μM forskolin (FSK), 3 mM Mn^{2+}, 2 mM fluoride + 0.1 mM Al^{3+} (AlF_4^-), 10-5 M isoproterenol (ISO), 10-6 M vasoactive intestinal peptide (VIP), and 10-5 M PGE_1 (and 10-4 M), PGE_2, and $PGF_{2\alpha}$. See text (data analysis) for definition of the stimulation index.

particulate with forskolin, AlF_4^- and Mn2+ is depicted in Figure 10.1a and in bovine whole tissue homogenate in Figure 10.1b. FSK sensitive AC was present as well as G-protein dependent activity that can be activated by F- (Gs^*) which in turn stimulates AC. Mn-ATP and free Mn^{2+} are known to be better substrate and activator of the catalytic unit relative to Mg^{2+} and amplify all AC-activities; Mn^{2+} increased basal activity in trabecular meshwork homogenate (Figure 10.1a and 10.1b). The presence of stimulatory G-protein is indicative for some receptor(s) linked to AC. Surprisingly, ISO (10^{-5}M) did not appreciably stimulate the bovine trabecular meshwork AC (Figure 10.1a). Further attempts to augment potential small effects of ISO by combining ISO with 2μM FSK and/or by using Mn^{2+} instead of Mg^{2+} were negative data (not shown). Positive controls using ISO were obtained with bovine ciliary processes particulate fraction from the same eyes (not shown) and with human trabecular meshwork tissue

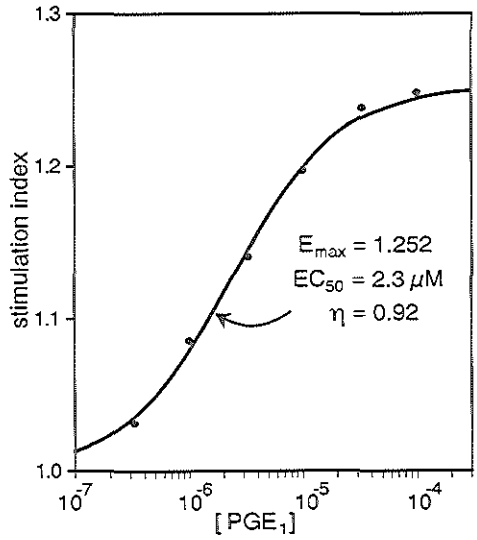

Figure 10.2. Dose-response curve for PGE_1-stimulated adenylyl cyclase in bovine trabecular tissue homogenate (five replicates per point). E_{max}, EC_{50}, and η(Hill coefficient) are calculated parameters from the fitted curve.

particulate preparation (see below). Stimulation or inhibition of AC was not found with norepinephrine, phenylephrine, p-aminoclonidine on bovine trabecular meshwork (not shown). Also no response was seen with the peptide VIP on this tissue preparation (Figure 10.1a).

Prostaglandins of the E-series stimulated bovine trabecular meshwork AC. Activation in the particulate fraction with PGE_1 is demonstrated in Figure 10.1a. Similar PGE_1-activation was found relative to its control baseline in whole tissue homogenate (Figure 10.1b), which includes the cytosol. Some AC acivity was also found in the collected supernatant, i.e. 0.75 $\pm$ 0.07 (n=4) compared to the basal activity of the corresponding, centrifuged, membranes. PGE_1-(10^{-5}M) stimulation was 1.24 $\pm$ 0.02 in the membranes and 1.25 $\pm$ 0.06 in the supernatant (relative to their control basals). This finding probably indicates that small particulate membrane fractions remain in the 27000 x g supernate. Among other PGs tested, PGE_2 activated bovine trabecular meshwork-AC to a similar extent as PGE_1 whereas $PGF_{2\alpha}$ had no effect (see Figure 10.1b). Dose-dependent stimulation with PGE_1 is depicted in figure 10.2, suggesting receptor mediated activation. GTP-dependency for the PGE_1 response is demonstrated by adding GDPßS to the tissue homogenate and leaving GTP out of the buffer. GDPßS

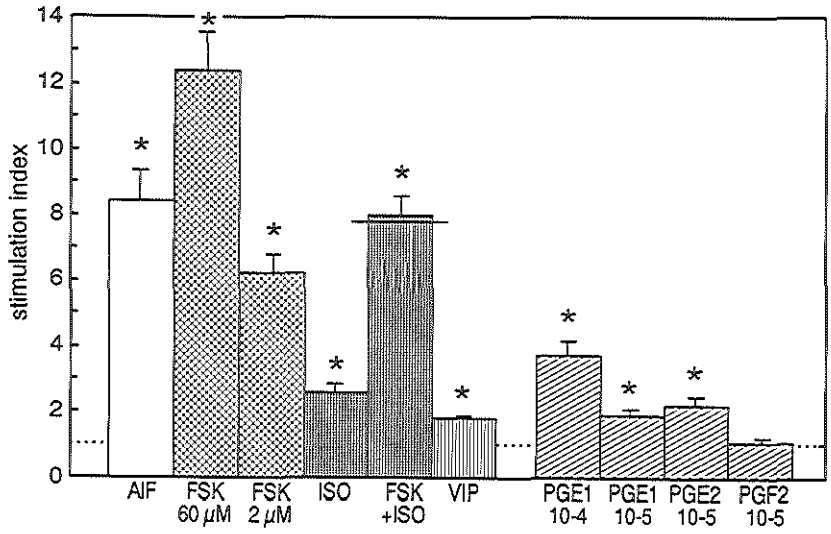

Figure 10.3 Adenylyl cyclase stimulation in membrane preparations of human trabecular meshwork in response to 2 mM fluoride + 0.1 mM Al^{3+} (AlF_4^-), 60 and 2 μM forskolin (FSK), 10-5 M isoproterenol (ISO), combination of 2 μM FSK with 10-5 M ISO, 10-6 M vasoactive intestinal peptide (VIP), 10-5 M PGE1 (and 10-4), PGE_2, and $PGF_{2\alpha}$.

irreversibly binds to and blocks activation of G-proteins. In two experiments (n=8) GDPβS significantly reduced basal activity to a stimulation index less than 1, i.e. 0.58± 0.04 ($p < 0.05$); PGE_1-stimulation index was reduced from 1.5 ± 0.1 in the presence of GTP to 0.68 ± 0.02 with GDPβS ($p < 0.05$). This further supports (EP-) receptor mediated AC activation.

One time-course experiment (n=4) done with full tissue homogenate showed near linearity for the basal AC activity and the PGE_1-response using assay times ranging from 5 up to 30 minutes. Basal activity was 1, 0.99, 1.00 and 1.04 in 5-, 10-, 20-, and 30-minute assays. Respective PGE_1 (10^{-5}M) stimulation was 1.31, 1.26, 1.27 and 1.22, indicating minimal loss of stimulatory capacity.

Dopamine, adenosine, cyclohexal adenosine nor 5'-N-ethylcarboxamidoadenosine (NECA) (dose 10^{-5}M) did not affect bovine AC activity.

Results of experiments with human trabecular tissue are shown in Figure 10.3. Basal specific activity of human trabecular meshwork particulate was 38.8 ± 8.8 pmol/min/mg protein (n=4). This is similar to the basal activity found for bovine trabecular meshwork preparations (see above). AC stimulation of human trabecular meshwork was evaluated in response to FSK, ISO, VIP and PGs. In contrast to bovine

trabecular meshwork a significant ISO as well as VIP response was present. Forskolin 2μM, however, did not potentiate the effect of ISO. With respect to PGs a similar, quantatively more pronounced, stimulation was found. The order of responsiveness was $PGE_2 = PGE_1 > PGF_{2\alpha}$, similar as observed for bovine trabecular meshwork tissue.

Discussion

Values for basal AC specific activity obtained in bovine and human trabecular meshwork tissue preparations were similar. Higher stimulation indices were seen in response to drugs or agents in human tissue as compared to bovine tissue, also implicating higher specific activities.

The results demonstrate the presence of PGE_1-sensitive AC in bovine and human trabecular meshwork particulate fractions. Human trabecular meshwork particulate revealed ISO- and VIP-sensitive AC, whereas these responses were not significant in bovine trabecular meshwork.

Activation with ISO is commonly observed most tissues containing AC but was notably absent in bovine trabecular meshwork particulate. Bartels (1988b) earlier reported that epinephrine in calf trabecular meshwork tissue incubations did not stimulate AC and suggested that ß-adrenoceptors were not present, but could not exclude α-adrenergic inhibition by G_i-protein. P-aminoclonidine and phenylephrine but were tested but neither a stimulatory nor inhibitory effect was observed. The lack of stimulatory responses to ISO, which is highly selective for ß-adrenoceptors, confirms that either few $ß_2$-receptors are present or that they are not coupled to AC. In contrast, bovine ciliary processes do have high levels $ß_2$-adrenoceptor stimulated AC (Elena et al, 1984).

In human trabecular meshwork membranes ISO did stimulate AC. Previously, ISO and epinephrine have been shown to stimulate cAMP production in human sclera-trabecular rings (Neufeld and Sears, 1974); human trabecular meshwork cells grown in primary culture, have been demonstrated to increase cAMP synthesis in response to ß-receptor agonists (Polansky and Alvaredo, 1985), and selective $ß_2$-adrenoceptors have been characterized by autoradiography (Jampelet al, 1987; Elena et al, 1990) in sections of human trabecular meshwork, as well as by radiolabeled ligand binding techniques for human cultured trabecular meshwork cells and human trabecular tissue (Wax et al, 1989). Recently, FSK-, ISO- and EPI-stimulated AC was reported in monkey trabecular meshwork membranes (Crawford et al, 1991). The present results reveal an ISO response in human trabecular meshwork membranes which we interpret

as stimulation by binding to β_2-receptors coupled to AC. Combining FSK with ISO induced an additive response without further potentiation. FSK is known to elicit greater activation of the AC catalytic unit if it is complexed with activated G_s alpha subunit than it does with the catalytic unit alone (Seaman, 1985). One may speculate whether the additivity and absence of a FSK-potentiated ISO response are indicative for the presence of different enzyme types of AC.

A variety of compounds which bind to receptors coupled to AC in various tissues was tested since no ISO response was present in bovine tissue, while the FSK as well as AlF_4^- stimulated AC response was indicative for stimulatory G-potein. For example VIP has been demonstrated to bind to VIP binding sites in rabbit ciliary epithelial cells and stimulates AC activity (Mittag et al, 1987b). In monkey cultured trabecular meshwork cells VIP increased dose-dependently cAMP production (Koh and Yue, 1988). In this study we demonstrate VIP sensitive AC in human trabecular meshwork particulate whereas no response was seen in bovine trabecular meshwork particulate. This accords with commonly observed co-existence on cells of VIP- and β_2-adrenoceptors.

Dopamine- or adenosine-receptor agonists had no effect in bovine trabecular meshwork. A significant finding was the AC activation with PGE_1 in bovine trabecular meshwork tissue. The dose-response relationship and GTP-dependency give evidence for a receptor mediated effect. The Hill coefficient of 0.92 is indicative for a single set of non-interactive ligand binding sites. The order of stimulation by the different PGs, $PGE_1 = PGE_2 > PGF2\alpha$, is suggestive for an EP-receptor. Discrimination between three currently distinguished EP-receptor subtypes remains to be established with sulprostone and EP1-antagonists. It seems reasonable to interprete the mechanism of PGE stimulation in human trabecular meshwork tissue also as EP-receptor mediated. This is based on analogy with the bovine results. Moreover, much of immediate post-receptor consequences of PGs is not known except for PGs of the E-series which in most tissues stimulate (but can sometimes inhibit) AC (Robertson, 1986). In vitro studies on cultured human trabecular meshwork cells have shown that the human trabecular meshwork cells are capable of synthesizing PGs; the order of magnitude was $PGE_2 > PGF_{2\alpha} >$ 6-keto-$PGF_{1\alpha}$ (Weinreb et al, 1988). Thus, together with the current results, a local physiological role for PGE and EP-receptors stimulating AC in trabecular meshwork seems likely. This may well contribute to controlling outflow facility. Earlier in vivo studies suggested that PGE_1 reduces intraocular pressure by increasing outflow facility (Camras, 1989). In a recent study we reported that phosphodiesterase inhibition with topical IBMX increased epinephrine's effect on C-value and that this effect was not only β_2- but also α_1-adrenoceptor dependent (Busch and Hoyng, 1990). It may be hypothesized that the Ca-signal (α-adrenergic stimulation with phenylephrine) generates PG (PGE_2) production

via phospholipase A_2, which activates AC. Various adrenergic agents stimulate prostaglandin synthesis in ocular tissues (Abdel-Latif, 1989) and endogenous PGs have been suggested to be involved in the ocular hypotensive response to catecholamines (Hoyng et al, 1982; Camras, 1989). Indomethacin pretreatment antagonized the effect of epinephrine to increase trabecular outflow facility (Anderson and Williams, 1990). PGE has been demonstrated to be a potent activator of AC in rabbit corneo-scleral rings (Neufeld and Sears, 1974).

It is concluded that both in bovine and human trabecular meshwork membranes AC is stimulated by PGE_1 and PGE_2, mediated by a prostaglandin E (EP-) receptor. In bovine trabecular meshwork $ß_2$-adrenoceptors may not be present or, if so, are not linked to AC. Human trabecular meshwork membranes contain AC which is responsive to ISO, and VIP. The findings indicate significant species differences in receptors coupled to AC in TM tissue. The results also suggest that AC may have a regulatory role in human trabecular meshwork controlling outflow facility by ß2-adrenoceptors, PGE_2 and VIP, all of which could have a local physiologic function in this tissue.

CHAPTER 11

SUMMARY

The bifunctional properties of adrenergic receptors include a binding site, and an effector component which transduces the signal to the cell via a second messenger system. The adenylyl cyclase(AC)/cyclic AMP(cAMP) second messenger system is thought to play an important role in the regulation of aqueous humour secretion by ciliary processes as well as the control of outflow mechanisms in the trabecular meshwork, particularly in response to adrenergic agents such as epinephrine. The purpose of the present study was to investigate in vivo in rabbits the role of AC/cAMP by augmenting the AC/cAMP signals of epinephrine and other adrenergic agents pharmacologically with isobutylmethylxanthine (IBMX). IBMX is a phosphodiesterase inhibitor which inhibits the metabolism of active cAMP to inactive AMP; theoretically, IBMX can be expected to increase $ß_2$-adrenergic responses since $ß_2$-adrenoceptors are coupled directly to AC.

Chapter 3. Topical IBMX alone had no effect on intraocular pressure (IOP), but IBMX enhanced the ocular hypotensive effect of the non-selective agonists epinephrine and norepinephrine and the selective $ß_2$-agonist isoproterenol. The main effect of IBMX was to increase the efficacy of these catecholamines. Thus an interaction between catecholamines and IBMX that reduces IOP has been demonstrated.

Chapter 4. The IBMX is not soluble but was suspended in hydroxypropyl methylcellulose for the experiments described in chapter 3. Two soluble forms of isobutylmethylxanthine were tested, i.e. the ethylenediamine salt of IBMX and complexes of IBMX and 2% cyclodextrin prepared in saline. Both soluble formulations increased the hypotensive effect of epinephrine in a dose-dependent manner and on the same order of magnitude as a suspension of IBMX. This indicated that the bioavailability of IBMX was not substantially improved by either of these soluble forms. It was concluded that the IBMX complex with cyclodextrin, having a neutral pH, was a suitable formulation for future experiments.

Chapter 5. Alterations in regional blood flow in the eye were studied in rabbits using the radiolabeled microsphere technique. The purpose was to investigate whether the epinephrine induced reduction of IOP, enhanced by IBMX, is based on a vascular mechanism since epinephrine is known to induce marked vasoconstriction in the anterior uvea and a marked vasoconstriction would tend to lower the IOP. However, application of IBMX in combination with epinephrine induced an increase in ocular blood flow, lasting up to 7.5 hours, in both the iris (3-fold) and the ciliary processes (2-fold). IBMX alone tended to increase the blood flow in the iris and ciliary body, and epinephrine had no effect on the ciliary processes. This indicated that the reduction of intraocular pressure seen after topical treatment with a combination of IBMX and epinephrine was not based on a vascular mechanism. The increase in ocular blood flow after application of a combination of IBMX and epinephrine may be indicative of the presence of vasodilative ß-adrenergic receptors in the ocular vasculature.

Chapter 6. Aqueous humour dynamics were studied to determine whether the mechanism by which IBMX potentiates the ocular hypotensive response to (dipivalyl) epinephrine consisted of increasing the outflow facility in the trabecular meshwork or reducing aqueous humour secretion in the ciliary processes. Outflow facility was measured by means of Schiötz indentation tonography and aqueous humour production by fluorophotometry in rabbits. Uveoscleral flow in rabbits is very low/neglible and was therefore not measured. Neither IBMX nor epinephrine affected the outflow facility; combination treatment with IBMX and epinephrine, however, increased the outflow facility by 43%. The slight reduction in aqueous humour formation after topical dipivalyl epinephrine was abolished by combination treatment with IBMX. The results indicate that phosphodiesterase inhibition with IBMX potentiates the ocular hypotensive effect of epinephrine by increasing the outflow facility and not by reducing aqueous humour secretion. These data support the accumulating evidence that AC plays an important role in the regulation of outflow facility.

Chapter 7. Potentiation of the ocular hypotensive response to catecholamines by IBMX was more pronounced for the non-selective adrenergic agonists epinephrine and norepinephrine than for the selective $ß_2$-agonist isoproterenol (chapter 3). This was a surprising finding since IBMX is expected to increase primarily the responses mediated by $ß_2$-adrenoceptors coupled to AC. The adrenoceptor selectivity of catecholamines for potentiation by IBMX was systematically investigated in this chapter by combination drug experiments using selective α_1(phenylephrine), α_2(B-HT920), and $ß_1$(dobutamine), $ß_2$(salbutamol,terbutaline)-adrenergic agonists. The order of responsiveness of the catecholamines to potentiation by IBMX can be summarized as: norepinephrine (α,ß) = epinephrine (ß,α) > isoproterenol (mainly $ß_2$) = salbutamol($ß_2$) > terbutaline ($ß_2$). IBMX had no effect on the ocular hypotensive

responses to dobutamine, phenylephrine and B-HT920. However, the addition of a subthreshold dose of phenylephrine to the salbutamol regimen further potentiated the hypotensive response to IBMX achieved with salbutamol. It is concluded that IBMX primarily enhances β_2- adrenoceptor/cAMP-mediated ocular hypotensive responses, but additional α_1-adrenergic receptor stimulation potentiates this effect. This is of interest since it may indicate that α_1-adrenoceptor stimulation, which evokes primarily a calcium second messenger response, induces an interaction with the β_2/AC/cAMP pathway.

Chapter 8. Further experiments (in the absence of IBMX) focussed on this interaction between α_1 and β_2-adrenergic signals by studying the combination of two selective adrenergic agonists. Enhanced ocular hypotensive responses were only found for the combination salbutamol (β_2) and phenylephrine (α_1). The outflow facility measured by tonography was not affected by either salbutamol or phenylephrine alone. Combination treatment, however, increased the C-value by 32%. These results provided confirmation of an interaction between α_1 and β_2-signalling systems that reduces IOP and increases outflow facility.

Chapter 9. It was shown that administration of exogenous cAMP, in the form of the cell permeable analogue 8-bromo-cAMP, reduced IOP. Analysis of the penetration of 8-bromo-cAMP into the aqueous humour indicated that the hypotensive effect depends on the mode of administration; subconjunctival, but not topical, application raised the level of 8-bromo-cAMP in the aqueous humour sufficiently to reduce IOP. A close temporal relationship with the effects on IOP could not be demonstrated since the maximum aqueous 8-bromo-cAMP levels occurred at two hours whereas the IOP response was seen between 2 and 5 hours.

The biochemical analysis of aqueous humour described in chapter 3 also focussed on the correlation between aqueous humour levels of endogenous cAMP and the IOP response. The aqueous levels of endogenous cyclic AMP were markedly elevated after combined epinephrine and IBMX treatment, indicating that adenylyl cyclase is stimulated by epinephrine whereas cAMP degradation is inhibited by the phosphodiesterase inhibition achieved with IBMX. A temporal relationship, however, was not present since potentiation of the pressure response occurred between 3 and 6 hours, whereas aqueous cAMP elevation was most pronounced 1 hour after medication. The absence of a close time relationship between cAMP levels and IOP response after epinephrine had already been mentioned by Boas et al (1981). This may indicate that initial AC activation is followed by a period in which AC is refractory whereas a sequence of events after initial AC stimulation leads to a delayed IOP response. The correlations between effects on IOP and those on aqueous cAMP responses suggest a role for cAMP in the reduction of IOP.

The finding that salbutamol, but also high doses of phenylephrine increased aqueous

cAMP levels (chapter 8) demonstrates that not only a β_2 but also an α_1/calcium signal stimulates AC. However, a combination of salbutamol and phenylephrine did not have a synergistic effect on cAMP responses which may be indicative of the presence of common AC. It is concluded that the results lend support to an interaction between α_1 and β_2-signals at the level of AC.

Chapter 10. Increased outflow facility has been demonstrated to play an important role in the reduction of IOP by both IBMX (chapter 6) and the interaction of α_1 and β_2-adrenergic stimulation (chapters 7,8). This suggests an important role for AC in the trabecular meshwork. Therefore, in vitro characteristics of the AC enzyme in trabecular meshwork membrane preparations were studied in chapter 10. Bovine and human trabecular meshwork membrane preparations were used. Bovine trabecular meshwork AC could be stimulated directly (forskolin) as well as by G-proteins (alumina fluoride) and prostaglandin E receptors. Little or no response was obtained with isoproterenol, which indicates that bovine trabecular meshwork may lack or have only a small number of β_2-adrenoceptors. Human trabecular meshwork AC stimulation was more pronounced. Human trabecular meshwork AC could be activated directly (forskolin) or by G-proteins (alumina fluoride), PGE_1, isoproterenol and VIP. This is indicative of the presence of β_2, VIP, and prostaglandin E- receptors coupled to AC. $Beta_2$, VIP, and prostaglandin E- receptors may play a local physiological role in the regulation of outflow facility in human trabecular meshwork with mediation of their effect by AC.

Epilogue

This thesis questions whether AC is a mediator of the ocular hypotensive response to adrenergic agents. Several arguments support a positive and others an inconclusive answer.

The hypotensive effect of IBMX is interpreted to be mainly due to phosphodiesterase-inhibition on the basis of the known properties of IBMX as well as the markedly enhanced aqueous cAMP levels and their correlation with the decrease in IOP found in the present studies. Antagonism for adenosine receptors is not likely to be an important pharmacological mechanism since IBMX had vasodilative effects whereas adenosine receptor antagonists are known to decrease ocular blood flow. Thus, IBMX proved to be a useful tool for the study of the role of AC/cAMP, providing evidence that AC is involved in mediation of the ocular hypotensive responses to epinephrine.

However, a more detailed answer is required regarding the observed differences in the effects on inflow and outflow facility. A proposed mechanism is presented in Figure 11.1. Outflow facility can be increased after strong AC/cAMP signals. This was demonstrated in this thesis consistently for IBMX and epinephrine as well as salbutamol and high doses of phenylephrine, and by others for isoproterenol, epinephrine and exogenous cAMP. Thus, it may be concluded that AC stimulation plays a role as mediator of the increase in outflow facility.
Interpretation of the role of AC stimulation in ciliary processes and its effect on aqueous humour formation remains complex. It may well be that effects on active

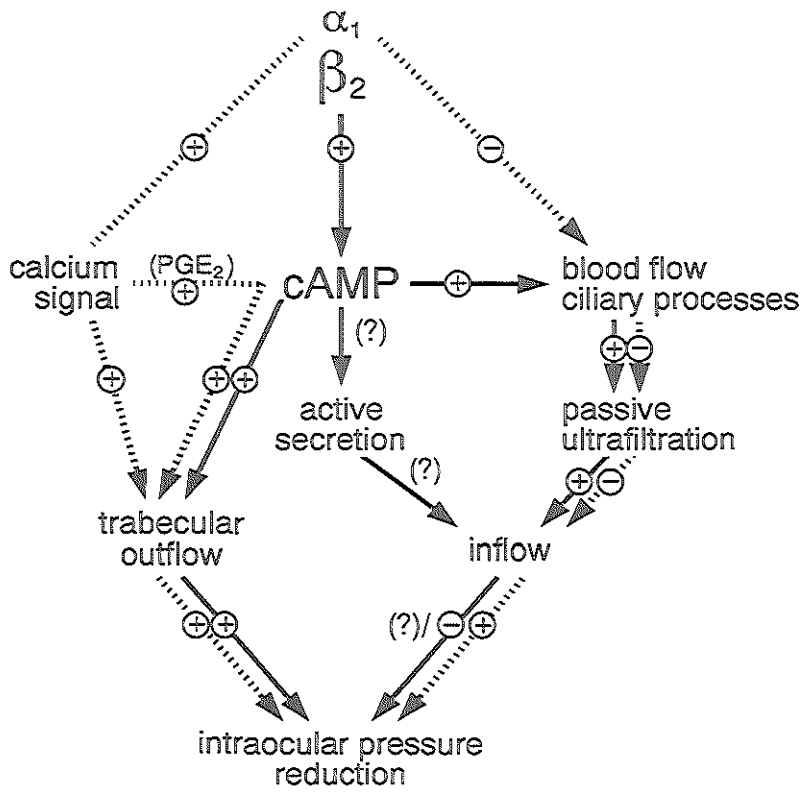

Figure 11.1 Schematic representation for the proposed mechanism by which adrenaline and combinations of a selective α_1 and β_2-adrenergic agonist reduce the intraocular pressure. Effects induced by β_2-adrenoceptor stimulation are indicated by straight lines, α_1-adrenoceptor dependent events by dotted lines. A stimulatory effect is marked by $\oplus$ and inhibitory by $\ominus$.

secretory processes in the ciliary epithelium are masked by concomitant effects on local haemodynamics. For example, the increase in blood flow in the iris and ciliary processes could well increase the ciliary perfusion rate which may in turn increase the rate of ultrafiltration. However, it is not really possible to determine whether a strong AC/cAMP signal inhibits active secretion in the ciliary epithelium and is counteracted by an increase in ultrafiltration due to vasodilation (IBMX with epinephrine) or that the reverse process occurs: the AC/cAMP signal increases active secretion but vasoconstriction (epinephrine) antagonizes this effect. The question of whether AC stimulation is involved in the reduction of the aqueous flow cannot therefore be answered conclusively. Whatever the real mechanism may be, a strong AC/cAMP signal did not reduce but tended to increase aqueous flow.
The present studies may contribute to our understanding of the ocular hypotensive effect of epinephrine. Combination drug studies of highly selective adrenoceptor agonists indicate that the hypotensive effect of epinephrine probably depends on its selective α_1 and $ß_2$-adrenergic properties. The increase in outflow facility induced by epinephrine may not merely depend on addition but instead on interaction after simultaneous $ß_2$ and α_1-adrenergic stimulation. The α_1/calcium signal could possibly stimulate AC in trabecular meshwork. Thus far, direct evidence for α_1-adrenergic stimulated AC in trabecular meshwork membrane preparations has not been found, but one may postulate that a calcium signal (α_1) generates prostaglandins and that PGE_2 in turn directly activate AC via EP-receptors. Other possible mechanisms may include the presence of calmodulin-sensitive AC, as in rabbit ciliary processes (Tormay, 1987), or protein kinase C that activates AC. Additional studies seem to be warranted.

On the basis of the animal studies presented topical treatment with a phosphodiesterase inhibitor may be useful in glaucoma if combined, for example, with epinephrine. Additional long-term studies are needed to determine whether tachyphylaxis and desensitization occur. Anticipated major advantages are:

1. IBMX will enhance the reduction of IOP by epinephrine (via its $ß_2$ and α_1-adrenoceptor-mediated ocular hypotensive effects)
2. the commonly accepted dose of epinephrine (1%) can be reduced. This could reduce the side effects of epinephrine, such as an irritating reactive hyperaemia. Irritation was never observed in the rabbit eyes treated with IBMX and epinephrine.

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SAMENVATTING

Adrenerge stoffen hebben in de medicamenteuze behandeling van glaucoom een belangrijke plaats. Aangrijpingspunten voor deze stoffen zijn adrenerge receptoren, die onderscheiden worden in 4 subtypes: α_1, α_2, $ß_1$, en $ß_2$-receptoren. Zowel selectieve en non-selectieve adrenerge agonisten en antagonisten verlagen de oogdruk. Zo is adrenaline (o.a. Diopine^R) een adrenerge agonist die voornamelijk bindt aan $ß_2$ en in geringe mate aan α_1-receptoren. Ook adrenerge antagonisten worden op grote schaal gebruikt om de oogdruk te verlagen bij glaucoom. Voorbeelden hiervan zijn timolol, levobunolol (beide $ß_{1,2}$), en betaxolol ($ß_1$). De pharmacologie en physiologie van deze adrenerge receptoren en oogdruk regulatie wordt echter maar ten dele begrepen. Dit proefschrift richt zich op de betekenis en functie van adenylaat cyclase als mediator voor oogdruk effecten van adrenerge stoffen. Adenylaat cyclase (AC) is een, algemeen voorkomend, intracellulair enzym systeem dat aan receptoren gekoppeld is en een agonist/receptor signaal doorgeeft in de cel.

Receptoren zijn intracellulair gekoppeld aan een "second messenger" systeem. Binding van een agonist aan de receptor activeert de receptor en dit leidt vervolgens tot activering van het second messenger systeem. Een paar, algemeen voorkomende second messenger systemen zijn beschreven. Adenylaat cyclase (AC) is zo'n systeem en is voor wat betreft de adrenerge receptoren gekoppeld aan $ß_1$, $ß_2$ en α_2-receptoren; AC gebruikt ATP als substraat om dit om te zetten in het biologisch actieve produkt cyclisch AMP (cAMP), i.e. het second messenger molecuul. Receptor activatie via $ß_1$ en $ß_2$-receptoren activeert het AC terwijl α_2-stimulatie de activiteit van AC remt. α_1-Receptoren activeren een geheel ander systeem, het calcium/protein kinase C second messenger systeem. Dit genereert calcium/inositol trifosfaat (IP_3)/diacyl glycerol (DAG) als second messenger moleculen.

AC/cAMP lijkt daarom een essentiële rol te spelen in zowel de physiologische als medicamenteus adrenerge oogdruk regulatie. Ook niet-adrenerge stoffen waarvan bekend is dat zij AC stimuleren (forskolin, vasoactive intestinal peptide, prostaglandine E) hebben sterke effecten op de oogdruk en bevestigen het belang van AC/cAMP. Dit leidde tot de probleemstelling voor dit proefschrift: medieert AC/cAMP het oogdruk verlagend effect van adrenerge stoffen? Met adrenaline in het

bijzonder is dit in konijnen bestudeerd door middel van combinatie-experimenten met isobutylmethylxanthine (IBMX). IBMX remt de afbraak van cAMP tot de inactieve metaboliet AMP door middel van remming van het enzym phosphodiesterase. IBMX versterkt daardoor cAMP afhankelijke effecten; in het algemeen zijn dat vooral de $ß_2$-adrenerg afhankelijke effecten. IBMX is chemisch en pharmacologisch verwant aan theophylline.

Hoofdstuk 1 en 2 zijn respectievelijk de algemene inleiding en beschrijving van de gebruikte materialen en methodes.

Hoofdstuk 3 laat in dose-response curves zien dat IBMX het oogdruk verlagende effect van adrenaline (ß,α), noradrenaline (α,ß) en isoproterenol ($ß_2$-selectief) sterk potentieert. IBMX zelf heeft geen effect. Hiermee is een interactie tussen catecholamines en IBMX aangetoond die de oogdruk verlaagt.

In **hoofdstuk 4** worden twee water-oplosbare vormen van IBMX getest omdat IBMX niet oplosbaar is en in hoofdstuk 3 gebruikt is als suspensie. Getest worden het ethyleendiamine zout van IBMX en IBMX in een 2% cyclodextraan oplossing dat leidt tot vorming van "inclusion complexes" . Beide oplossingen potentiëren het oogdruk verlagende effect van adrenaline dosis-afhankelijk en in dezelfde mate als de IBMX-suspensie. Geconcludeerd wordt dat de biologische beschikbaarheid niet wezenlijk verbetert. IBMX in een 2% cyclodextraan oplossing, dat een neutrale pH heeft, is geschikt voor toekomstige experimenten.

Hoofdstuk 5 bestudeert lokale hemodynamische effecten in de ciliaire processen met behulp van radioactief gelabelde microsferen. Het doel was om na te gaan of het potentiërende effect van IBMX op de oogdrukdaling na adrenaline toediening verklaard kon worden op grond van lokaal hemodynamische veranderingen. Adrenaline werkt vasoconstrictief, en vasoconstrictie zou kunnen leiden tot een verminderde kamerwater productie. Er werd echter een sterke en langdurige (tot 7.5 uur na medicatie) toename van de doorbloeding waargenomen in de ciliaire processen (2-voudig) en iris (3-voudig) na combinatie behandeling van IBMX met adrenaline; IBMX en adrenaline afzonderlijk hadden weinig effect. Dit betekent dat het oogdrukdaling potentiërende effect van IBMX niet verklaard kan worden op grond van hemodynamische veranderingen. De resultaten leveren een aanwijzing voor het bestaan van vasodilatoire ß-receptoren.

Hoofdstuk 6 laat zien dat het oogdrukdaling potentiërende effect van IBMX op adrenaline teweeg wordt gebracht door een afname van de weerstand in het trabekelsysteem en niet door verminderde kamerwater productie. De uitstroomcapaciteit die door middel van tonografie gemeten was, nam 30-40% toe, terwijl een bescheiden inhibitie van kamerwater productie na adrenaline alleen juist werd opgeheven door toevoeging van IBMX (gemeten met fluorophotometrie).

Hoofdstuk 7 bestudeert systematisch de adrenoceptor selectiviteit voor het oogdruk

verlagende effect van IBMX. Een niet begrepen bevinding in hoofdstuk 3 was namelijk dat IBMX meer effect had met (nor)adrenaline (α,ß) dan met isoproterenol ($ß_2$); met IBMX werd vooral een versterkend effect op selectief $ß_2$-adrenerge stimuli verwacht. Combinatie experimenten met selectieve α_1, α_2, $ß_1$, en $ß_2$- agonisten laten zien dat IBMX de $ß_2$-adrenerg geinduceerde oogdrukdaling (terbutaline, salbutamol) versterkt. Echter, een additionele α_1-stimulus (fenylephrine) potentiëert dit effect van IBMX nog meer. Dit verklaart dus waarom IBMX een beter effect heeft met niet-selectieve agonisten (nor)adrenaline (ß,α) dan met isoproterenol ($ß_2$). Het suggereert bovendien dat een interactie tussen twee wezenlijk verschillende "second messenger" signalen ($ß_2$/AC/cAMP en α_1/calcium/IP_3/CAM) optreedt die de oogdruk verlaagt.
Hoofdstuk 8 gaat nader in op deze interactie tussen het $ß_2$ en α_1-signaal. Individueel geven zowel een selectief α_1(fenylephrine), α_2(B-HT920), $ß_1$(dobutamine), als een $ß_2$(salbutamol, terbutaline)-stimulus oogdrukdaling. Bij combinatie van twee van deze stoffen/signalen werd een additief oogdruk verlagend effect alleen gevonden voor salbutamol ($ß_2$) met fenylephrine (α_1). Bovendien werd een potentiërend effect op de afvoerweerstand in de kamerhoek aangetoond omdat salbutamol en fenylephrine alleen geen effect hadden maar in combinatie de weerstand met 32% verlaagden. Dit bevestigt het bestaan van een specifieke interactie tussen $ß_2$ en α_1-adrenerge stimulatie die de oogdruk en de weerstand in de kamerhoek verlaagt (zie Figuur 11.1).
Hoofdstuk 9 laat zien dat een exogeen cel-permeabel cyclisch AMP derivaat, 8-bromo-cAMP, de oogdruk verlaagt. Dit geldt voor toediening als subconjunctivaal depot en niet in de vorm van lokaal druppelen. De reden hiervoor is dat met een subconjunctivaal depot tienvoudig hogere 8-bromo-cAMP concentraties in het kamerwater bereikt worden, en blijkbaar nodig zijn. Een sterke tijd-effect relatie was niet aanwezig omdat de piek concentratie op 2 uur gevolgd wordt door een oogdrukverlaging tussen 2 en 5 uur. Zo was ook in hoofdstuk 3 de piek concentratie van endogeen cAMP in het kamerwater (na IBMX met adrenaline) maximaal op één uur terwijl het oogdruk verlagend effect tussen 3 en 6 uur optrad. Het ontbreken van een sterke tijd-effect relatie is eerder door Boas (1981) voor adrenaline beschreven. Desondanks is een correlatie tussen kamerwater cAMP concentratie en oogdruk effect duidelijk aanwezig. Een verklarend mechanisme kan bijvoorbeeld zijn dat initiële AC stimulatie gevolgd wordt door een refractaire periode (receptor ontkoppeling), en dat intracellulaire processen leiden tot een latere oogdrukdaling.

Het effect op de uitstroom capaciteit vormde het belangrijkste fysiologisch mechanisme voor het oogdruk dalend effect van IBMX en de beschreven interactie tussen $ß_2$ en α_1-stimulatie. **Hoofdstuk 10** probeert hiervoor een biochemisch substraat te vinden. In vitro werd het AC enzym systeem in membraan fracties van humaan en runder trabekulair kamerhoekweefsel bestudeerd. Het runder AC enzym werd gestimuleerd rechtstreeks aangrijpend op de katalytische plaats (met forskolin), via G-

eiwitten (met fluoride ionen) en via prostaglandine E receptoren. De geringe of afwezige stimulatie via $ß_2$-receptoren gaf aan dat mogelijk weinig of geen $ß_2$-receptoren aanwezig zijn. AC stimulatie in humaan trabekel weefsel was sterker; niet alleen met PGE maar ook met $ß_2$, en vasoactive intestinal peptide(VIP)-stimulatie. Deze resultaten laten zien dat $ß_2$-adrenerge, maar ook prostaglandine E, en VIP-receptoren gekoppeld zijn aan AC in trabekel weefsel. Zij kunnen van fysiologische betekenis zijn voor het reguleren van de weerstand in de kamerhoek via cAMP stimulatie. Een direct verband tussen α_1-stimulatie en AC stimulatie werd niet aangetoond; echter, indirect zou α_1-stimulatie via phospholipase A_2 activatie en PGE_2 productie AC kunnen stimuleren (zie Figuur 11.1).

Epiloog

De werkingsmechanismen via welke medicamenteuze glaucoombehandeling met adrenerge stoffen de oogdruk verlaagt, zijn voor een groot deel onbekend. In dit proefschrift wordt de vraag gesteld in hoeverre het adenylaat cyclase/cAMP systeem een rol speelt als tussenschakel in het verlagen van de oogdruk met adrenerge stoffen. Hiertoe wordt gebruik gemaakt van de stof IBMX die AC/cAMP-signalen versterkt door middel van het remmen van de afbraak van cAMP. De uitgevoerde dierexperimentele studies bevestigen dat AC/cAMP een belangrijke rol speelt in de overdracht van adrenerge effecten op vooral de uitstroom capaciteit in de kamerhoek. Het combineren van IBMX met adrenerge stoffen is een bruikbare methode om AC/cAMP signalen van adrenaline en andere ß-adrenerge stoffen te versterken. Biochemisch blijkt dit uit sterk gestegen kamerwaterconcentraties van cAMP en physiologisch uit potentiëring van oogdrukverlagende effecten van vooral $ß_2$-adrenerge stoffen. Onderscheid moet echter worden gemaakt tussen effecten op het trabekel systeem (verantwoordelijk voor de weerstand in het afvoersysteem van kamerwater) en de ciliaire processen (verantwoordelijk voor productie van kamerwater). IBMX heeft een duidelijk potentiërend effect op de afvoerweerstand verlagende eigenschappen van $ß_2$ (en zelfs α_1)-adrenerge stimulatie (zie Figuur 11.1).
Beduidend moeilijker is de interpretatie van effecten op kamerwater productie. Enerzijds geeft adrenaline afzonderlijk een bescheiden remming van kamerwater productie; in combinatie met IBMX echter wordt deze afname opgeheven en is het netto effect nul. Dit kan veroorzaakt worden doordat de kamerwaterproductie de resultante is van een balans tussen actieve en passieve secretie. Dit zijn achtereenvolgens actieve secretie van kamerwater uit ciliair epitheelcellen en ultrafiltratie dat waarschijnlijk afhankelijk is van bloeddoorstroming en colloid

osmotische gradienten in de ciliaire processen. Een toegenomen perfusie zou kunnen leiden tot toename van de ultrafiltratie. Twee interpretaties zijn mogelijk op grond van de experimenten waarin de doorbloeding van de ciliaire processen en de kamerwaterproductie gemeten zijn (zie Figuur 11.1):

1. $ß_2$/AC/cAMP stimulatie remt actieve secretie (adrenaline), maar de 2-3 voudig toegenomen perfusie van de ciliaire processen bij zeer sterke $ß_2$-stimulatie (IBMX + adrenaline) vergroot de ultrafiltratie. Of:
2. $ß_2$/AC/cAMP stimulatie versterkt actieve secretie van het ciliaire epitheel (IBMX + adrenaline), maar adrenaline remt de kamerwater productie door vasoconstrictie in ciliaire processen en vermindering van de ultrafiltratie.

De rol van Ac/cAMP in de overdracht van adrenerge effecten op de kamerwaterproductie kan op grond hiervan niet eenduidig beantwoord worden. Wel kan geconcludeerd worden dat een sterk $ß_2$/AC/cAMP-signaal in ieder geval niet leidt tot remming van de kamerwater productie.

De studies in dit proefschrift geven meer inzicht in de werking van adrenaline als oogdruk verlagend medicament (zie Figure 11.1). Specifiek de $ß_2$- en α_1-adrenerge eigenschappen lijken verantwoordelijk te zijn voor het verlagen van de oogdruk door vergroting van van de uitstroomcapaciteit in de kamerhoek. Combinatie experimenten met selectieve adrenerge agonisten geven aan dat dit niet alleen berust op additie, maar juist op interactie tussen beide receptor signalen. Een hypothetisch biochemisch mechanisme hiervoor kan zijn dat adrenaline AC direct activeert via $ß_2$ receptoren die direct aan AC koppelen en indirect via α_1-stimulatie gevolgd door prostaglandine E synthese. PGE_2 stimuleert dan AC direct via PGE_2-receptoren, zoals werd aangetoond in membraan fracties van humaan kamerhoek weefsel. Er zijn geen aanwijzingen gevonden dat de α_2-adrenoreceptor stimulerende eigenschap van adrenaline een rol van betekenis speelt in het verlagen van de oogdruk.

Bij medicamenteuze behandeling van glaucoom kan het zinvol zijn, gelet op de beschreven dierexperimentele resultaten, om adrenaline te combineren met een fosfodiesterase remmer zoals IBMX. Te verwachten voordelen van deze combinatie zijn:

1. IBMX versterkt de oogdruk verlagende eigenschappen van adrenaline en
2. de klinisch gebruikelijke adrenaline dosering van 1% kan verlaagd worden. Bijwerkingen als reactieve hyperemie kunnen daardoor wellicht afnemen.

Gebleken is dat toevoegen van IBMX het vasoconstrictieve effect van adrenaline opheft en verandert in een toeneming van de doorbloeding in intraoculaire weefsels met het vermogen tot autoregulatie. Deze toeneming van de intraoculaire doorbloeding kan een voordeel zijn bij glaucoom patiënten met normale oogdruk en een kritische papillaire doorbloeding.

CURRICULUM VITAE

Geboren in 1959 te Nijmegen werd in 1978 het eindexamen V.W.O. (Canisius College, Nijmegen) behaald, in 1984 het doctoraal geneeskunde en in 1986 het artsexamen (R.U. Groningen). Als artsassistent algemene chirurgie, niet in opleiding, gedurende 1 jaar werkzaam in het R.K. Ziekenhuis Groningen (Dr L Vos, chirurg). Van april 1988 tot april 1992 werkzaam als wetenschappelijk medewerker aan dit proefschrift bij Dr PhFJ Hoyng, oogarts, op de afdeling Experimentele Oogheelkunde van het Interuniversitair Oogheelkundig Instituut (directeur Dr AJ Otto, oogarts), waarvan 5 maanden (1988) op het Pharmacia Laboratorium voor glaucoom onderzoek (J Stjernschantz MD PhD, Uppsala, Zweden) en 5 maanden (1991) op de afdeling Oogheelkunde en Farmacologie van het Mount Sinaï Medical Center (hoofd Prof ThW Mittag PhD, New York, V.S.). Van januari 1990 tot maart 1992 tevens werkzaam (deeltijd) als arts-assistent EHBO/oogheelkunde in het Leyenburg en Oogziekenhuis 's Gravenhage (Dr La Lau, oogarts). Met ingang van april 1992 in opleiding tot oogarts in Rotterdam (Prof PTVM de Jong, oogarts). Toekenning van de eerste prijs (met Dr PhFJ Hoyng) voor glaucoomonderzoek, gepresenteerd op poster, door de European Glaucoma Society, Amsterdam mei 1992.