PÄIVI ALAJUUMA

Calcitonin Gene-Related Peptide

Characterization of Binding Sites and Structure-Activity Relationships in the Eye and Effects on Intraocular Pressure

■

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere, on May 14th, 2004, at 12 o'clock.

Acta Universitatis Tamperensis 1006

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital, Department of Ophthalmology
Finland

Supervised by
Docent Hannu Uusitalo
University of Tampere

Reviewed by
Professor Johan Stjernschantz
University of Uppsala
Professor Arto Urtti
University of Kuopio

Distribution

University of Tampere
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 1006
ISBN 951-44-5962-8
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 342
ISBN 951-44-5963-6
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2004

To Petteri and Tuomas

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numerals I-V.

I Heino P, Oksala O, Luhtala J and Uusitalo H. Localization of calcitonin gene-related peptide binding sites in the eye of different species. Curr Eye Res 1995;14:783-790.

II Alajuuma P, Oksala O and Uusitalo H. Amylin competes for binding sites of CGRP in the chamber angle and uvea of monkey, cat and pig eye. J Ocul Pharmacol Ther 2003;19(6):555-568.

III Heino P, Oksala O, Palkama A, Valo T, Vihavainen S, Koskinen A and Uusitalo H. Binding of CGRP analogs and their effect on adenylate cyclase activity in porcine iris-ciliary body. J Ocul Pharmacol Ther 1998;14(6):543-554.

IV Oksala O, Heino P, Uusitalo H and Palkama A. Effect of intracameral and intravitreal injection of calcitonin gene-related peptide on the intraocular pressure and outflow facility of aqueous humor in the rabbit. Exp Eye Res 1998;67:411-415.

V Alajuuma P, Oksala O, Alaranta S, Palkama A and Uusitalo H. Effect of simultaneous CGRP and $PGF_{2\alpha}$ on the outflow facility in the rabbit eye. Ophthalmic Res 2002;34:309-313.

ABBREVIATIONS

AC	Adenylate cyclase
AR42J	Rat pancreatic acinar cell line
ATP	Adenosine 5'-triphosphate
BAB	Blood-aqueous barrier
BSA	Bovine serum albumin
C	Outflow facility
cAMP	Cyclic adenosine 3',5'-monophosphate
cGMP	Cyclic guanosine 3'5'-monophosphate
CGRPα and β	Calcitonin gene-related peptide α and β
CHO-P	Chinese hamster ovary cell line
COS-7	African green monkey kidney cell line
CRLR	Calcitonin receptor-like receptor
CT	Calcitonin
ΔP	Change in intraocular pressure
EC_{50}	Effective concentration 50%, the concentration at which 50% of maximum response is achieved
EDTA	Ethylenediamine tetraacetic acid
F	Infusion rate
GTP	Guanosine 5'-triphosphate
HEK-293	Human embryonic kidney cell line
HPLC	High-pressure liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Inhibitory concentration 50%, the concentration at which 50% of binding is inhibited
IOP	Intraocular pressure
KS-4	Preosteoblast cell line
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleinic acid
NO	Nitric oxide
OHS-4	Human osteosarcoma cell line
P_{CO2}	Partial pressure of carbon dioxide
PEI	Polyethylenimine
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}$-IE	Prostaglandin $F_{2\alpha}$ -isopropyl ester
PKA	Proteinkinase A
P_{O2}	Partial pressure of oxygen
h/rCGRP	Human/rat calcitonin gene-related peptide
RAMP1-3	Receptor activity modifying proteins 1-3
RCP	Receptor component protein
RP-HPLC	Reverse-phase high-pressure liquid chromatography
RT	Room temperature
SEM	Standard error of mean
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid

ABSTRACT

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide existing in α and β forms. It is widely distributed in the central and peripheral nervous systems. CGRP has several functions in the tissues but is probably best known as a potent vasodilator. In the eye, CGRP-immunoreactive nerves have been found in several tissues, including cornea, ciliary body, iris blood vessels, trabecular meshwork and choroid. In the rabbit eye it has been reported to induce vasodilatation and further, to disrupt the blood-aqueous barrier (BAB), leading to an increase in intraocular pressure (IOP). In contrast, in the cat eye CGRP has been reported to reduce IOP probably by increasing the conventional outflow of aqueous humour.

In the present study, CGRP binding sites in the eye were localized and further characterized using different forms of CGRP fragments, analogs and amylins. The structure-activity relationship of CGRP has been investigated by receptor binding and measurement of adenylate cyclase enzyme activity. To evaluate the effect of CGRP on the aqueous humour dynamics in the rabbit eye, manometrical measurement of IOP and the two-level constant pressure infusion method were used in the presence and absence of $PGF_{2\alpha}$.

CGRP binding sites were found in the ciliary muscle, ciliary processes and limbal conjunctiva of the monkey, pig, cat and guinea pig, in the iris of the pig, cat and guinea pig, in the chamber angle in the cat, in the choroid of the monkey, pig and guinea pig and in the retina of the pig. Specific CGRP binding in the anterior part of the monkey, feline and porcine eye was displaced by cat, human and rat amylins. This effect was greatest in feline and porcine eyes, where ^{125}I-CGRP was displaced from different anterior tissues by all three amylins. The carboxyterminal end of the CGRP peptide was found to be responsible for the binding, while the aminoterminal end of the peptide was responsible for adenylate cyclase (AC) enzyme activation. The temperature-dependent conformation of the peptide structure seems to play a role in the affinity of the CGRP to its receptors. In addition to the known effects of CGRP in the eye, e.g. vasodilatation and breakdown of the BAB, CGRP induced a prolonged IOP decrease after intravitreal administration in the rabbit and increased the outflow facility after both intracameral and intravitreal administration. $PGF_{2\alpha}$ had no clear additive effect on the increased outflow facility induced by CGRP.

It is concluded that CGRP has a marked effect on the aqueous humour dynamics in the eye by affecting at least the aqueous humour outflow. The specific binding sites found in other ocular tissues indicate that CGRP may also have other functions in the eye. Although the CGRP receptor has a

complex structure and function, structure-activity studies with the peptide provide information on the essential parts of the CGRP molecule important for further development of active compounds mimicking or modulating the effects of this potent peptide.

1 INTRODUCTION

Glaucoma is a progressive optic neuropathy involving characteristic structural and functional changes in the optic nerve. Increased intraocular pressure (IOP) is the most prominent risk factor in glaucoma; 67 million people in the world suffer from this disorder and it is one of the leading causes of blindness in the world. The mechanisms leading to glaucomatous damage have not yet been clarified, although both mechanical and vascular theories have been proposed. The current treatment in glaucoma is addressed to lowering IOP, which has been shown to reduce the progression of the disease (2000, Anderson 2003, Lee and Wilson 2003, Leske et al. 2003). In the treatment of glaucoma, a decrease in IOP is achieved either by drugs, laser treatment or surgery or a combination of these. At present, the drugs most commonly used in glaucoma treatment are prostaglandin analogs and β-blockers. Prostaglandins are endogenous lipids and $PGF_{2\alpha}$ analogs lower the intraocular pressure particularly effectively by increasing the aqueous humour uveoscleral outflow (Crawford and Kaufman 1987, Nilsson et al. 1989, Alm and Villumsen 1991, Toris et al. 1993, Stjernschantz and Alm 1996). The first prostaglandin analog (latanoprost) came into clinical use in the mid-nineties while β-blockers have been used since the late 1970s.

Calcitonin gene-related peptide (CGRP) is a peptide of 37 amino acids. It belongs to the calcitonin peptide family together with calcitonin, amylin and adrenomedullin. It is localized in motor and sensory neurons (Rosenfeld et al. 1983, Gibson et al. 1984), which are widely distributed in the peripheral and central nervous systems (Rosenfeld et al. 1983). It exerts various biological effects, including vasodilatation (Brain et al. 1985). In the eye CGRP immunoreactivity has been detected in the sensory neurons in the anterior uvea of several species (Terenghi et al. 1985, Uusitalo et al. 1989). CGRP has been shown to act as a mediator in ocular neurogenic inflammation (Unger et al. 1985). In the late 1980s it was found to lower IOP by increasing the outflow facility in the cat eye (Oksala and Stjernschantz 1988a, Oksala and Stjernschantz 1988b). An increase in outflow facility after CGRP administration has since also been demonstrated in the monkey (Almegård and Andersson 1990).

Based on pharmacological studies CGRP has at least two different receptor subtypes (CGRP1 and CGRP2) and their regional distribution may also vary within the tissue (Foulkes et al. 1991). Although pharmacological evidence of the existence of these two CGRP receptor subtypes was already reported more than ten years ago, it was not until 1996 that the CGRP receptor was cloned (Aiyar et al. 1996). The receptor itself appears to possess a complicated structure. The cloned CGRP receptor evidently represents a CGRP1 subtype consisting of a calcitonin receptor-like receptor (CRLR), a receptor activity-modifying protein 1 (RAMP1) and a receptor component protein (RCP).

The CGRP2 receptor has not yet been cloned (Juaneda et al. 2000). There is also cross-reactivity among the calcitonin family of peptides at their receptors and it has been shown, for example, that CGRP and amylin can share receptors (Galeazza et al. 1991, Giuliani et al. 1992).

The challenge entailed in the use of a peptide as a drug is usually the poor penetration of such a high-molecular-weight compound into the eye. In experimental studies this problem has been overcome by injecting CGRP intracamerally into the eye. In the clinic, however, in most cases this is impossible. A more practical solution would be a CGRP analog which has the IOP-lowering effect of CGRP with better pharmacokinetic properties. To further explore whether this is a viable option, more information on the structure-activity relationship of the CGRP molecule as well as the CGRP receptors should be available. The present study was undertaken to further localize and characterize the CGRP receptors in the eye, to study the structure-activity relationship of the CGRP molecule and the possibility of modifying the peptide structure without affecting its activity, and to further investigate the effects of CGRP on IOP and outflow facility in the rabbit eye.

2 REVIEW OF THE LITERATURE

2.1 The Eye

The eye can be divided structurally and functionally into two parts. The anterior part of the eye consists of the eyelids, cornea, iris, ciliary body and lens, while the posterior part consists of retina, optic nerve and the supporting structures sclera and choroid. The retina and optic nerve, which form an essential part of the posterior segment, constitute part of the central nervous system. Functionally, the anterior part is responsible for the optical properties of the eye, while the posterior part is the receptive structure dealing with visual information. The anatomy of the human eye is shown in Figure 1.

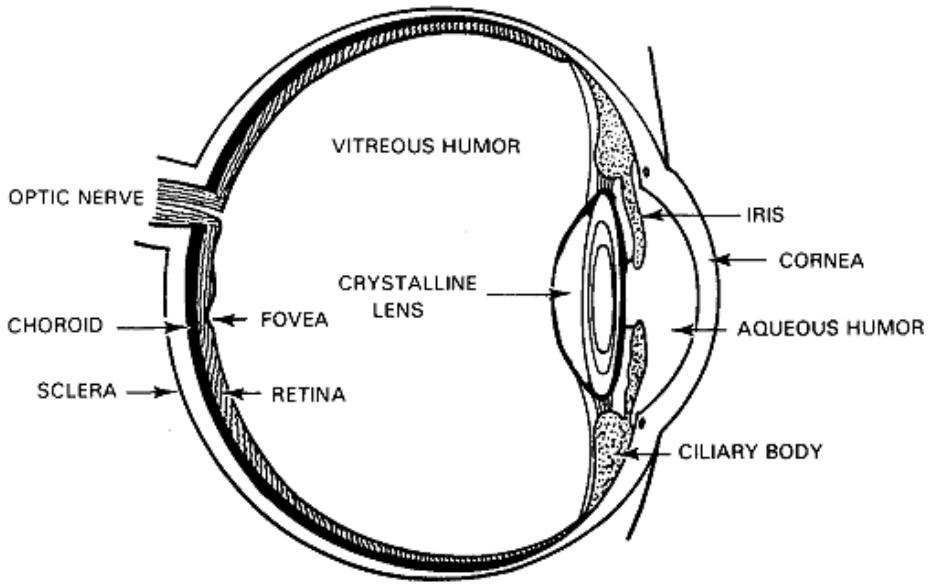

Figure 1. Anatomy of the human eye.

2.2 Blood Circulation in the Eye

The eye is nourished by two vascular systems: the retinal and the uveal (ciliary) vessels (Cioffi et al. 2003). Both systems originate from the ophthalmic artery, which in humans divides into the central retinal artery, the posterior ciliary arteries, and several anterior ciliary arteries (Hayreh 1962). The retinal circulation is to a marked extent autoregulated, which means that the flow is independent of the perfusion pressure in a certain range (Flammer et al. 2002). The retinal vascular endothelial cells as well as the retinal pigmented epithelial cells are connected to each other by tight junctions forming a blood-retinal barrier. This barrier prevents large proteins like albumin from entering into the eye (Cioffi et al. 2003). Another barrier, the blood-aqueous barrier, is located in the anterior part of the

eye. It is formed by the nonpigmented epithelial cells in the ciliary processes connected to each other by tight junctions and by the endothelial cells in the iris blood vessels with similar junctions.

The uveal blood vessels establish the vascular beds of the iris, the ciliary body and the choroid (Cioffi et al. 2003). These vessels are responsible for most of the blood supply to the eye. The choroidal circulation is poorly autoregulated and compared to the retinal flow the blood flow is more dependent on the perfusion pressure (Flammer et al. 2002). Most of the orbital venous circulation enters the superior ophthalmic vein. From the retinal veins the blood is drained into the central retinal vein, and from most of the choroid the blood is drained into the vortex venous system (Cioffi et al. 2003).

2.2.1 Regulation of Blood Circulation

The vasculature of the uvea is abundantly supplied by vasoactive autonomic nerves. The sympathetic nerves are derived from the superior cervical sympathetic ganglion and follow the path of the blood vessels to innervate all parts of the uvea (Flammer et al. 2002, Cioffi et al. 2003), while the parasympathetic nerves enter the eye through the oculomotor and facial nerves and through the ophthalmic and maxillary division of the trigeminal nerve (Flammer et al. 2002). The oculomotor nerve has been shown to innervate mainly the blood vessels in the iris and ciliary body, whereas the innervation in the choroid is less well characterised. However, the choroidal vessels are known to be innervated mainly by the facial nerve. Moreover, the sensory nerves derived from the trigeminal ganglion are also linked to the regulation of the uveal blood flow (Cioffi et al. 2003).

Sympathetic stimulation reduces the blood flow in the uvea in several species, while the retinal blood flow remains unaffected (Bill 1962, Alm and Bill 1973, Alm 1977). This neurogenic vasoconstriction is mediated via adrenergic $\alpha_1$receptors at least in the anterior uvea of rats and cats (Koss and Gherezghiher 1993, Kawarai and Koss 1998). One of the main physiologic roles of the sympathetic nerves in the regulation of ocular blood flow is to maintain the flow at a suitable level during elevations in blood pressure (Bill and Geijer 1977).

The role of the parasympathetic nerves in the eye remains somewhat obscure. Oculomotor nerve stimulation can both increase and reduce the blood flow in the anterior uvea depending on the tissues and species in question (Cioffi et al. 2003), while stimulation of the facial nerve causes mainly vasodilatation in the anterior uvea and choroid in rabbits, cats and monkeys (Stjernschantz and Bill 1980, Nilsson et al. 1985). The vasoconstriction induced in the iris by oculomotor nerve stimulation evidently comprises both cholinergic and aminergic components. The cholinergic transmitter is

muscarinic while the nature of the aminergic component remains unknown (Bill et al. 1976, Stjernschantz and Bill 1979).

Mechanical or electrical stimulation of the ocular sensory nerves in rabbits causes an increase in vascular permeability, miosis and a rise in intraocular pressure (Eakins 1977). Further, these effects are not mediated by prostaglandins but apparently by substance P and CGRP (Bill et al. 1979, Oksala and Stjernschantz 1988a). In the monkey eye, CGRP has been shown to increase the ocular blood flow in conjunctiva, ciliary body and sclera, while cholecystokinin has no effect on the blood flow but causes miosis in the eye (Almegård and Andersson 1993).

2.3 Aqueous Humour Dynamics and Intraocular Pressure

The role of the aqueous humour is to nourish the avascular cornea and lens, as well as the trabecular meshwork. As a transparent and colourless medium, it also functions as a suitable optical medium in the anterior eye. It differs from plasma in that it has a low protein and a high ascorbate concentration (Kinsey and Reddy 1964, Stjernschantz et al. 1973, Lam and Lee 1975, Johnsen et al. 1985). A low protein concentration is maintained by the blood–aqueous barrier preventing large molecules in the blood from entering the aqueous humour (Bill 1968, Uusitalo et al. 1973, Eakins 1977). Aqueous humour is actively secreted by the nonpigmented epithelial cells of the ciliary processes to the posterior chamber of the eye. It then flows through the pupil to the anterior chamber, and leaves the eye via two possible routes: the trabecular meshwork pathway (conventional outflow) or the uveoscleral pathway (unconventional outflow) (Gabelt and Kaufman 2003). The trabecular meshwork pathway is pressure-dependent and accounts for the main part of the aqueous humour outflow, while the uveoscleral pathway is pressure-insensitive and is responsible for 10 to 30% of the total outflow depending on the species in question. The uveoscleral outflow appears to be greater in younger compared to older individuals (Bill and Phillips 1971, Toris et al. 1999).

In a healthy eye, the intraocular pressure is approximately 10-21 mmHg. The level of pressure is determined mainly by the aqueous humour outflow. The juxtacanalicular tissue in the anterior chamber angle constitutes the main resistance to the flow of aqueous humour into the canal of Schlemm, which is connected to the scleral and episcleral veins (Gabelt and Kaufman 2003). Since in steady state the net outflow of aqueous humour has to equal its net inflow, and the latter, largely, is pressure-insensitive, it follows that IOP is determined by the resistance in the outflow pathways and the episcleral venous pressure. In most cases of ocular hypertension the resistance in the anterior chamber angle is increased, leading to a higher IOP.

Acute breakdown of the blood-aqueous barrier (BAB), which leads to an increase in aqueous humour inflow, may also raise the IOP. The BAB breakdown-induced elevation of IOP is based on a net increase in ultrafiltration of blood plasma. When tonography or constant-pressure infusion methods are used to measure the outflow facility (Gabelt and Kaufman 2003) the ultrafiltration is reduced by the increase in IOP brought about by the measurement. This phenomenon, known as pseudofacility, exaggerates the true outflow facility.

2.4 Glaucoma

Glaucoma is a chronic, progressive optic neuropathy involving characteristic structural and functional changes. The most important risk factor for glaucoma is a high IOP. Glaucoma can be divided into different types, for example, primary open-angle glaucoma, normal tension glaucoma, angle-closure glaucoma, congenital glaucoma and secondary glaucoma. The most common types of the disorder in Finland are primary open-angle glaucoma (glaucoma simplex) and exfoliation glaucoma (glaucoma capsulare), which is also the most common type of secondary glaucoma (Forsius 1988). During the progress of the disease, the axons of the retinal ganglion cells are damaged at the level of the optic disk, and the ganglion cells die, probably due to apoptosis (Garcia-Valenzuela et al. 1995, Quigley et al. 1995, Kerrigan et al. 1997, Okisaka et al. 1997). Ultimately, this leads to the destruction of the optic nerve head and the loss of sight. Two theories have been presented regarding the reason for this destruction. The mechanical theory assumes that it is a direct effect of an elevated IOP on the neuronal structures, while the vascular theory assumes that the neuronal damage in glaucoma is a consequence of a poor blood supply due e.g. to a high IOP (Fechtner and Weinreb 1994).

Glaucoma is treated by reducing the IOP with drugs, by laser treatment or by surgery. Drugs lower it either by increasing the aqueous humour outflow, or reducing aqueous humour formation. At present the most commonly used drugs are prostaglandin analogs and β-blockers such as timolol. Prostaglandins increase the uveoscleral outflow probably by remodelling the extracellular matrix of the ciliary muscle (Lindsey et al. 1996, Weinreb et al. 1997, Ocklind 1998), while timolol, a nonselective β-adrenergic antagonist, lowers the pressure by inhibiting aqueous humour formation (Zimmerman et al. 1977, Yablonski et al. 1978).

2.5 Calcitonin Peptide Family

Calcitonin, CGRP, amylin and adrenomedullin comprise the peptide family of calcitonin. Although these peptides together have only partial similarities in their primary structures (Table 1), they share very similar secondary structures, including an intramolecular disulfide bond, an α-helix and an amidated C terminal. All are widely distributed in various tissues, including the peripheral and central nervous systems, and they also share some common biological activities. They can act through each other's receptors, although they also have their own distinct receptors (Poyner et al. 2002).

Table 1. Amino Acid Sequence of human CGRPα, CGRPβ, amylin, calcitonin and adrenomedullin

PEPTIDE	NH_2 AMINO ACIDS $CONH_2$
	1 10 20 30 37
Human CGRPα	**A C D T A T C V T H R L A G L L S R S G G V V K N N F V P T N V G S K A F**
Human CGRPβ	**A C** N **T A T C V T H R L A G L L S R S G G** M **V K S N F V P T N V G S K A F**
Human Amylin	K **C** N **T A T C** A **T** Q **R L A** N F L V H **S** S N N F G A I L S S **T N V G S** N T Y
Human Calcitonin	C G N L S **T C** M L G T Y T Q D F N K F H T F P Q T A I G V G A P
	1 15 24 34 44 52
Human Adrenomedullin	Y R Q S M N N F Q G L R S F G **C** R F G **T C** T V Q K **L A** H Q I Y Q F T D K D **K** D **N** V A **P** R S K I **S** P Q G Y

Amino acids printed bold are identical with human CGRPα. Human adrenomedullin $_{15-52}$ shares nine amino acids with human CGRPα, and has biological activity similar to complete adrenomedullin (Eguchi et al. 1994).

2.5.1 CGRP

CGRP is a 37-amino acid peptide, existing in two different forms, CGRPα and CGRPβ (CGRP I and II). CGRPα is generated from the alternative splicing of the calcitonin gene mRNA (Amara et al. 1982, Rosenfeld et al. 1984), while CGRPβ is a gene product of its own gene (Amara et al. 1985, Steenbergh et al. 1985). The tissue-specific alternative splicing of the mRNA leads in thyroid C cells to the production of calcitonin, and in neuronal cells to the production of CGRP. The processing of mRNA is regulated by multiple important sequence elements in DNA, and poorly known protein factors. The important step in the regulatory process is the recognition and inclusion of exon 4 of the calcitonin/CGRP gene to produce calcitonin mRNA, or nonrecognition and exclusion of it to produce CGRP mRNA (Lou et al. 1994). The sequence element located in the intron downstream to exon 4 takes part in this inclusion, and seems to be regulated by a splicing regulator protein called TIAR (Zhu et al. 2003).

CGRPα is located mostly in sensory neurons while CGRPβ is situated predominantly in enteric neurons (Mulderry et al. 1988). Human CGRPα and β differ in only three amino acids, at positions 3, 22 and 25 Asp->Asn, Val->Met and Asn->Ser, respectively (Table1). Generally, CGRP is a highly conserved molecule, its primary structure differing in only three to five amino acids in bovine, rat, porcine and chicken CGRP compared to human CGRPα (Collyear et al. 1991). It is degraded by endopeptidases. A mast cell tryptase has been demonstrated to cleave the CGRP in the human skin (Brain and Williams 1988, Walls et al. 1992) as well as in the human lung, in which the tryptase hydrolyzes CGRP at positions 18 (Arg) and 24 (Lys) (Tam and Caugney 1990). Both CGRP and substance P have been reported to be hydrolyzed by the endopeptidase isolated from human cerebrospinal fluid. This endopeptidase cleaves CGRP at positions 16(Leu) and 17(Ser) (Greves et al 1989). More recently, matrix metalloproteinases (MMPs) have also been shown to have a role in degrading vasoactive peptides, in addition to their well known role in the breakdown of extracellular matrix proteins. MMP-2 cleaves the CGRP specifically at positions 14(Gly) and 15(Leu) in rat mesenteric arteries, reducing its vasodilatory potency 20-fold (Fernandez-Patron et al. 2000).

2.5.1.1 Localization and Function of CGRP

CGRP has been shown to have several functions. It is a potent vasodilator (Brain et al. 1985, Brain et al. 1986), it regulates calcium metabolism (Grunditz et al. 1986), and inhibits gastric acid secretion (Hughes et al. 1984). CGRP binding sites are found e.g. in the central nervous system (Skofitsch and Jacobowitz 1985, Inagaki et al. 1986, Chatterjee and Fisher 1991), in the cardiovascular (Wimalawansa and MacIntyre 1988) and pulmonary systems (Carstairs 1987). CGRP is co-localized, for example, with substance P in rat sensory neurons in the trigeminal ganglia (Terenghi et al. 1985) as well as in primary sensory neurons of cat and rat dorsal root ganglia (Gibson et al. 1984).

In the eye, CGRP immunoreactivity is found in the sensory nerve fibres in the human ciliary body, iris blood vessels, cornea (Stone and McGlinn 1988, Uusitalo et al. 1989), choroid and trabecular meshwork (Stone and McGlinn 1988). Furthermore, CGRP immunoreactivity has been found in neuronal cells in the trigeminal ganglia of the rat (Terenghi et al. 1985) and guinea pig (Uusitalo et al. 1989), as well as in sensory nerve fibres in the dilator and sphincter muscles of the iris, in the ciliary body and uveal blood vessels of the rat, in the iris of cat and monkey (Terenghi et al. 1985), in the ciliary body and iris of the guinea pig (Terenghi et al. 1985, Uusitalo et al. 1989) and in the cornea of guinea pig (Terenghi et al. 1985, Uusitalo et al. 1989), and pigeon (Harti et al. 1989).

Exogenous CGRP has been reported to cause vasodilatation, breakdown of the BAB, a rise in IOP (Unger et al. 1985, Wahlestedt et al. 1986, Krootila et al. 1988, Oksala 1988) and an increase in cAMP content in the aqueous humour (Krootila 1988) in the rabbit eye. In the cat eye, CGRP has been found to cause vasodilation and reduction of IOP by facilitating aqueous outflow (Oksala and Stjernschantz 1988a, Oksala and Stjernschantz 1988b). An increase in outflow facility (Almegård and Andersson 1990) and an increase in blood flow in the conjunctiva, ciliary body and sclera have also been determined with CGRP in the monkey eye (Almegård and Andersson 1993).

2.5.1.2 CGRP Receptors

The results of pharmacological studies indicate that the effects of CGRP are mediated via two different receptors, named CGRP1 and CGRP2. These receptors have been distinguished from each other by sensitivity to the antagonistic properties of CGRP 8-37. The CGRP1 receptor has been shown to be more sensitive to CGRP 8-37 than the CGRP2 receptor. CGRP1 receptors are present e.g. in the guinea pig atrium, while CGRP2 receptors can be found, for instance, in the rat vas deferens (Dennis et al. 1989, Dennis et al. 1990). The regional distribution of these receptor subtypes can also differ within one tissue (Foulkes et al. 1991) and they may evince multiple affinity forms which are regulated by guanine nucleotides (Chatterjee and Fisher 1991, van Rossum et al. 1993, Chatterjee and Fisher 1995). The binding affinity of CGRP to its receptors in different tissues is of picomolar to nanomolar magnitude (Skofitsch and Jacobowitz 1985, Inagaki et al. 1986, Yoshizaki et al. 1987, Sexton et al. 1988, Dennis et al. 1990). For example, in rat cerebellum membranes, rat brain, guinea pig atria and guinea pig vas deferens van Rossum and coworkers have demonstrated CGRP to have both high- and low-affinity binding sites with binding affinity constants of 10-100 pM and 1-10 nM, respectively, and total binding capacities of about 1-100 fmol/mg protein (van Rossum et al. 1993). In the porcine iris-ciliary body membranes CGRP has been shown to have an affinity constant of about 1 nM and a binding capacity of 42 pmol/g membrane wet weight (Malminiemi and Malminiemi, 1992).

The structure of the CGRP receptor would appear to be complex. The active CGRP receptor consists of a calcitonin receptor-like receptor (CRLR), a receptor activity-modifying protein 1 (RAMP1), and a receptor component protein (RCP) (Table 2)(Aiyar et al. 1996, Luebke et al. 1996, McLatchie et al. 1998). CRLR is a 461-amino-acid-long protein having seven hydrophopic regions indicating a seven-transmembrane domain (Aiyar et al. 1996) generally found in G protein-coupled receptors. RAMP1 is a 177-amino-acid-long protein having a single transmembrane domain close to the carboxyterminal end. If CRLR is expressed with RAMP2 or RAMP3, the receptor behaves as an adrenomedullin

receptor (Poyner et al. 2002). The length and the single transmembrane domain structure of RAMP2 and RAMP3 are identical with RAMP1 (McLatchie et al. 1998). It would appear that the role of RAMPs is to transport CRLR to the plasma membrane and that their expression can regulate the ligand specificity of cell receptors to different calcitonin family peptides (McLatchie et al. 1998). RCP is the third protein most probably needed for the proper function of the CGRP receptor. Luebke and coworkers found this 146-amino-acid-long protein in 1996 in the guinea pig organ of Corti (Luebke at al. 1996). It is an intracellular protein (Evans et al. 2000) containing no hydrophobic regions and no consensus sequences for lipid attachments, suggesting its association through ionic interactions. Its role seems to be to couple the receptor to the cellular signalling pathway (Prado et al. 2002). It has been demonstrated also in ocular tissues such as in the iris, ciliary processes, lens, retina and choroid of the rabbit eye, and it has been shown to be essential for signal transduction at ocular CGRP receptors (Rosenblatt et al. 2000).

Table 2. Components needed to form functional CGRP and Amylin receptors

	$CGRP_1$	$CGRP_2$	AMYLIN
RECEPTOR COMPONENTS	CRLR+RAMP1+RCP	UNKNOWN	CTr +RAMP1 OR CTr +RAMP3 (CTr +RAMP2)

CRLR; calcitonin receptor-like protein, RAMP; receptor activity-modifying protein, RCP; receptor component protein, CTr; calcitonin receptor. Modified from Juaneda and co-workers (Juaneda et al. 2000) .

2.5.1.3 Signalling Pathways of CGRP

CGRP appears to have several signalling pathways in cells, a phenomenon generally found in class II G protein-coupled receptors (Segre and Goldring 1993). Ono and co-workers first reported in 1989 that CGRP increases intracellular Ca^{2+} in cultured heart cells. CGRP has also been reported to activate the adenylate cyclase (AC) enzyme in cultured endothelial cells of the human umbilical vein (Crossman et al. 1987), in rat striated muscle (Kobayashi et al. 1987), and in human embryonic kidney 293 (HEK-293) cells (Aiyar et al. 1999). In these cells it also independently increased intracellular calcium release (Aiyar et al. 1999). However, in human osteosarcoma (OHS-4) cells CGRP has been found not to increase intracellular cAMP, but the intracellular calcium content (Drissi et al. 1999). Further, it has been reported to activate ATP-sensitive K^+ channels in pig coronary artery smooth muscle (Wellman et al. 1998), in rabbit mesenteric artery smooth muscle cells (Quayle et al.

1994), and both ATP-sensitive K^+ channels and Ca^{2+}-activated K^+ channels in pig coronary artery smooth muscle cells (Miyoshi and Nakaya 1995). All of these actions are mediated by adenylate cyclase (cAMP-PKA pathway) (Quayle et al. 1994, Miyoshi and Nakaya 1995, Wellman et al. 1998).

At least two different pathways have been introduced for CGRP-related vasodilatation. First, CGRP has the ability to release nitric oxide (NO) from the vascular endothelium e.g. in the human internal mammary artery (Raddino et al. 1997) and in the rat aorta (Gray and Marshall 1992b). NO then further stimulates guanylate cyclase e.g. in the rat thoracic aorta (Gray and Marshall 1992a), resulting in elevated cGMP levels. The other possibility is that CGRP acts directly by stimulating adenylate cyclase releasing cAMP (Brain and Cambridge 1996). Recently, Champion and co-workers have reported CGRP to act directly through hAmylin-(8-37) and hCGRP-(8-37) –sensitive receptors in the rat isolated mesenteric resistance artery, instead of releasing NO from the endothelium or through ATP-sensitive K^+ channels (Champion et al. 2001).

CGRP has been reported to relax the rabbit iris dilator muscle by a release of cAMP (Yousufzai and Abdel-Latif 1998) and in addition without cAMP involvement (Haruno et al. 1996). In the intact rabbit ophthalmic artery the CGRP-induced relaxation was mediated through NO. In the endothelium-denuded rabbit ophthalmic artery CGRP caused hyperpolarization through activation of ATP-sensitive K^+ channels. However, the hyperpolarization was not considered to be the main mechanism of CGRP-induced relaxation in that study (Zschauer et al. 1992). In cultured human nonpigmented ciliary epithelial cells CGRP has been seen to stimulate the production of cAMP by a mechanism involving protein kinase C (Crook and Yabu 1992). Intravenous and intracameral administrations of CGRP increase the aqueous humour cAMP concentration in the rabbit eye, which would suggest that cAMP is at least one of the mediators of the CGRP effects in the eye (Krootila et al. 1991).

2.5.2 Amylin

Amylin (islet amyloid polypeptide, IAPP) is a 37-amino acid peptide whose structure is closely related to that of CGRP. Human amylin, for example, has approximately 40% sequence homology with human CGRPα (Table 1). It is a gene product of a single gene in chromosome 12 (Mosselman et al. 1988). It has been found predominantly in the pancreatic islet ß-cells of both normal and type II diabetic subjects (Westermark et al. 1987), as well as in islet ß-cells in a number of animal species, including cat, dog, mouse and rat (Johnson et al. 1988). Amylin is also expressed in sensory neurons in the trigeminal, jugular-nodose and dorsal root ganglia in the rat (Mulder et al. 1995) as well as in the stomach, intestinal tract and lungs (Ferrier et al. 1989). It is co-localized in the pancreatic islet ß-

cells with insulin (Lukinius et al. 1989, Kahn et al. 1990) and is assumed to have an important role in glucose homeostasis. Amylin and CGRP each induce hyperlactemia and hyperglycemia, as well as hypotension and hypocalcemia in fasted, anesthetized rats (Young et al. 1993). In rodents amylin has been shown to reduce food intake in food-deprived animals (Bouali et al. 1995) and to inhibit gastric emptying in both diabetic and control mice (Young et al. 1995, Young et al. 1996). Furthermore, both CGRP and amylin have been shown to inhibit basal and insulin-stimulated glycogen synthesis in striated muscle (Cooper et al. 1988, Leighton and Cooper 1988). Amylin has also been shown to reduce spontaneous motor activity and to increase body temperature in rats (Bouali et al. 1995).

2.5.2.1 Amylin Receptors

Amylin receptors are also reported to exist in multiple affinity forms. High-affinity binding sites for amylin with a dissociation constant of 27 pM have been identified e.g. in the rat brain nucleus accumbens region (Beaumont et al. 1993). Both high- and low-affinity binding sites for amylin with dissociation constants of 70 pM and 8 nM, and a binding capacity of about 1 and 500 fmol/ mg protein, respectively, have been demonstrated e.g. in the monkey kidney (Chai et al. 1997).

The amylin receptor consists of a calcitonin (CT) receptor and receptor activity-modifying proteins (RAMPs) (Table 2). Co-transfection of the CT receptor and RAMP1 or RAMP3 induces amylin receptors in African green monkey kidney (COS-7) cells (Christopoulos et al. 1999), and in rabbit aortic endothelial cells (Muff et al. 1999). However, co-transfection with all three RAMPs has also been reported to induce amylin receptors in Chinese hamster ovary (CHO-P) cells (Tilakaratne et al. 2000). In COS-7 cells different CT receptor and RAMP combinations give rise to different amylin receptor phenotypes with respect to CGRP; the CT receptor and RAMP1 bind both amylin and CGRP while the CT receptor and RAMP3 bind only amylin (Christopoulos et al. 1999). In addition to the RAMPs, the CT receptor isoform as well as the host cell environment modulate the physiological activity of the amylin receptor (Tilakaratne et al. 2000). Cross-reactivity has been reported among the calcitonin family of peptides at their receptors (Poyner et al. 2002) and it is thus not surprising that amylin has also been found to act through the CGRP receptor. Amylin has been reported to induce e.g. vasodilatation in the perfused rat mesenteric arterial bed by stimulating the CGRP receptor, being however ten times less potent than CGRP (Westfall and Curfman-Falvey 1995). It competes both high- and low-affinity binding sites for CGRP e.g. in the rat brain, liver and muscle in nanomolar concentrations, while the affinity constants for these receptors with CGRP are of picomolar and nanomolar magnitude, respectively (Galeazza et al. 1991). In porcine iris-ciliary body membranes rat and human amylins displace 50% of ^{125}I-CGRP binding by about 4 and 70 nM concentrations,

respectively, compared to that of 1.8 nM of CGRP (Malminiemi and Malminiemi, 1992). Furthermore, CGRP has been demonstrated to displace 50% of amylin binding in pig and guinea pig lung tissue equally with amylin itself by about 5 nM concentration. However, in pig and guinea pig nucleus accumbens membranes it displaces 50% for amylin low- and high-affinity binding sites by about 170 and 55 nM concentrations compared to 2 and 0.2 nM of amylin (Aiyar et al. 1995).

2.5.2.2 Signalling Pathways of Amylin

Amylin has been reported to act via several different second messengers depending on the tissue in question. It stimulates AC, increasing the cAMP level e.g. in skeletal muscle (Pittner et al. 1995), and in hepatocytes, although in these cells about 400-fold higher concentrations compared to CGRP are needed to obtain half-maximal activation. This stimulation is mediated via receptors common with CGRP (Houslay et al. 1994). It has also been observed to act through cGMP to transmit its anorectic effect in the area postrema neurons. This cGMP formation is independent of NO formation (Riediger et al. 2001). Furthermore, amylin increases the intracellular Ca^{2+} level in rat acinar AR42J cells. This increase is not induced by CGRP or salmon calcitonin, indicating that the effect is mediated through a distinct amylin receptor (Huang et al. 1996).

2.6 Prostaglandins

Prostaglandins are fatty acids found throughout the human body. They are synthesized mainly from arachidonic acid, as shown in Figure 2. Arachidonic acid is released from the phospholipid layer primarily by phospholipase A_2 although it may also be released by diacyl glycerol lipase subsequent to hydrolysis by phospholipase C.

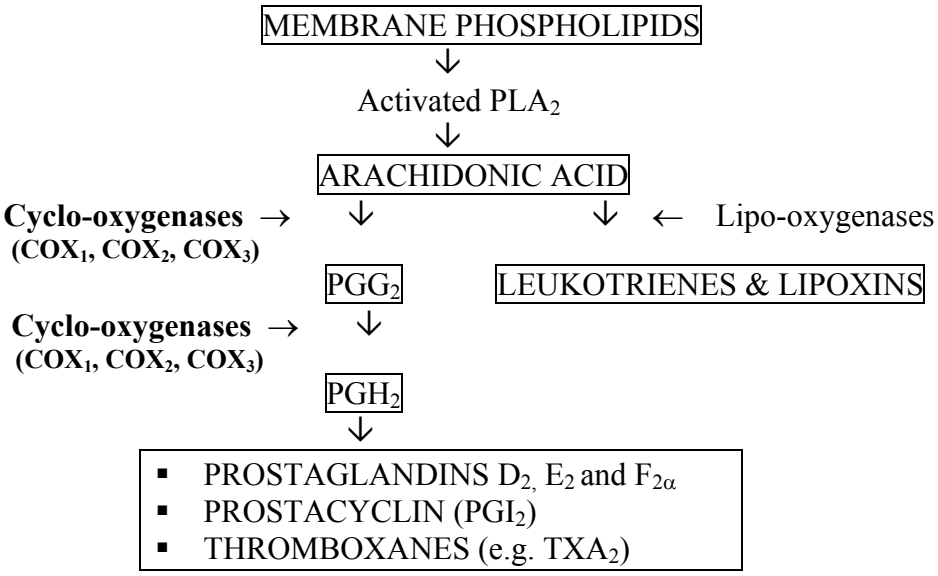

Figure 2. Biosynthesis of eicosanoids. PLA_2; phospholipase A_2, PGG_2; prostaglandin G_2, PGH_2; prostaglandin H_2, TXA_2; thromboxane A_2.

The prostanoids (prostaglandins and thromboxane A_2) act through specific seven-transmembrane G-protein-coupled receptors, which are classified as DP for PGD_2, EP for PGE_2, FP for $PGF_{2\alpha}$, IP for PGI_2 and TP for thromboxane (Kennedy et al. 1982). The distribution of the FP receptor in the eye was first demonstrated in the monkey by *in situ* hybridization and immunohistochemistry. It was found to be expressed e.g. in the corneal epithelium and endothelium, lens epithelium, ciliary muscle and epithelium, iridial melanocytes and in the retina, including the ganglion cells (Ocklind et al. 1996). FP receptors are expressed in several human ocular tissues, e.g. in the corneal epithelium (Schlotzer-Schrehardt et al. 2002), in the iris blood vessels, stromal and smooth muscle cells (Mukhopadhyay et al. 2001, Schlotzer-Schrehardt et al. 2002) and in the iridial melanocytes (Wentzel et al. 2003), in the choroid (Schlotzer-Schrehardt et al. 2002), in nonpigmented (Mukhopadhyay et al. 1997, Schlotzer-Schrehardt et al. 2002) and pigmented (Schlotzer-Schrehardt et al. 2002) ciliary epithelium, in the ciliary muscle (Chen et al. 1997a, Mukhopadhyay et al. 1997, Schlotzer-Schrehardt et al. 2002) and in the trabecular meshwork (Anthony et al. 1998, Schlotzer-Schrehardt et al. 2002).

In the eye prostaglandin-like activity was discovered in the mid-50s when Ambache found that iris extract contracted smooth muscle preparations and caused miosis in the rabbit eye (Ambache 1955, Ambache 1956, Ambache 1957). This compound, called irin, was subsequently identified as a prostaglandin (Änggård and Samuelsson, 1964). The first studies with prostaglandins in the rabbit eye showed an increase in IOP and breakdown of BAB (Waitzman and King 1967, Beitch and Eakins 1969, Starr 1971, Kass et al. 1972, Neufeld et al. 1972). Subsequently it was observed that in the

rabbit lower doses of prostaglandins induced initial hypertension followed by a prolonged reduction in IOP (Camras et al. 1977). Contradictory early results with prostaglandins have since then been explained by the choice of the animal species used. Bito has reported that the rabbit is a highly sensitive model with respect to ocular irritation (Bito 1984). The IOP-reducing capacity of $PGF_{2\alpha}$ was further confirmed in monkeys (Camras and Bito 1981), non-glaucomatous human volunteers (Giuffre 1985) and in patients suffering from ocular hypertension or glaucoma (Camras et al. 1989).

In the early 1990s it was reported that phenyl-substituted $PGF_{2\alpha}$ analogs, devoid of irritant effect, reduced IOP in primates (Stjernschantz and Resul, 1992). One of these, phXA34 (epimeric mixture containing latanoprost) was shown to reduce IOP in healthy volunteers (Alm and Villumsen 1991) as well as in patients with ocular hypertension, without irritant effect or effect on BAB (Camras et al. 1992, Villumsen and Alm 1992). The IOP reduction caused by $PGF_{2\alpha}$, and its analogs $PGF_{2\alpha}$-IE and latanoprost, is based on an increase in the uveoscleral outflow (Crawford and Kaufman 1987, Gabelt and Kaufman 1989, Nilsson et al. 1989, Toris et al. 1993), which is most probably to be attributed to an effect on the extracellular matrix in the ciliary muscle (Lütjen-Drecoll and Tamm 1988, Lütjen-Drecoll and Tamm 1989, Ocklind 1998, Sagara et al. 1999). Further, prostaglandins have been reported to increase the production of matrix metalloproteinases 1, 2, 3 and 9 (Weinreb et al. 1997), as well as the proenzymes of MMP-1 and MMP-3 (Lindsey et al. 1996) in human ciliary smooth muscle cells, resulting in a decrease in the extracellular matrix components in the ciliary muscle (Weinreb et al. 1997).

3 AIMS OF THE STUDY

1. To identify and characterize CGRP binding sites in the eye in different species.
2. To investigate the structure-activity relationship of CGRP in the eye.
3. To investigate the effect of CGRP on the intraocular pressure and outflow facility in rabbits in the presence and absence of $PGF_{2\alpha}$.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents and Test Compounds

Human (2-[^{125}I]iodohistidyl^{10}) calcitonin gene-related peptide, specific activity 2000 Ci/mmol, adenosine 5'-[alpha-^{32}P]triphosphate and [8-^{3}H]adenosine 3',5',cyclic phosphate were purchased from Amersham International, Amersham, UK. Unlabelled rat and human CGRPα, cat, human and rat amylins, rCGRP 29-37 and rCGRP 30-37 were obtained from Peninsula Laboratories, Inc., Belmont, California, U.S.A. rCGRP(tyr22) 22-37 was obtained from Star Biochemicals, Inc., Torrance, California, USA and rCGRP(tyr0) 28-37 from Multiple Peptide System, California, USA. Thyrocalcitonins, hCGRP $_{8-37}$, trizma-base, bovine serum albumin (BSA), bacitracin, aprotinin, polyethylenimine (PEI), GTP, ATP, cAMP, IBMX, maleic acid, Alumina, hematoxylin and eosin were purchased from Sigma Chemical Company, St. Louis, U.S.A., $PGF_{2\alpha}$ and Dowex 50wx8 from Fluka Chemie AG, Buchs, Switzerland, and sucrose from BDH Limited, Dorset, UK. The films used for autoradiography were Hyperfilm-^{3}H from Amersham and Ektascan NB from Kodak. The developer was Kodak D-19 and the fixer Kodak Unifix. The acetic acid, $KAl(SO_4)_2 \cdot 12\ H_2O$, chloral hydrate, citric acid crystals, HCl and EDTA were obtained from Baker, Deventer, Holland, and $NaJO_3$, $MgSO_4 \cdot 7H_2O$, TCA and imidazole from Merck, Darmstadt, Germany. For synthesis of peptide fragments (Study III) Fmoc-Ala, -Arg(Mtr), -Asn, -Asp(OtBu), -Cys(tBu), -Glu(OtBu), -Gln, -Gly, -His(Trt), -IIe, -Leu, -Lys(Boc), -Phe, -Pro, -Ser(tBu), -Thr(tBu) and Val were purchased from Du Pont de Nemours (Deutschland) GmbH, Bad Homburg, Germany and Bachem AG, Regensdorf, Switzerland. Pentafluorophenol, diisopropylcarbodiimide, piperidine, trifluoroacetic acid, 2(2-aminoethoxy)ethanol, diphenylsulfoxide, anisole, 1,2-ethanedithiol, 2-mercaptoethanol and methyltrichlorosilane were obtained from Aldrich, Steinheim, Germany. 9-Fluorenylmethyl chloroformate and 1-hydroxybenzotriazole hydrate were obtained from Lancaster, Lancashire, UK, HPLC grade S acetonitrile from Rathburn Chemicals Ltd, Peeblesshire, UK, and scintillation fluid Opti Phase HiSafe II from Fisons Chemicals, Loughborough, UK.

4.1.2 Anesthetics and Equipment

Urethane was purchased from Sigma, St Louis, USA, Rompun® (xylaeine: 20 mg/ml, Vet. Inject) from Bayer AG, Leverkusen, Germany and Ketalar® (ketamine: 10 mg/ml, Vet. Inject) from Parke-Davis Scandinavia AB, Solna, Sweden. Mebunat® (pentobarbital: 60 mg/ml, Vet. Inject) was obtained from Orion, Espoo, Finland, Confortid® (indometasin: 50 mg, injection solution, 10 mg/kg b.w.),

from Dumex, Copenhagen, Denmark and Oftan® Obucain from Santen Oy, Tampere, Finland. Blood pressure and IOP were monitored using pressure transducers P-50, Gould/Statham, Bilthoven, the Netherlands and a Grass Model 79-D polygraph, Quincy, MA, USA. In study IV, IOP was also measured using a Digilab® Modular 1 pneumatonometer from Biorad, Cambridge, MA, USA and pH, P_{co2}, and P_{o2} of the arterial blood were checked using ABL 30 blood gas analyzer from Radiometer, Copenhagen, Denmark.

4.1.3 Tissues and Experimental Animals

Pig eyes were obtained from a local slaughterhouse, kept on ice and frozen in dry ice-cooled isopentane (I, II), and iris-ciliary bodies were dissected and frozen in liquid nitrogen (III) within 3-5 hours postmortem. All tissues were stored at -70°C or lower temperature. Cat, guinea pig and monkey eyes were enucleated after an overdose of pentobarbital and immediately frozen and stored as previously described. All studies were approved by the Local Committee on Animal Welfare and Use. Cynomolgus monkey eyes were obtained from the National Laboratory Animal Centre, University of Kuopio, Finland from animals sacrificed for other purpose. In *in vivo* studies albino New Zealand White rabbits of both sexes weighing 2 - 3.5 kg (IV,V) were used.

4.2 Methods

4.2.1 Autoradiography

The frozen eyes were cut into 20-μm serial sections in a cryostat. Two eyes of each species were studied using two sections on every slide. The tissue sections were stored at -20°C until used for experiments. No prior fixation was used.

Sections were pre-incubated for 10 minutes at room temperature (RT) with 50 mM Tris-buffer, pH 7.4, containing 0.33% PEI. The actual incubation was performed in a humidified incubation chamber at RT for 90 minutes. A 50 mM Tris-buffer, pH 7.4, containing 1% BSA, 0.1 mg/ml bacitracin and 0.05 mg/ml aprotinin was used as incubation buffer; 0.08 nM radio-labelled CGRP, (in study II with increasing concentrations of cat, human and rat amylins as displacers), was added to the tissue sections. As controls, rat CGRP (0.5 μM) was used for the tissue sections of cat and pig, and human CGRP (0.5 μM) for monkey tissue sections to determine nonspecific binding of CGRP. After 90 minutes the sections were washed three times for 2 minutes with ice-cold 5 mM Tris-buffer, pH 7.4. The sections were dried under airflow for at least two hours and continuing overnight in a desiccator containing silica gel. The following day tissue slides were placed in the cassette and Ektascan or

Hyperfilm-^{3}H used with one-week and six-week exposure times at +4°C, respectively. The films were developed using a filtered Kodak D-19 developer at RT for 4 minutes and thereafter rinsed in 0.1% acetic acid. Films were fixed in Kodak Unifix for ten minutes, and thereafter washed under running tap water for at least 20 minutes and dried. Tissue slides were stained routinely by hematoxylin-eosin after autoradiography. The displacement results were evaluated semiquantitatively by two persons.

4.2.2 Synthesis of Analogs

Synthesis of CGRP fragments and analogs was carried out by the solid-phase technique using Fmoc/OtBu chemistry on 2,4-dimethoxy-benzhydrylamine resin (RapidAmide) purchased from Du Pont de Nemours (Deutschland) GmbH, Bad Homburg, Germany (Aherton and Sheppard 1989). Peptides were synthesized on a Du Pont RaMPS Multiple Peptide Synthesis System. All amino acids were L-configuration. The completeness of each coupling cycle was determined by the ninhydrin test on a small sample of the resin. The purity and homogeneity of the final peptides were checked by thin-layer chromatography (Merck silica gel 60 F254) and reverse-phase high-pressure liquid chromatography (RP-HPLC). In RP-HPLC gradient elution of water and acetonitrile containing 0.1% TFA was used. The RP-HPLC system consisted of a Waters Model 660 Solvent Programmer, two Waters 501 HPLC Pumps, a Waters 486 Tunable Absorbance detector, and a Waters 746 Data Module. The detector wavelength was set at 215 nm. HPLC grade S acetonitrile was used as eluent. Ultra-pure water was obtained using Water-I Model D 2200 (Barnstead, Division of Sybron Corp.) equipment. For analytical purposes Nucleosil ODS (particle size 5µ, 4.6 x 250 mm) or µBondapak C-18 (particle size 10µ, 3.9 x 300 mm) columns and a Rheodyne injector having a loop volume of 20 µl were used. For semipreparative scale purification of peptides a Nucleosil ODS (particle size 5µ, 10 x 250 mm) column and an injector loop of 300 µl were used.

4.2.3 Binding Assay

Thawed porcine iris-ciliary bodies were homogenized with a glass-glass homogenizer in ice-cold 10 mM Tris buffer, pH 7.4, containing 0.32 M sucrose. The homogenate was centrifuged at 1000 g for 10 minutes at +4°C, and the supernatant then further centrifuged at 48000 g for 30 minutes at +4°C. The pellet was re-suspended and re-centrifuged as above, whereafter the membranes were weighed and re-suspended in the homogenate buffer, yielding a final concentration of 30 mg / ml, and stored at -70^{o}C until used.

In the binding assay, 0.8 mg of membranes were incubated in 400 µl of 50 mM Tris buffer, pH 7.4, containing 1% BSA, 0.1 mg/ml bacitracin, and 0.05 mg/ml aprotinin, in the presence of different concentrations of CGRP analogs for 10 minutes at +4°C, or +37°C. The nonspecific binding was analysed using unlabelled 0.5 µM rat CGRP in the incubation solution. ^{125}I-CGRP was added to the incubation solution, giving a final concentration of 0.08 nM, and incubated for 2 hr / 1 hr at +4°C / +37°C. Incubation was terminated by centrifugation for 3 minutes at 11500 g. The pellets were counted in a Wallac Compugamma (LKB Wallac, Turku, Finland) counter, and analysed using either binding program EBDA / LIGAND (McPhearson 1985), or Multicalc Software (LKB Wallac, Turku, Finland). All binding experiments were performed in triplicate.

4.2.4 Adenylate Cyclase Assay

The AC stimulation assay was performed using a combination of the methods of Osborne and Barnett (1991) and Salomon (1979). The porcine iris-ciliary body was homogenized immediately prior to analysis in 2 mM Tris-maleate buffer, containing 2 mM EDTA, pH 7.4. In the assay 2 mg of iris-ciliary body homogenate in a final volume of 210 µl was used. The peptides studied were added to the incubation solution containing 2 mM $MgSO_4$, 0.2 mM EDTA, 0.1 mM GTP, and 0.24 mM IBMX in 80 mM Tris buffer, pH 7.4. Incubation was initiated by adding 1µCi ^{32}P-ATP and cold ATP (final conc. 1 mM), and continued for 10 minutes at +37^{o}C. The assay was stopped by adding 15% TCA and 0.5 mM cold cAMP. After adding ^{3}H-cAMP (approximately 5000 cpm) for detection of column recovery, the test tubes were centrifuged at 11500 g for 3 minutes, whereafter the supernatants were removed. cAMP was separated chromatographically using Dowex and Alumina columns, and radioactivity measured by liquid scintillation according to the method of Salomon (Salomon 1979). The stimulation of AC was calculated as percentage of the basal AC activity in the tissue. All measurements were performed in triplicate.

4.2.5 General Anesthesia and Intracameral Administration of Test Compounds

In study IV the animals were anesthetized with 25% urethane and kept warm with a thermostated heating pad. In study V the animals were either anesthetized with intravenous 25% urethane alone, or anesthesia was initiated with a Rompun/Ketalar combination and maintained with pentobarbital sodium (Table 1,V). After adequate anesthesia had been induced, a femoral artery was cannulated and connected to a pressure transducer for blood pressure monitoring. All animals were also tracheotomized to assure adequate ventilation. The pH, P_{co2}, and P_{o2} of the arterial blood were checked routinely a few times during the course of the experiment (IV).

About 30 minutes prior to the cannulation of the eyes, the animals were pre-treated with intravenous indomethacin to prevent release of endogenous prostaglandins. In addition, the eyes were also topically anesthetized with oxybuprocaine. The eyes were cannulated with three needles (27G) connected to polyethylene tubings. One cannula was used for continuous IOP monitoring, one for injection of the test compound or isotonic saline, and one for infusion of the fluid for outflow measurement. The experimental eye of each animal received a slow intracameral injection of 5 µl of rat CGRPα (5 µg; IV), or of either rat CGRPα or $PGF_{2\alpha}$ or both (20 ng and 50 ng; V), administered with Hamilton precision syringes. The fellow eye served as control, receiving the same volume of isotonic saline.

4.2.6 Intravitreal Administration of Test Compound and Pneumatonometer IOP Measurement

The intravitreal injections were carried out in unanesthetized animals under topical anesthesia with oxybuprocaine. The needle was inserted into the vitreous body through the pars plana and 5 µg CGRP (in 50 µl) was inserted. The IOP was measured with a pneumatonometer. The tonometer was specially calibrated for IOP measurement in rabbits using simultaneous manometric and pneumatonometric pressure measurements according to the closed stopcock method (Hammond and Bhattacherjee, 1984). Before each pressure measurement the cornea was anesthetized with oxybuprocaine. The IOP was measured before and 6, 10, and 24 hours after the injection of CGRP. Thereafter IOP was measured once daily for 10 consecutive days.

4.2.7 Measurement of IOP and Aqueous Humour Outflow after Intracameral and Intravitreal Administration of Test Compounds

IOP was measured manometrically in both eyes one hour after intracameral injection of the test compound, i.e. immediately prior to the outflow measurements, which took about 20 minutes. In study IV, IOP and aqueous humour outflow facility were also determined 24 and 72 hours after intravitreal injections of 1 and 5 µg CGRP.

The outflow facility of aqueous humour was measured by the two-level constant pressure infusion method (Bárány 1964). A mock solution of aqueous humour (Sperber and Bill 1984) was infused from a reservoir into the anterior chamber to increase IOP 5 to 7 mmHg above the preinfusion level, and the reservoir was adjusted to give a steady-state IOP. The infusion rate (F_1) and the increase in IOP (ΔP_1) were registered over a 4- to 8-minute period. IOP was then again raised about 5 to 7

mmHg above the previous level (10 to 14 mmHg above the preinfusion level). Figures for the infusion rate (F_2), and the increase in IOP (ΔP_2) from preinfusion level were then calculated under steady-state conditions. In order to ensure a technically successful experiment, IOP had to return to the preinfusion level ±1 mmHg after the second infusion. The outflow facility was calculated based on the formula $C=F/\Delta P$. C was calculated for both pressure levels.

4.2.8 Aqueous Humour Protein Concentration

The protein concentration in the aqueous humour was measured 24 and 72 hours after intravitreal injection of 5 μg CGRP. Paracentesis was performed with a 27-gauge needle and the aqueous humour samples were stored at -70°C until analysed (Lowry et al. 1951). The results are expressed as arithmetical mean ± SEM.

4.2.9 Statistical Analysis

Student's paired t-test was used in the *in vivo* studies when the experimental eye was compared with the saline-treated control eye of the same animal (IV, V). The unpaired t-test was used when the effects in different animals were compared (V). $P \leq 0.05$ was considered a statistically significant.

5 RESULTS

5.1 GCRP Binding Sites in the Eye of Monkey, Pig, Cat and Guinea Pig (I)

Specific CGRP binding sites were found in the ciliary muscle, ciliary processes and limbal conjunctiva in monkey, pig, cat and guinea pig. In addition, specific binding appears to be species-dependent in choroid, retina, anterior chamber angle and in the blood vessels of the iris. Both arteries and veins show specific binding of CGRP, although the binding was more intensive in arteries.

5.1.1 Monkey

In the monkey, the ciliary muscle, ciliary processes and some choroidal blood vessels showed marked binding of CGRP. A low number of specific binding sites were seen in the limbal conjunctiva. No specific binding of CGRP was found in the iris blood vessels, anterior chamber angle or retina.

5.1.2 Pig

A particularly high number of CGRP binding sites were found in the porcine ciliary processes, retina and some of the choroidal blood vessels, and a moderate number in the ciliary muscle, in the blood vessels of the iris and in the limbal conjunctival epithelium and stroma, while a low number were found in the iris stroma. No specific binding sites were detected in the anterior chamber angle.

5.1.3 Cat

In the cat, intensive specific binding of CGRP was observed in ciliary muscle, ciliary processes and blood vessels of the iris and in the anterior chamber angle. Only some CGRP binding sites were found in the limbal conjunctiva. No binding sites were found in the retina and choroid.

5.1.4 Guinea pig

A high number of CGRP binding sites were found in the ciliary muscle and ciliary processes, in the iris, and in the choroid of the guinea pig. A moderate number of specific binding sites were found in the limbal conjunctiva. No binding sites were detected in the anterior chamber angle or the retina.

5.2 Displacement of ^{125}I-CGRP Binding by Cat, Human and Rat Amylins in the Eye of Monkey, Cat and Pig (II)

In the monkey, a 1000 nM concentration of cat and rat amylins slightly inhibited the binding of iodinated CGRP in the ciliary muscle and ciliary processes. Human amylin, however, did not affect the binding of ^{125}I-CGRP in the ciliary muscle or ciliary processes at any of the concentrations studied (up to 1000 nM).

Cat amylin clearly competed for the binding of iodinated CGRP in the anterior chamber angle at a 1 nM concentration as well as at 100 nM in the ciliary muscle, ciliary processes and iris of the cat. In the ciliary muscle, ciliary processes and iris the displacement did not further increase at higher concentrations, while in the anterior chamber angle it was further increased at a 100 nM concentration. Human amylin clearly displaced ^{125}I-CGRP from the anterior chamber angle at 1 nM, ciliary muscle at 100 nM, and ciliary processes and iris at 1000 nM concentrations. The displacement from the anterior chamber angle and ciliary muscle was further increased at 100 nM and 1000 nM. Cat and human amylins did not displace iodinated CGRP in the limbal conjunctiva. In the iris and limbal conjunctiva, rat amylin slightly competed for the binding of ^{125}I-CGRP at 10-1000 nM, and clearly in the ciliary muscle and ciliary processes at 100 nM and 1000 nM concentrations, respectively.

In the pig, cat amylin displaced iodinated CGRP from the ciliary muscle, ciliary processes and iris slightly, and from the limbal conjunctiva clearly at a 100 nM concentration. At a 1000 nM concentration, the displacement in these tissues was practically complete. Human amylin clearly competed for the binding sites of CGRP in the ciliary muscle, ciliary processes, iris and limbal conjunctiva at a 1000 nM concentration, and further, rat amylin clearly competed in the ciliary muscle and ciliary processes and particularly clearly in the iris and limbal conjunctiva at the same concentration.

5.3 Structure-Activity Relationship of CGRP Molecule (III)

Several CGRP analogs as well as thyrocalcitonins were tested for the displacement of ^{125}I-CGRP binding in the porcine iris-ciliary body as well as AC stimulation in the same tissue. The amino acid sequences of the analogs tested differed from that of human CGRP with respect to one or several amino acids, or the analogs lacked, or had modified sequences of amino acids compared to human

CGRP. More detailed information on the amino acid sequences of the compounds tested is presented in Table 1 of publication III.

5.3.1 Binding Affinity

CGRP analogs which displaced ^{125}I-CGRP at +4°C in the porcine iris-ciliary body to the same extent (IC_{50} 1-10 nM) as human CGRP were rat CGRP, $CGRP_{8-37}$, SH892, SH992, SH1292, TV5591, TV5691 and TV6191. Lower affinities (IC_{50} 10-50 nM) were observed with SH792, TV5491, TV5791 and SH192, while SH1092, SH1192, TV5891, TV6091, $rCGRP(tyr0)_{28-37}$ and chicken, eel and salmon thyrocalcitonins had only weak affinities (IC_{50} 200-1000 nM) at +4°C. The rest of the analogs tested exhibited at least 1000 times lower affinity to the CGRP receptor than human CGRP at +4°C.

Only human CGRP, rat CGRP, SH892 and SH1292 retained almost the same affinity (IC_{50} < 10 nM) at +37°C as at +4°C, while the affinity of analogs SH992, TV5591, TV5691 and TV6191 clearly decreased (IC_{50} 20-50 nM) at the higher temperature. Moreover, analogs SH792, TV5791 and SH192 had 10 to 80 times lower affinities (IC_{50} 200-1600 nM) at +37°C and $CGRP_{8-37}$, in fact, 300 times lower affinity (IC_{50} ~300 nM). TV5491, which showed moderate affinity at +4°C, failed to bind to CGRP receptors at +37°C. Finally, analogs SH1092, SH1192, TV5891, TV6091 and $rCGRP(tyr0)_{28-37}$, which already exhibited low affinity at +4°C, were considered to have at most only very weak affinity (IC_{50} >2000 nM) to the receptors at +37°C.

5.3.2 Stimulation of Adenylate Cyclase

In addition to human and rat CGRP, only SH892, SH992, TV5691, TV6191 and TV5491 stimulated AC in porcine iris-ciliary body homogenate at +37°C. These CGRP analogs exhibited EC_{50} values of 40 nM, 100 nM, 150 nM, 150 nM and 200 nM, respectively, compared to 38 nM and 20 nM for human and rat CGRP. Even though SH892 and SH992 had quite good EC_{50} values, their efficacy was inferior (about 1/3 and 1/6) to that of CGRP.

5.4. Intracameral and Intravitreal Injections of CGRP (IV)

5.4.1 IOP and Aqueous Humour Outflow after Intracameral Injection of CGRP

The intraocular pressure measured immediately prior to the outflow facility measurement did not differ significantly between 5 μg CGRP and saline-injected eyes (experimental eyes: 23.5±2.2;

control eyes: 20.6± 2.2 mmHg). There was, however, a tendency towards increased IOP in the CGRP-injected eyes and in this respect these results are in agreement with the earlier findings of Oksala and Stjernschantz (1988a), in which an intracameral injection of CGRP increased the intraocular pressure in rabbits. Intracameral injection of CGRP increased the outflow facility significantly at both pressure levels 0.746±0.076 μl/min/mmHg and 0.188±0.038 μl/min/mmHg, respectively, and 0.622±0.057μl/min/mmHg and 0.216±0.049 μl/min/mmHg, respectively, (experimental eye and control eye), about one hour after the injection.

5.4.2 IOP after Intravitreal Injection of CGRP

The IOP was already decreased at 6 hours after intravitreal injection of 5 μg CGRP, but a significant decrease was not seen until day 1. The duration of the IOP reduction was 9 days. Only mild hyperemia in the anterior segment of the eye was observed 2 to 6 hours after the injection of CGRP.

5.4.3 IOP and Aqueous Humour Outflow after Intravitreal Injection of CGRP

Twenty-four hours after the injection, CGRP caused a dose-dependent significant increase in the outflow facility of aqueous humour with 1 and 5 μg doses. However, 72 hours later there was no longer a significant increase in the outflow facility. At the same time IOP was lowered significantly both at 24 hours and at 72 hours after intravitreal injections of 1 μg and 5 μg CGRP. The maximum decrease in IOP was reached 72 hours after the injection.

5.4.4 Protein Concentration in Aqueous Humour after Intravitreal Injection of CGRP

Twenty-four hours after the administration of 5 μg CGRP the protein concentration in the aqueous humour was possibly slightly increased in the experimental and control eyes (58 ± 9.4 and 105 ± 51.9 mg/100ml, respectively). This increase was apparently due to the injection *per se*, as no CGRP was administered to the control eyes; 72 hours after the injections the protein concentrations had returned to normal in both experimental and control eyes (37 ± 1.7 and 32 ± 2.2 mg/100 ml, respectively).

5.5 Effects of Simultaneous Intracameral Injection of CGRP and $PGF_{2\alpha}$ on IOP and Aqueous Humour Outflow in Rabbit (V)

IOP measured immediately prior to the aqueous humour outflow measurement was significantly decreased after an intracameral injection of 20 ng CGRP ($P \leq 0.05$). In contrast, IOP significantly

increased after intracameral injection of 20 ng $PGF_{2\alpha}$ ($P \leq 0.05$). No significant effects on IOP were found with higher doses of CGRP or $PGF_{2\alpha}$. No iridial hyperemia was seen with the doses used.

Both the 20 ng and 50 ng intracameral CGRP doses, as well as the 50 ng but not the 20 ng dose of $PGF_{2\alpha}$ increased outflow facility significantly ($P \leq 0.05$) when compared to the saline-injected fellow eyes. Simultaneous administration of CGRP and $PGF_{2\alpha}$ increased the outflow facility significantly ($P \leq 0.01$ and $P \leq 0.05$ at different pressure levels) at a 50 ng dose when compared with the saline-injected fellow eye. Moreover, when the outflow facility of simultaneously administered CGRP and $PGF_{2\alpha}$ was compared with that of $PGF_{2\alpha}$ given alone, a significant increase ($P \leq 0.01$) was found at 50 ng doses.

6 DISCUSSION

6.1 CGRP Binding Sites in the Eye

6.1.1 Ciliary Muscle

All the species tested, i.e. monkey, pig, cat and guinea pig, were found to have CGRP binding sites in the ciliary muscle. CGRP immunoreactivity has also been shown to be present in nerve fibres in the ciliary body in several species, including man (Terenghi et al. 1985, Stone and McGlinn 1988, Uusitalo et al. 1989). Our findings support these results and indicate a specific role for CGRP in the eye. In the ciliary muscle CGRP might affect muscle tone and further, aqueous humour outflow.

6.1.2 Anterior Chamber Angle

Only the cat was found to possess specific binding sites for CGRP in the anterior chamber angle. The aqueous humour outflow has previously been reported to increase in the cat (Oksala and Stjernschantz 1988b) and monkey (Almegård and Andersson 1990) eye after CGRP administration. Furthermore, our data show that CGRP also induces an increase in aqueous humour outflow (outflow facility) in the rabbit (Oksala et al. 1998). The increase in aqueous humour outflow in the cat and rabbit eye is most probably caused by an increase in trabecular outflow (Oksala and Stjernschantz 1988b, Oksala et al. 1998). This is in good agreement with our finding of specific CGRP binding sites in the cat chamber angle, as well as the reported immunoreactive nerve fibres in the trabecular meshwork in the human and monkey eye (Stone and McGlinn 1988).

6.1.3 Ciliary Processes and Iris

Monkey, pig, cat and guinea pig had CGRP binding sites in the ciliary processes and pig, cat and guinea pig in the iris. The distribution of the CGRP binding sites in the ciliary processes suggests their relation to the blood vessels. Similarly, specific binding sites for CGRP were also found in the iris blood vessels of pig, cat and guinea pig. This is well in agreement with previous studies showing that CGRP has binding sites in systemic blood vessels (Wimalawansa and MacIntyre 1988, Foulkes et al. 1991) and is a potent vasodilator (Brain et al. 1985, Brain et al. 1986). These findings are further supported by data showing that CGRP induces vasodilatation in the rabbit (Unger et al. 1985, Wahlestedt et al. 1986, Krootila et al. 1988, Oksala and Stjernschantz 1988a), cat (Oksala 1988) and monkey eye (Almegård and Andersson 1993). The vasoactive role of CGRP in the ciliary processes is probably also important with respect to the integrity of the blood-aqueous barrier, which is reported to be disrupted in the rabbit after CGRP administration (Unger et al. 1985, Wahlestedt et al. 1986, Krootila et al. 1988, Oksala and Stjernschantz 1988a).

6.1.4 Limbal Conjunctiva

The specific binding sites found in the limbal conjunctiva of all species studied may indicate a role of CGRP in the regulation of epithelial cell regeneration, this in view of the fact that the limbal conjunctiva serves as a germinative region for the corneal epithelium. A role for CGRP in the limbal area is further supported by findings of CGRP-immunoreactive nerve fibres in the limbal area in the guinea pig (Kuwayama and Stone 1987) and rat (Luhtala et al. 1991). Furthermore, Gallar and co-workers demonstrated that in the rabbit prolonged treatment with capsaicin, which has been shown to cause a depletion of neuropeptides, including CGRP, from primary afferent neurons, induces a significant delay in epithelial migration rates and in wound closure times in the cornea (Gallar et al. 1990).

6.1.5 Choroid and Retina

CGRP binding sites were found in the choroid of monkey, pig and guinea pig, and in the retina of the pig. CGRP has not been shown to have any effect on the blood flow in the choroid of the rabbit (Krootila et al. 1988), cat (Oksala 1988) or monkey (Almegård and Andersson 1993), and a possible physiological role of CGRP in the choroid remains to be established. In the chicken, CGRP has been found in retinal amacrine cells (Kiyama et al. 1985), and in the rabbit it has been reported to play a role in modulating retinal responses to photic stimulation (Cao et al. 1993). Moreover, in the monkey and rat CGRP-like immunoreactive nerves are associated with the central retinal artery, although the immunoreactivity is lost when the artery enters the optic disk (Ye et al. 1990). The precise role of CGRP in the retina remains unknown.

6.1.6 Autoradiography as a Method

Degradation of the peptide ligand by endogenous peptidases in the tissue, and nonspecific binding of the ligand e.g. to glass may constitute problems in autoradiography. In our studies these problems were minimized by using bacitracin and aprotinine as peptidase-inhibitors, and bovine serum albumin to prevent nonspecific binding. A further disadvantage of the autoradiographic method is that it does not recognize low-affinity receptors, occupied receptors or spare receptors. Sometimes when the distributions of the endogenous peptides and their receptors are compared they may differ significantly from each other. This is referred to as a mismatch problem (Palacios and Dietl 1987). The reason for this discrepancy can be the different techniques used, for example, the immunohistochemical method to demonstrate the localization of the endogenous peptide and

autoradiography for studying the distribution of their receptors. Furthermore, different receptor subtypes and affinities may explain these differences (Palacios and Dietl 1987).

6.2 The Displacement of ^{125}I-CGRP by Cat, Human and Rat Amylins

Our results show that cat, human and rat amylins can compete for the binding of CGRP in the anterior part of the eye in the monkey, cat and pig. Galeazza and co-workers (1991) also noted the ability of amylin to compete for CGRP binding in the rat brain, skeletal muscles and liver, although amylin exhibited lower affinity than CGRP. Similarly, in the guinea pig isolated left atrium and urinary bladder, and the rat vas deferens, rat amylin mimics the effect of CGRP *in vivo*, although it is less potent than CGRP (Giuliani et al. 1992). Although the primary sequence of amylins differs by 19 to 21 amino acids from that of human CGRPα, the secondary structures of CGRP and amylin exhibit similarities such as the intramolecular disulfide bond, the α-helix and the amidated C terminal (Poyner et al. 2002). It was evident here that the ability of amylins to displace CGRP binding varied between different species, being weakest in the monkey eye. This species specificity is also found with respect to amylin receptors in the mouse, pig and guinea pig lung tissue (Bhogal et al. 1995).

Amylin or amylin receptors have not previously been identified in the eye. It has, however, been reported that both amylin and CGRP apparently possess their own receptors even though they can also act through each others' receptors. The amylin receptor consists of a CT receptor and one of the various RAMPs (Chen et al. 1997b, Tilakaratne et al. 2000) and it would appear that different CT and RAMP combinations produce different receptor phenotypes binding only amylin (CT+RAMP3), or both amylin and CGRP (CT+RAMP1) (Christopoulos et al. 1999). The CGRP receptor for its part must consist of a CRLR, RAMP1 and RCP to function properly (Aiyar et al. 1996, Luebke et al. 1996, McLatchie et al. 1998). As both are found in the sensory nerves, e.g. in the rat trigeminal ganglia (Terenghi et al. 1985, Mulder et al. 1995) it seems possible, although not yet shown, that amylin is also expressed in the eye.

6.3 Structure-Activity Relationship of CGRP Molecule

6.3.1 Binding Affinity

The structure-activity relationship of the CGRP molecule was studied using different CGRP analogs in the porcine iris-ciliary body. It was found that the carboxyterminal end of the CGRP molecule is important for receptor binding and the aminoterminal end for AC stimulation. In general, an increase in temperature from +4°C to +37°C reduced the binding affinity of the CGRP analogs. Even small

changes in the carboxyterminal end of the peptide affect the binding affinity of the molecule. An isosteric replacement of asparagines from positions 25 or 26 by leucine, as in TV5791 and TV5891, respectively, accordingly weakens the binding affinity of these analogs nearly 20 and 1000 times. Furthermore, in the aminoterminus, deletion of the first seven amino acids does not alter the binding affinity at +4°C but attenuates it at +37°C about 60 times compared to native CGRP. Accordingly, this deletion abolishes the ability of the CGRP molecule to stimulate AC enzyme and changes the molecule from agonist (CGRP) to antagonist ($CGRP_{8-37}$). This antagonistic property has been shown in several *in vitro* studies, e.g. in rat liver plasma membranes (Chiba et al. 1989). In our study we showed that even a minor change in the aminoterminus can eliminate the ability of the peptide to stimulate adenylate cyclase, although the binding affinity remains comparable to that of CGRP. This is demonstrated, for example, with a CGRP analog having an isosteric replacement of threonine for valine at position 6 (TV5591), or an analog having a substitution of amino acids in positions 3-6 (Asp->Tyr, Thr->Ile, Ala->Gln, Thr->Asn) as in SH1292. In the case of TV5591 it was shown that the appropriate steric size does not suffice to keep the peptide active, probably because compared to threonine the valine has a different hydrophobicity and ability to form hydrogen bonds.

6.3.2 Stimulation of Adenylate Cyclase

Elimination of the first and the third amino acids of the CGRP molecule reduced the stimulation of AC. Furthermore, a change of the fourth amino acid threonine to valine (SH892) resulted in reduced efficacy of the peptide to 1/3 of that of the native CGRP. N-terminal cysteine loop replacement for Thr-Pro-Pro-Thr amino acids, as in SH992, does not much affect EC_{50} value of the analog, although it reduces its maximum response to 1/6 of that of CGRP. Presumably it allows the peptide to form an α-helix which is needed for a correct three-dimensional structure of the peptide. Lynch and Kaiser (1988) have suggested that for this three-dimensional structure amino acids 8-18 form an α-helical region and amino acids 18-23 a β-turn. The importance of the aminoterminus for adenylate cyclase-stimulating activity is further emphasized by Maggi and co-workers (1990), who demonstrated that truncated CGRP analogs, or analogs lacking the disulfide bridge, were unable to stimulate AC in a preosteoblast cell line (KS-4). The same investigators have, however, also shown that *in vivo* short aminoterminal fragments such as 1-12, 1-15 and 1-22 still exert some hypotensive effects in rats (Maggi et al. 1990), while even shorter fragments, 1-8, 2-8 and 1-10, proved unable to bind to the CGRP receptor in the porcine iris-ciliary body (Malminiemi and Malminiemi 1992). Further, in our present study we showed that CGRP analog 1-18 (SH392) retains some binding affinity, while CGRP analog 1-15 (SH492) does not.

6.4 The Effect of CGRP on IOP and Outflow Facility

Intracameral injection of CGRP has previously been reported to increase intraocular pressure and cause a breakdown of the blood-aqueous barrier in the rabbit eye (Unger et al. 1985, Oksala and Stjernschantz 1988a), while in the cat IOP is reduced by CGRP probably due to increased outflow facility with little or no effect on BAB (Oksala and Stjernschantz 1988a, Oksala and Stjernschantz 1988b). In our study intracameral injection of 5 μg CGRP was found to induce a significant increase in outflow facility in the rabbit, although it also slightly increased the IOP (IV). This increase in outflow facility may be considered to be due to an increase in trabecular outflow, although it may involve a pseudofacility component based on the breakdown of BAB, as previously reported (Unger et al. 1985, Oksala and Stjernschantz 1988a). In previous studies, CGRP has generally been used in intracameral doses of 500 –5000 ng (Unger et al. 1985, Krootila et al. 1988, Oksala and Stjernschantz 1988a). The low doses used in study V also seem to be sufficient to increase the outflow facility in the rabbit. The possible role of pseudofacility in the increase of gross outflow facility after the 5 μg dose of CGRP is most probably negligible after such low doses as 20 or 50 ng. Such a conception is supported by the fact that no iridial hyperemia was seen with low doses of CGRP (V) and that the threshold dose of CGRP inducing the breakdown of BAB and an increase in IOP is envisaged as about 100 ng (Oksala and Stjernschantz 1988a).

IOP decreased six hours after the intravitreal injection of 5 μg CGRP and remained significantly decreased for nine days. Taniguchi and associates have also reported a 12 hours' decrease in IOP in the rabbit after low-dose (about 76 ng) intravitreal administration of CGRP (Taniguchi et al. 1999). Further, intravitreal administration of 1 and 5 μg CGRP significantly increased the aqueous humour outflow. The aqueous humour protein concentration was not significantly increased after intravitreal administration of CGRP, and it would thus appear that the increase in outflow facility is not based on pseudofacility. The increase in outflow facility had disappeared 72 h after the intravitreal administration of CGRP, whereas the decrease in IOP remained much longer, indicating a distinct mechanism of action for CGRP.

6.5 The Effect of Simultaneous Injection of CGRP and $PGF_{2\alpha}$ on Outflow Facility

The ability of CGRP to enhance the outflow facility in the rabbit was further studied after simultaneous administration of CGRP and $PGF_{2\alpha}$ (V). $PGF_{2\alpha,}$ is reported to elicit an increase in IOP, anterior uveal vasodilatation, breakdown of BAB and miosis in the rabbit (Cole and Unger 1973). However, at the 50 ng dose used in our study, it evidently only increased the outflow facility in the

rabbit eye. When the outflow facilities C_1 and C_2 (at different pressure levels) were analysed, CGRP apparently tended to increase the outflow in a pressure-dependent manner (C1=C2), indicating an increase in trabecular outflow. Such a conclusion is also supported by our previous study in the rabbit (IV) (Oksala et al. 1998). $PGF_{2\alpha,}$ in contrast, seemed to increase the uveoscleral outflow (C1>C2), as also shown in earlier studies in different species such as the rabbit (Poyer et al. 1992), the monkey (Crawford and Kaufman 1987, Gabelt and Kaufman 1989, Nilsson et al. 1989) and humans (Toris et al. 1993). Thus CGRP seems to mediate the increase in outflow via the trabecular outflow, while the $PGF_{2\alpha}$ increases the uveoscleral outflow. The simultaneous administration of CGRP and $PGF_{2\alpha}$ induced only a slight additive effect on the outflow facility at 50 ng doses in the rabbit eye. This increase in outflow was mainly based on CGRP. This is probably due to the species used in the present study, the rabbit having less developed ciliary muscle compared to some other species (Bito 1984). The uveoscleral outflow has been reported to account for only about 10% of the total outflow in rabbits according to Bill (1966), and groups under Goh (1989), and Poyer (1992).

7 CONCLUSIONS

This thesis was undertaken to identify, localize and characterize CGRP receptors in the eye, to study the structure-activity relationship of CGRP, and to study the effect of CGRP on IOP and outflow facility alone and in combination with $PGF_{2\alpha}$ in the rabbit.

The main findings were:

1. Specific CGRP binding sites are located mainly in the vasculature as well as in the chamber angle. These findings support the physiological findings showing that CGRP is a potent vasoactive agent with effect also on IOP.
2. The carboxyterminal end of the CGRP molecule is mainly responsible for the binding of the molecule, while the aminoterminal end is responsible for the activation of AC enzyme. The binding affinity was found to be temperature-dependent. Furthermore, there are sensitive regions in the molecule where even minor changes in the primary structure cause marked changes in biological activity and/or receptor binding.
3. The CGRP receptor in the eye also binds amylins, suggesting a possible role for endogenous amylins in the eye. However, whether they act as agonists or antagonists at the receptor remains to be established.
4. Intravitreal injection of CGRP induced a prolonged decrease in IOP in the rabbit eye. The main mechanism of action would appear to be related to an increase in trabecular outflow.
5. When administered simultaneously with CGRP, $PGF_{2\alpha}$ did not significantly potentiate the IOP- lowering effect of CGRP.

CGRP has been shown to exert significant effects in the eye related to blood flow and IOP. CGRP receptors (binding sites), furthermore, have been demonstrated in relevant structures of the eye such as the vasculature and chamber angle. Due to its large size it has been impossible for the molecule to enter into the eye through the cornea after topical administration, and intracameral or intravitreal administrations have been used to study the effects of this peptide. However, for the medical use of CGRP this problem should be resolved. Although CGRP is a relatively large neuropeptide with complex tertiary structure and receptor binding/activating properties, it is not inconceivable in the future that truncated analogs of CGRP, or peptidomimetics, may be developed which could be potentially useful in the treatment of glaucoma.

8 ACKNOWLEDGEMENTS

This study was carried out at the Research Center of Santen Oy, Tampere and in the Department of Ophthalmology in Tampere University Hospital. It was financially supported by the Finnish Technology Development Centre, the Sigrid Juselius Foundation of Finland, the Väinö and Hilkka Kiltti Foundation of Finland and the Finnish Association for Eye Research, which help is gratefully acknowledged.

I wish to express my deepest gratitude to my supervisor Professor Hannu Uusitalo, MD, PhD for his guidance and encouragement in this undertaking. His extensive knowledge of the area of ophthalmic research has taught me a great deal, and his patience and support during these years have made this thesis possible.

I would like to express my sincere thanks to Professor Lotta Salminen, MD, PhD, who has supported me, and gave me a possibility to make my postgraduate studies in the field of ophthalmology. I also wish to thank Santen Oy, President Jyrki Liljeroos, MSc, for making the facilities and equipment available for me, and Vice President of Research and Development, Kari Lehmussaari, MSc for his encouragement and support in preparing this thesis.

I am deeply grateful to my official reviewers Professor Johan Stjernschantz, MD, PhD and Professor Arto Urtti, PhD, for their detailed assessments, and their valuable suggestions and constructive criticism in reviewing the manuscript. I was fortunate to have such highly reputed experts as my reviewers.

I am grateful to all my co-authors who have taken part in the original studies for their fruitful collaboration. I would like to thank Dr. Olli Oksala, PhD for introducing me to the interesting molecule CGRP and especially to *in vivo* studies in the field of ophthalmology. I also wish to thank Professor Arto Palkama, MD, PhD, for sharing this project at the very beginning and supporting and kindly coaxing me to complete this thesis, Professor Ari Koskinen, PhD, for valuable help in the field of peptide chemistry, Ms Sari Vihavainen, MSc and Ms Tuula Valo MSc for synthesizing the peptide analogs studied, and Mr Jussi Luhtala, MD and Dr. Sakari Alaranta, PhD for sharing their study interests in the field of ophthalmology. I am also particularly thankful to both Ms Sirkka Ahoniemi and Ms Marja Mali for their skilful and expert technical assistance in these studies.

Further, I wish to thank all of my colleagues at Santen Oy for their help and the interest they have shown for my thesis, and I am also thankful to Mr Robert MacGilleon, MA for revising the English of the thesis and the original publications.

I want to thank my parents Toini and Antti for their encouragement and for making this thesis possible by taking care of Tuomas, and thus allowing me the time to finalize the work during my maternity leave; also my sister Kirsi and her family for sharing all kinds of feelings during this process, my brother Arto for supporting me throughout these years, and all of my friends who in various ways have shared this experience with me.

Finally, I owe my deepest gratitude to my husband Petteri for his patience and support during this process, and our son Tuomas, who has taugh me the deepest meaning of life.

Tampere, April 16, 2004

Päivi Alajuuma

9 REFERENCES

(2000): The Advanced Glaucoma Intervention Study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration.The AGIS Investigators. Am J Ophthalmol 130:429-440.

Aherton K and Sheppard R (1989): Solid phase peptide synthesis- a practical approach. IRL Press, Oxford .

Aiyar N, Baker E, Martin J, Patel A, Stadel JM, Wilette RN and Barone FC (1995): Differential calcitonin gene-related peptide (CGRP) and amylin binding sites in nucleus accumbens and lung: potential models for studying CGRP/amylin receptor subtypes. J Neurochem 65:1131-1138.

Aiyar N, Disa J and Stadel JM (1999): Calcitonin gene-related peptide receptor independently stimulates 3',5'-cyclic adenosine monophosphate and Ca2+ signaling pathways. Mol Cell Biochem 197:179-185.

Aiyar N, Rand K and Elshourbagy NA (1996): A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J Biol Chem 271:11325-11329.

Alm A (1977): The effect of sympathetic stimulation on blood flow through t,e uvea, retina and optic nerve in monkeys (Macacca irus). Exp Eye Res 25:19-24.

Alm A and Bill A (1973): The effect of stimulation of the cervical sympathetic chain on retinal oxygen tension and on uveal, retinal and cerebral blood flow in cats. Acta Physiol Scand 88:84-94.

Alm A and Villumsen J (1991): PhXA34, a new potent ocular hypotensive drug. A study on dose-response relationship and on aqueous humor dynamics in healthy volunteers. Arch Ophthalmol 109:1564-1568.

Almegård B and Andersson SE (1990): Outflow facility in the monkey eye: effects of calcitonin gene-related peptide, cholecystokinin, galanin, substance P and capsaicin. Exp Eye Res 51:685-689.

Almegård B and Andersson SE (1993): Vascular effects of calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) in the monkey eye. J Ocul Pharmacol 9:77-84.

Amara SG, Arriza JL and Leff SE (1985): Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229:1094-1097.

Amara SG, Jonas V and Rosenfeld MG (1982): Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298:240-244.

Ambache N (1955): Irin, a smooth-muscle contracting substance present in rabbit iris. J Physiol 129:65-66.

Ambache N (1956): Trigeminomimetic action of iris extracts in rabbits. J Physiol 132:49-50.

Ambache N (1957): Properties of irin, a physiological constituent of the rabbit's iris. J Physiol 135:114-132.

Anderson DR (2003): Collaborative normal tension glaucoma study. Curr Opin Ophthalmol 14:86-90.

Änggård E and Samuelsson B (1964): Smooth muscle stimulating lipids in sheep iris, identification of prostaglandin $F_{2\alpha}$, prostaglandins, and related factors. Biochem Pharmacol 13:281-283.

Anthony TL, Pierce KL and Stamer WD (1998): Prostaglandin F2 alpha receptors in the human trabecular meshwork. Invest Ophthalmol Vis Sci 39:315-321.

Bárány E (1964): Simultaneous measurement of changing intraocular pressure and outflow facility in the vervet monkey by constant pressure infusion. Invest Ophthalmol Vis Sci 3:135-143.

Beaumont K, Kenney MA, Young AA and Rink TJ (1993): High affinity amylin binding sites in rat brain. Mol Pharmacol 44:493-497.

Beitch BR and Eakins KE (1969): The effects of prostaglandins on the intraocular pressure of the rabbit. Br J Pharmacol 37:158-167.

Bhogal R, Smith DM and Owji AA (1995): Binding sites for islet amyloid polypeptide in mammalian lung: species variation and effects on adenylyl cyclase. Can J Physiol Pharmacol 73:1030-1036.

Bill A (1962): Autonomic nervous control of uveal blood flow. Acta Physiol Scand 56:70-81.

Bill A (1966): The routes for bulk drainage of aqueous humour in rabbits with and without cyclodialysis. Doc Ophthalmol 20:157-169.

Bill A (1968): Capillary permeability to and extravascular dynamics of myoglobin, albumin and gammaglobulin in the uvea. Acta Physiol Scand 73:204-219.

Bill A and Geijer C (1977): Effect of blood flow on albumin turnover in the choroid of unanaesthetised rabbits. Bibl Anat:47-50.

Bill A and Phillips CI (1971): Uveoscleral drainage of aqueous humour in human eyes. Exp Eye Res 12:275-281.

Bill A, Stjernschantz J and Alm A (1976): Effects of hexamethonium, biperiden and phentolamine on the vasoconstrictive effects of oculomotor nerve stimulation in rabbits. Exp Eye Res 23:615-622.

Bill A, Stjernschantz J and Mandahl A (1979): Substance P: release on trigeminal nerve stimulation, effects in the eye. Acta Physiol Scand 106:371-373.

Bito LZ (1984): Species differences in the responses of the eye to irritation and trauma: a hypothesis of divergence in ocular defense mechanisms, and the choice of experimental animals for eye research. Exp Eye Res 39:807-829.

Bouali SM, Wimalawansa SJ and Jolicoeur FB (1995): In vivo central actions of rat amylin. Regul Pept 56:167-174.

Brain SD and Cambridge H (1996): Calcitonin gene-related peptide: vasoactive effects and potential therapeutic role. Gen Pharmacol 27:607-611.

Brain SD and Williams TJ (1988): Substance P regulates the vasodilator activity of calcitonin gene-related peptide. Nature 335:73-75.

Brain SD, MacIntyre I and Williams TJ (1986): A second form of human calcitonin gene-related peptide which is a potent vasodilator. Eur J Pharmacol 124:349-352.

Brain SD, Williams TJ and Tippins JR (1985): Calcitonin gene-related peptide is a potent vasodilator. Nature 313:54-56.

Camras CB and Bito LZ (1981): Reduction of intraocular pressure in normal and glaucomatous primate (Aotus trivirgatus) eyes by topically applied prostaglandin F2 alpha. Curr Eye Res 1:205-209.

Camras CB, Bito LZ and Eakins KE (1977): Reduction of intraocular pressure by prostaglandins applied topically to the eyes of conscious rabbits. Invest Ophthalmol Vis Sci 16:1125-1134.

Camras CB, Schumer RA and Marsk A (1992): Intraocular pressure reduction with PhXA34, a new prostaglandin analogue, in patients with ocular hypertension. Arch Ophthalmol 110:1733-1738.

Camras CB, Siebold EC and Lustgarten JS (1989): Maintained reduction of intraocular pressure by prostaglandin F2 alpha-1-isopropyl ester applied in multiple doses in ocular hypertensive and glaucoma patients. Ophthalmology 96:1329-36; discussion 1336-1337.

Cao W, Drumheller A and Zaharia M (1993): Effects of calcitonin gene-related peptide on the rabbit electroretinogram. Neuropeptides 24:151-157.

Carstairs JR (1987): Distribution of calcitonin gene-related peptide receptors in the lung. Eur J Pharmacol 140:357-358.

Chai SY, Christopoulos G, Cooper ME and Sexton PM (1997). Characterization of binding sites for amylin, calcitonin, and CGRP in primate kidney. Am J Physiol 274:F51-F62.

Champion HC, Pierce RL and Bivalacqua TJ (2001): Analysis of responses to hAmylin, hCGRP, and hADM in isolated resistance arteries from the mesenteric vascular bed of the rat. Peptides 22:1427-1434.

Chatterjee TK and Fisher RA (1991): Multiple affinity forms of the calcitonin gene-related peptide receptor in rat cerebellum. Mol Pharmacol 39:798-804.

Chatterjee TK and Fisher RA (1995): Multiple affinity and guanine nucleotide sensitive forms of the calcitonin gene related peptide (CGRP) receptor. Can J Physiol Pharmacol 73:968-973.

Chen W, Andom T and Bhattacherjee P (1997a): Intracellular calcium mobilization following prostaglandin receptor activation in human ciliary muscle cells. Curr Eye Res 16:847-853.

Chen WJ, Armour S and Way J (1997b): Expression cloning and receptor pharmacology of human calcitonin receptors from MCF-7 cells and their relationship to amylin receptors. Mol Pharmacol 52:1164-1175.

Chiba T, Yamaguchi A and Yamatani T (1989): Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). Am J Physiol 256:E331-E335.

Christopoulos G, Perry KJ and Morfis M (1999): Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol 56:235-242.

Cioffi G, Granstam E and Alm A (2003): Ocular circulation. In: Adler's physiology of the eye, pp. 747-784. Eds. P Kaufman and A Alm, Mosby, Inc., St. Louis, Missouri.

Cole DF and Unger WG (1973): Prostaglandins as mediators for the responses of the eye to trauma. Exp Eye Res 17:357-368.

Collyear K, Girgis SI and Saunders G (1991): Predicted structure of the bovine calcitonin gene-related peptide and the carboxy-terminal flanking peptide of bovine calcitonin precursor. J Mol Endocrinol 6:147-152.

Cooper GJ, Leighton B and Dimitriadis GD (1988): Amylin found in amyloid deposits in human type 2 diabetes mellitus may be a hormone that regulates glycogen metabolism in skeletal muscle. Proc Natl Acad Sci U S A 85:7763-7766.

Crawford K and Kaufman PL (1987): Pilocarpine antagonizes prostaglandin F2 alpha-induced ocular hypotension in monkeys. Evidence for enhancement of uveoscleral outflow by prostaglandin F2 alpha. Arch Ophthalmol 105:1112-1116.

Crook RB and Yabu JM (1992): Calcitonin gene-related peptide stimulates intracellular cAMP via a protein kinase C-controlled mechanism in human ocular ciliary epithelial cells. Biochem Biophys Res Commun 188:662-670.

Crossman D, McEwan J and MacDermot J (1987): Human calcitonin gene-related peptide activates adenylate cyclase and releases prostacyclin from human umbilical vein endothelial cells. Br J Pharmacol 92:695-701.

Dennis T, Fournier A and Cadieux A (1990): hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. J Pharmacol Exp Ther 254:123-128.

Dennis T, Fournier A, St Pierre S and Quirion R (1989): Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J Pharmacol Exp Ther 251:718-725.

Drissi H, Lieberherr M and Hott M (1999): Calcitonin gene-related peptide (CGRP) increases intracellular free Ca2+ concentrations but not cyclic AMP formation in CGRP receptor-positive osteosarcoma cells (OHS-4). Cytokine 11:200-207.

Eakins KE (1977): Prostaglandin and non-prostaglandin mediated breeakdown of the blood-aqueous barrier. Exp Eye Res 25:483-498.

Eguchi S, Hirata Y, Iwasaki H, Sato K, Watanabe TX, Inui T, Nakajima K, Sakakibara S and Marumo F (1994): Structure-activity relationship of adrenomedullin, a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. Endocrinology 135:2454-2458.

Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR and Dickerson IM (2000): CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. J Biol Chem 275:31438-31443.

Fechtner RD and Weinreb RN (1994): Mechanisms of optic nerve damage in primary open angle glaucoma. Surv Ophthalmol 39:23-42.

Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW and Davidge ST (2000): Vascular matrix metalloproteinase-2-dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. Circ Res 87:670-676.

Ferrier GJ, Pierson AM and Jones PM (1989): Expression of the rat amylin (IAPP/DAP) gene. J Mol Endocrinol 3:R1-R4.

Flammer J, Orgul S and Costa VP (2002): The impact of ocular blood flow in glaucoma. Prog Retin Eye Res 21:359-393.

Forsius H (1988): Exfoliation syndrome in various ethnic populations. Acta Ophthalmol Suppl 184:71-85.

Foulkes R, Shaw N and Bose C (1991): Differential vasodilator profile of calcitonin gene-related peptide in porcine large and small diameter coronary artery rings. Eur J Pharmacol 201:143-149.

Gabelt BT and Kaufman PL (1989): Prostaglandin F2 alpha increases uveoscleral outflow in the cynomolgus monkey. Exp Eye Res 49:389-402.

Gabelt B and Kaufman P (2003): Aqueous humor hydrodynamics. In: Adler's physiology of the eye, pp. 237-289. Eds. P Kaufman and A Alm, Mosby, Inc., St. Louis, Missouri.

Galeazza MT, O'Brien TD and Johnson KH (1991): Islet amyloid polypeptide (IAPP) competes for two binding sites of CGRP. Peptides 12:585-591.

Gallar J, Pozo MA and Rebollo I (1990): Effects of capsaicin on corneal wound healing. Invest Ophthalmol Vis Sci 31:1968-1974.

Garcia-Valenzuela E, Shareef S and Walsh J (1995): Programmed cell death of retinal ganglion cells during experimental glaucoma. Exp Eye Res 61:33-44.

Gibson SJ, Polak JM and Bloom SR (1984): Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. J Neurosci 4:3101-3111.

Giuffre G (1985): The effects of prostaglandin F2 alpha in the human eye. Graefes Arch Clin Exp Ophthalmol 222:139-141.

Giuliani S, Wimalawansa SJ and Maggi CA (1992): Involvement of multiple receptors in the biological effects of calcitonin gene-related peptide and amylin in rat and guinea-pig preparations. Br J Pharmacol 107:510-514.

Goh Y, Araie M and Nakajima M (1989): Effect of topical prostaglandin D2 on the aqueous humor dynamics in rabbits. Graefes Arch Clin Exp Ophthalmol 227:476-481.

Gray DW and Marshall I (1992a): Human alpha-calcitonin gene-related peptide stimulates adenylate cyclase and guanylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide. Br J Pharmacol 107:691-696.

Gray DW and Marshall I (1992b): Nitric oxide synthesis inhibitors attenuate calcitonin gene-related peptide endothelium-dependent vasorelaxation in rat aorta. Eur J Pharmacol 212:37-42.

Grunditz T, Ekman R and Håkanson R (1986): Calcitonin gene-related peptide in thyroid nerve fibers and C cells: effects on thyroid hormone secretion and response to hypercalcemia. Endocrinology 119:2313-2324.

Hammond BR and Bhattacherjee P (1984): Calibration of the Alcon applanation pneumatonograph and Perkins tonometer for use in rabbits and cats. Curr Eye Res 3:1155-1158.

Harti G, Sharkey KA and Pierau FK (1989): Effects of capsaicin in rat and pigeon on peripheral nerves containing substance P and calcitonin gene-related peptide. Cell Tissue Res 256:465-474.

Haruno I, Yoshitomi T and Harada Y (1996): Calcitonin gene-related peptide induced relaxation of the rabbit iris dilator muscle. Curr Eye Res 15:105-110.

Hayreh S (1962): The ophthalmic artery. III. Branches. Brit J Ophthal 46:212-247.

Houslay MD, Morris NJ and Savage A (1994): Regulation of hepatocyte adenylate cyclase by amylin and CGRP: a single receptor displaying apparent negative cooperatively towards CGRP and simple saturation kinetics for amylin, a requirement for phosphodiesterase inhibition to observe elevated hepatocyte cyclic AMP levels and the phosphorylation of Gi-2. J Cell Biochem 55:66-82.

Huang Y, Fischer JE and Balasubramaniam A (1996): Amylin mobilizes [Ca2+]i and stimulates the release of pancreatic digestive enzymes from rat acinar AR42J cells: evidence for an exclusive receptor system of amylin. Peptides 17:497-502.

Hughes JJ, Levine AS and Morley JE (1984): Intraventricular calcitonin gene-related peptide inhibits gastric acid secretion. Peptides 5:665-667.

Inagaki S, Kito S and Kubota Y (1986): Autoradiographic localization of calcitonin gene-related peptide binding sites in human and rat brains. Brain Res 374:287-298.

Johnsen H, Ringvold A and Blika S (1985): Ascorbic acid determination in serum and aqueous humour by high-performance liquid chromatography. Acta Ophthalmol 63:31-34.

Johnson KH, O'Brien TD and Hayden DW (1988): Immunolocalization of islet amyloid polypeptide (IAPP) in pancreatic beta cells by means of peroxidase-antiperoxidase (PAP) and protein A-gold techniques. Am J Pathol 130:1-8.

Juaneda C, Dumont Y and Quirion R (2000): The molecular pharmacology of CGRP and related peptide receptor subtypes. Trends Pharmacol Sci 21:432-438.

Kahn SE, D'Alessio DA and Schwartz MW (1990): Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. Diabetes 39:634-638.

Kass MA, Podos SM and Moses RA (1972): Prostaglandin E 1 and aqueous humor dynamics. Invest Ophthalmol 11:1022-1027.

Kaufman PL (1986): Effects of intracamerally infused prostaglandins on outflow facility in cynomolgus monkey eyes with intact or retrodisplaced ciliary muscle. Exp Eye Res 43:819-827.

Kawarai M and Koss MC (1998): Sympathetic vasoconstriction in the rat anterior choroid is mediated by alpha1-adrenoceptors. Eur J Pharmacol 363:35-40.

Kennedy I, Coleman RA and Humphrey PP (1982): Studies on the characterisation of prostanoid receptors: a proposed classification. Prostaglandins 24:667-689.

Kerrigan LA, Zack DJ and Quigley HA (1997): TUNEL-positive ganglion cells in human primary open-angle glaucoma. Arch Ophthalmol 115:1031-1035.

Kinsey VE and Reddy DVN (1964): Chemistry and dynamics of aqueous humor. In: The rabbit in eye research, pp. 215-316. Ed. JH Prince, Springfield Ill. Publisher Charles C Thomas.

Kiyama H, Katayama Y and Hillyard CJ (1985): Occurrence of calcitonin gene-related peptide in the chicken amacrine cells. Brain Res 327:367-369.

Kobayashi H, Hashimoto K and Uchida S (1987): Calcitonin gene related peptide stimulates adenylate cyclase activity in rat striated muscle. Experientia 43:314-316.

Koss MC and Gherezghiher T (1993): Adrenoceptor subtypes involved in neurally evoked sympathetic vasoconstriction in the anterior choroid of cats. Exp Eye Res 57:441-447.

Krootila K (1988): CGRP in relation to neurogenic inflammation and cAMP in the rabbit eye. Exp Eye Res 47:307-316.

Krootila K, Uusitalo H and Palkama A (1988): Effect of neurogenic irritation and calcitonin gene-related peptide (CGRP) on ocular blood flow in the rabbit. Curr Eye Res 7:695-703.

Krootila K, Uusitalo H and Palkama A (1991): Intraocular and cardiovascular effects of calcitonin gene-related peptide (CGRP)-I and -II in the rabbit. Invest Ophthalmol Vis Sci 32:3084-3090.

Kuwayama Y and Stone RA (1987): Distinct substance P and calcitonin gene-related peptide immunoreactive nerves in the guinea pig eye. Invest Ophthalmol Vis Sci 28:1947-1954.

Lam KW and Lee PF (1975): Analysis of ascorbate concentration in the aqueous humor by high-pressure liquid chromatography. Invest Ophthalmol 14:947-950.

Le Greves P, Nyberg F, Hokfelt T and Terenius L (1989): Calcitonin gene-related peptide is metabolized by an endopeptidase hydrolyzing substance P. Regul Pept 25:277-286.

Lee BL and Wilson MR (2003): Ocular Hypertension Treatment Study (OHTS) commentary. Curr Opin Ophthalmol 14:74-77.

Leighton B and Cooper GJ (1988): Pancreatic amylin and calcitonin gene-related peptide cause resistance to insulin in skeletal muscle in vitro. Nature 335:632-635.

Leske MC, Heijl A and Hussein M (2003): Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. Arch Ophthalmol 121:48-56.

Lindsey JD, Kashiwagi K and Boyle D (1996): Prostaglandins increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. Curr Eye Res 15:869-875.

Lou H, Cote GJ and Gagel RF (1994): The calcitonin exon and its flanking intronic sequences are sufficient for the regulation of human calcitonin/calcitonin gene-related peptide alternative RNA splicing. Mol Endocrinol 8:1618-1626.

Lowry O, Rosebrough N and Farr A (1951): Protein measurement with the folin phenol reagent. J Biol Chem 193:256-275.

Luebke AE, Dahl GP and Roos BA (1996): Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay. Proc Natl Acad Sci U S A 93:3455-3460.

Luhtala J, Palkama A and Uusitalo H (1991): Calcitonin gene-related peptide immunoreactive nerve fibers in the rat conjunctiva. Invest Ophthalmol Vis Sci 32:640-645.

Lukinius A, Wilander E and Westermark GT (1989): Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets. Diabetologia 32:240-244.

Lütjen-Drecoll E and Tamm E (1988): Morphological study of the anterior segment of cynomolgus monkey eyes following treatment with prostaglandin F2 alpha. Exp Eye Res 47:761-769.

Lütjen-Drecoll E and Tamm E (1989): The effects of ocular hypotensive doses of PGF2 alpha-isopropylester on anterior segment morphology. Prog Clin Biol Res 312:437-446.

Lynch B and Kaiser ET (1988): Biological properties of two models of calcitonin gene related peptide with idealized amphiphilic alpha-helices of different lengths. Biochemistry 27:7600-7607.

Maggi CA, Rovero P and Giuliani S (1990): Biological activity of N-terminal fragments of calcitonin gene-related peptide. Eur J Pharmacol 179:217-219.

Malminiemi OI and Malminiemi KH (1992): [125I]calcitonin gene-related peptide binding in membranes of the ciliary body-iris block. Curr Eye Res 11:1079-1085.

McLatchie LM, Fraser NJ and Main MJ (1998): RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393:333-339.

McPhearson GA (1985): A collection of radioligand binding analysis program. In: Kinetic, EBDA, Ligand, Lowry, pp. 1-127. Eds., Elsevier Science Publishers BV, Amsterdam.

Miyoshi H and Nakaya Y (1995): Calcitonin gene-related peptide activates the K+ channels of vascular smooth muscle cells via adenylate cyclase. Basic Res Cardiol 90:332-336.

Mosselman S, Hoppener JW and Zandberg J (1988): Islet amyloid polypeptide: identification and chromosomal localization of the human gene. FEBS Lett 239:227-232.

Muff R, Buhlmann N and Fischer JA (1999): An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology 140:2924-2927.

Mukhopadhyay P, Bian L and Yin H (2001): Localization of EP(1) and FP receptors in human ocular tissues by in situ hybridization. Invest Ophthalmol Vis Sci 42:424-428.

Mukhopadhyay P, Geoghegan TE and Patil RV (1997): Detection of EP2, EP4, and FP receptors in human ciliary epithelial and ciliary muscle cells. Biochem Pharmacol 53:1249-1255.

Mulder H, Leckstrom A and Uddman R (1995): Islet amyloid polypeptide (amylin) is expressed in sensory neurons. J Neurosci 15:7625-7632.

Mulderry PK, Ghatei MA and Spokes RA (1988): Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience 25:195-205.

Neufeld AH, Jampol LM and Sears ML (1972): Aspirin prevents the disruption of the blood-aqueous barrier in the rabbit eye. Nature 238:158-159.

Nilsson SF, Linder J and Bill A (1985): Characteristics of uveal vasodilation produced by facial nerve stimulation in monkeys, cats and rabbits. Exp Eye Res 40:841-852.

Nilsson SFE, Samuelsson M, Bill A and Stjernschantz J (1989): Increased uveoscleral outflow as a possible mechanism of ocular hypotension caused by prostaglandin $F_{2\alpha}$-isopropyl ester in the cynomolgus monkey. Exp Eye Res 48:707-716.

Ocklind A (1998): Effect of latanoprost on the extracellular matrix of the ciliary muscle. A study on cultured cells and tissue sections. Exp Eye Res 67:179-191.

Ocklind A, Lake S, Wentzel P, Nister M and Stjernschantz J (1996). Localization of the prostaglandin $F_{2\alpha}$ receptor messenger RNA and proteins in the cynomolgus monkey eye. Invest Ophthalmol Vis Sci 37:716-726.

Okisaka S, Murakami A and Mizukawa A (1997): Apoptosis in retinal ganglion cell decrease in human glaucomatous eyes. Jpn J Ophthalmol 41:84-88.

Oksala O (1988): Effects of calcitonin gene-related peptide and substance P on regional blood flow in the cat eye. Exp Eye Res 47:283-289.

Oksala O and Stjernschantz J (1988a): Effects of calcitonin gene-related peptide in the eye. A study in rabbits and cats. Invest Ophthalmol Vis Sci 29:1006-1011.

Oksala O and Stjernschantz J (1988b): Increase in outflow facility of aqueous humor in cats induced by calcitonin gene-related peptide. Exp Eye Res 47:787-790.

Oksala O, Heino P and Uusitalo H (1998): Effect of intracameral and intravitreal injection of calcitonin gene-related peptide on the intraocular pressure and outflow facility of aqueous humor in the rabbit. Exp Eye Res 67:411-415.

Ono K, Delay M and Nakajima T (1989): Calcitonin gene-related peptide regulates calcium current in heart muscle. Nature 340:721-724.

Osborne NN and Barnett NL (1991): Calcitonin gene-related polypeptide stimulates c-AMP production in the iris/ciliary body complex. Exp Eye Res 53:131-133.

Palacios JM and Dietl MM (1987): Regulatory peptide receptors: visualization by autoradiography. Experientia 43:750-761.

Pittner R, Beaumont K and Young A (1995): Dose-dependent elevation of cyclic AMP, activation of glycogen phosphorylase, and release of lactate by amylin in rat skeletal muscle. Biochim Biophys Acta 1267:75-82.

Poyer JF, Gabelt B and Kaufman PL (1992): The effect of topical PGF2 alpha on uveoscleral outflow and outflow facility in the rabbit eye. Exp Eye Res 54:277-283.

Poyner DR, Sexton PM and Marshall I (2002): International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54:233-246.

Prado MA, Evans-Bain B and Dickerson IM (2002): Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction. Biochem Soc Trans:30:460-464.

Quayle JM, Bonev AD and Brayden JE (1994): Calcitonin gene-related peptide activated ATP-sensitive K+ currents in rabbit arterial smooth muscle via protein kinase A. J Physiol 475:9-13.

Quigley HA, Nickells RW and Kerrigan LA (1995): Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. Invest Ophthalmol Vis Sci 36:774-786.

Raddino R, Pela G and Manca C (1997): Mechanism of action of human calcitonin gene-related peptide in rabbit heart and in human mammary arteries. J Cardiovasc Pharmacol 29:463-470.

Riediger T, Schmid HA and Lutz T (2001): Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP. Am J Physiol Regul Integr Comp Physiol 281:R1833-1843.

Rosenblatt MI, Dahl GP and Dickerson IM (2000): Characterization and localization of the rabbit ocular calcitonin gene-related peptide (CGRP)-receptor component protein (RCP). Invest Ophthalmol Vis Sci 41:1159-1167.

Rosenfeld MG, Amara SG and Evans RM (1984): Alternative RNA processing: determining neuronal phenotype. Science 225:1315-1320.

Rosenfeld MG, Mermod JJ and Amara SG (1983): Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 304:129-135.

Sagara T, Gaton DD and Lindsey JD (1999): Topical prostaglandin F2alpha treatment reduces collagen types I, III, and IV in the monkey uveoscleral outflow pathway. Arch Ophthalmol 117:794-801.

Salomon Y (1979): Adenylate cyclase assay. Adv Cyclic Nucleotide Res 10:35-55.

Schlotzer-Schrehardt U, Zenkel M and Nusing RM (2002): Expression and localization of FP and EP prostanoid receptor subtypes in human ocular tissues. Invest Ophthalmol Vis Sci 43:1475-1487.

Segre GV and Goldring SR (1993): Receptors for secretin calcitonin. parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G protein linked receptor family. Trends Endocrinol Metab 4:309-314.

Sexton PM, McKenzie JS and Mendelsohn AO (1988): Evidence for a new subclass of calcitonin/calcitonin gene-related peptide binding site in rat brain. Neurochem Int 12:323-335.

Skofitsch G and Jacobowitz DM (1985): Autoradiographic distribution of 125I calcitonin gene-related peptide binding sites in the rat central nervous system. Peptides 6:975-986.

Sperber GO and Bill A (1984): A method for near-continuous determination of aqueous humor flow; effects of anaesthetics, temperature and indomethacin. Exp Eye Res 39:435-453.

Starr MS (1971): Further studies on the effect of prostaglandin on intraocular pressure in the rabbit. Exp Eye Res 11:170-177.

Steenbergh PH, Hoppener JW and Zandberg J (1985): A second human calcitonin/CGRP gene. FEBS Lett 183:403-407.

Stjernschantz J and Alm A (1996): Latanoprost as a new horizon in the medical management of glaucoma. Curr Opin Ophthalmol 7:11-17.

Stjernschantz J and Bill A (1979): Effect of intracranial stimulation of the oculomotor nerve on ocular blood flow in the monkey, cat, and rabbit. Invest Ophthalmol Vis Sci 18:99-103.

Stjernschantz J and Bill A (1980): Vasomotor effects of facial nerve stimulation: Noncholinergic vasodilatation in the eye. Acta Physiol Scand 109:45-50.

Stjernschantz J and Resul B (1992): Phenyl substituted prostaglandin analogues for glaucoma treatment. Drugs Future 17:691-704.

Stjernschantz J, Uusitalo R and Palkama A (1973): The aqueous proteins of the rat in normal eye and after aqueous withdrawal. Exp Eye Res 16:215-221.

Stone RA and McGlinn AM (1988): Calcitonin gene-related peptide immunoreactive nerves in human and rhesus monkey eyes. Invest Ophthalmol Vis Sci 29:305-310.

Tam EK and Caughey GH (1990): Degradation of airway neuropeptides by human lung tryptase. Am J Respir Cell Mol Biol 3:27-32.

Taniguchi T, Nakai Y and Karim Z (1999): Biphasic intraocular pressure response to calcitonin gene-related peptide. Curr Eye Res 19:432-438.

Terenghi G, Polak JM and Ghatei MA (1985): Distribution and origin of calcitonin gene-related peptide (CGRP) immunoreactivity in the sensory innervation of the mammalian eye. J Comp

Neurol 233:506-516.

Tilakaratne N, Christopoulos G and Zumpe ET (2000): Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. J Pharmacol Exp Ther 294:61-72.

Toris CB, Camras CB and Yablonski ME (1993): Effects of PhXA41, a new prostaglandin F2 alpha analog, on aqueous humor dynamics in human eyes. Ophthalmology 100:1297-1304.

Toris CB, Yablonski ME and Wang YL (1999): Aqueous humor dynamics in the aging human eye. Am J Ophthalmol 127:407-412.

Unger WG, Terenghi G and Ghatei MA (1985): Calcitonin gene-related polypeptide as a mediator of the neurogenic ocular injury response. J Ocul Pharmacol 1:189-199.

Uusitalo H, Krootila K and Palkama A (1989): Calcitonin gene-related peptide (CGRP) immunoreactive sensory nerves in the human and guinea pig uvea and cornea. Exp Eye Res 48:467-475.

Uusitalo R, Palkama A and Stjernschantz J (1973): An electron microscopical study of the blood-aqueous barrier in the ciliary body and iris of the rabbit. Exp Eye Res 17:49-63.

van Rossum D, Menard DP and Quirion R (1993): Effect of guanine nucleotides and temperature on calcitonin gene-related peptide receptor binding sites in brain and peripheral tissues. Brain Res 617:249-257.

Villumsen J and Alm A (1992): PhXA34--a prostaglandin F2 alpha analogue. Effect on intraocular pressure in patients with ocular hypertension. Br J Ophthalmol 76:214-217.

Wahlestedt C, Beding B and Ekman R (1986): Calcitonin gene-related peptide in the eye: release by sensory nerve stimulation and effects associated with neurogenic inflammation. Regul Pept 16:107-115.

Waitzman MB and King CD (1967): Prostaglandin influences on intraocular pressure and pupil size. Am J Physiol 212:329-334.

Walls AF, Brain SD, Desai A, Jose PJ, Hawkings E, Church MK and Williams TJ (1992): Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide. Biochemical Pharmacology 43:1243-1248.

Weinreb RN, Kashiwagi K and Kashiwagi F (1997): Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells. Invest Ophthalmol Vis Sci 38:2772-2780.

Wellman GC, Quayle JM and Standen NB (1998): ATP-sensitive K+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. J Physiol 507:117-129.

Wentzel P, Bergh K, Wallin Ö, Niemelä P and Stjernschantz J (2003): Transcription of prostanoid receptor genes and cyclooxygenase enzyme genes in cultivated human iridial melanocytes from eyes of different colours. Pigment Cell Res 16:43-49.

Westermark P, Wernstedt C and Wilander E (1987): Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. Proc Natl Acad Sci U S A 84:3881-3885.

Westfall TC and Curfman-Falvey M (1995): Amylin-induced relaxation of the perfused mesenteric arterial bed: meditation by calcitonin gene-related peptide receptors. J Cardiovasc Pharmacol 26:932-936.

Wimalawansa SJ and MacIntyre I (1988): Calcitonin gene-related peptide and its specific binding sites in the cardiovascular system of rat. Int J Cardiol 20:29-37.

Yablonski ME, Zimmerman TJ and Waltman SR (1978): A fluorophotometric study of the effect of topical timolol on aqueous humor dynamics. Exp Eye Res 27:135-142.

Ye XD, Laties AM and Stone RA (1990): Peptidergic innervation of the retinal vasculature and optic nerve head. Invest Ophthalmol Vis Sci 31:1731-1737.

Yoshizaki H, Takamiya M and Okada T (1987): Characterization of picomolar affinity binding sites for [125I]-human calcitonin gene-related peptide in rat brain and heart.Biochem Biophys Res Commun 146:443-451.

Young AA, Gedulin BR and Rink TJ (1996): Dose-responses for the slowing of gastric emptying in a rodent model by glucagon-like peptide (7-36) NH2, amylin, cholecystokinin, and other possible regulators of nutrient uptake. Metabolism 45:1-3.

Young AA, Gedulin B and Vine W (1995): Gastric emptying is accelerated in diabetic BB rats and is slowed by subcutaneous injections of amylin. Diabetologia 38:642-648.

Young AA, Rink TJ and Wang MW (1993): Dose response characteristics for the hyperglycemic, hyperlactemic, hypotensive and hypocalcemic actions of amylin and calcitonin gene-related peptide-I (CGRP alpha) in the fasted, anaesthetized rat. Life Sci 52:1717-1726.

Yousufzai SY and Abdel-Latif AA (1998): Calcitonin gene-related peptide relaxes rabbit iris dilator smooth muscle via cyclic AMP-dependent mechanisms: cross-talk between the sensory and sympathetic nervous systems. Curr Eye Res 17:197-204.

Zimmerman TJ, Harbin R and Pett M (1977): Timolol and facility of outflow. Invest Ophthalmol Vis Sci 16:623-624.

Zhu H, Hasman RA, Young KM, Kedersha NL and Lou H (2003): U1 snRNP-dependent function of TIAR in the regulation of alternative RNA processing of the human calcitonin/CGRP pre-mRNA. Mol Cell Biol 23:5959-5971.

Zschauer A, Uusitalo H and Brayden JE (1992): Role of endothelium and hyperpolarization in CGRP-induced vasodilation of rabbit ophthalmic artery. Am J Physiol 263:H359-H365.

ORIGINAL PUBLICATIONS

Permission from the Publisher:

Swets & Zeitlinger Publishers (I)
Mary Ann Liebert, Inc. Publishers (II, III)
Elsevier (IV)
S. Karger AG, Basel (V)