Experimental investigation of the ophthalmological effects of the pituitary adenylate cyclase activating polypeptide (PACAP)

Ph.D. thesis

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I. Introduction

PACAP (pituitary adenylate cyclase activating polypeptide)

Pituitary adenylate cyclase activating polypeptide was isolated from ovine hypothalami in 1989 based on its potential to increase adenylate cyclase activity in the pituitary gland. PACAP is a member of the secretin/glucagon/VIP family, and has 67% sequence similarity with VIP. It occurs in two amidated forms, with 38 and 27 amino acid residues. The primary structure of PACAP-38 is identical among all mammalian species examined, and it also shows marked similarity with lower vertebrates and invertebrates, with differences in only 1-4 amino acids. This suggest that the structure of PACAP has remained very conserved throughout evolution and it may reflect its importance in fundamental functions in the nervous system. PACAP is localized not only in the central but in the peripheral nervous system and in non-neural tissues as well, such as in the endocrine glands, cardiovascular, urogenital, respiratoric and gastrointestinal tracts.

The biological actions of PACAP

PACAP is a pleitropic peptide, with various functions in the nervous system and peripheral organs. Since its discovery, it became evident that it is more than „just" a hypothalamo-hypophyseal regulator. In the nervous system, it has important roles in the development, synaptic plasticity, neuronal excitability, it influences various behavioral symptoms and stimualtes cognitive functions. Its neuroprotective effects have been proven in numerous cell cultures and in vivo systems. In vitro, it protects cells against various toxic agents, like glutamate, ethanol, oxidative stress, ceramide, hypoxia. In vivo, it protects in animal models of traumatic brain injury, Parkinson`s disease and stroke. Its endocrine effects are also well-known: it plays a central regulatory role in hypothalamic-hypophyseal hormone secretion, and functions of the adrenal gland and gonads. In additions, PACAP plays a role in circadian rhythmic functions, in smooth muscle contraction, secretion of exocrine glans, influences central and peripheral pain responses and has general anti-inflammatory effects.

PACAP receptors

The receptors for PACAP are G protein-coupled receptors and can be divided into two main groups: PAC1 receptor, which binds PACAP with higher affinity than VIP, and VPAC receptors (VPAC1 and VPAC2), wich bind PACAP and VIP with similar affinities.

PACAP and its receptors occur in various tissues of the eye. PACAP immunoreactivity has been found in the lacrimal gland, choroid, iris, ciliary body, conjuntiva, sclera, cornea. PACAP is present in the trigeminal, sphenopalatine and ciliary ganglia.

PACAP in the eye

Numerous studies have described the presence of PACAP and its receptors in the whole retina and in the different layers. PACAP immunpositive fibers can be observed in the plexiform layers, while immunopositive cell bodies are displayed in the ganglion cell layer and inner nuclear layer. PACAP immunoreactivity displays circadian alteration in the chicken retina. In mammals, the retinohypothalamic tract originates from a subset of retinal ganglion cells, and it mainly synapses in the suprachiasmatic nucleus of the hypothalamus, which is the biological clock of mammalian species. A subset of these ganglion cells is intrinsically photosensitive due to the expression of melanopsin. Melanopsin is exclusively expressed in PACAP-containing cells. The PACAP expression of ganglion cells is under the control of dopamine. PACAP immunoreactivity appears at early stages of retinal development. PACAP can be detected in the inner nuclear layer of embryonic chicken retinas from embryonic day 8 (E8). In the rat, PACAP mRNA appears in the ganglion cell layer at E20.

The selective PACAP receptors are responsible for approximately 80% of PACAP binding in the retina. Radioligand binding studies have revealed the existence of PACAP receptors also in the human fetal retina, which has been confirned by the detection of PACAP receptor mRNA in RT-PCR experiments. Retinoblastoma cells also contain PACAP receptors. PAC1 receptor mRNA and protein expression have been described in all layers of neonatal rat retina. Similarly, PAC1 receptor and its mRNA can be detected in chicken retinas already at E6. The mRNA for all PACAP receptors has been reported in the retinal pigment epithelium.

Strong expression of PAC1 receptor mRNA is in the ganglion cell layer, inner nuclear layer and nerve fiber layer, while weaker expression is in the inner- and outer plexiform, the outer nuclear layers and the outer segments of the photoreceptors. Other studies have confirmed the presence of VPAC receptors in the retina. In culture, PAC1 receptor expression has been shown in the Muller cells. PACAP occurs not only in the retina, but also in the iris, ciliary body and conjuctiva. It is known that PACAP acts on vasodilation, smooth muscle contraction, inflammatory reactions and cAMP production in the eye. It is also expressed in the trigeminal, ciliary and sphenopalatine ganglia, through which the secretion of the glands can be influenced by PACAP.

The aims of the thesis are: (1) to investigate the effects of PACAP on the protein composition of the tear and whether PACAP occurs in the tear and in the aqeous humor; (2) to investigate the effects of PACAP on corneal regeneration; and (3) to investigate whether PACAP has protective effects in UV light-induced retinal degeneration.

I.1. The regulation of the lacrimal gland is complex, influenced both by hormonal and neuronal (sympathetic and parasympathetic) effects. Tear production is increased upon mechanical or chemical irritation, or strong light. Emotions can also evoke increased tear production involving higher cortical centers. Stimuli are conveyed from the oculomotor nucleus, ciliary ganglion through the lacrimal branch of the trigeminal nerve (parasympathetic) or from the superior cervical ganglion (sympathetic). The ratio of the water phase increases with higher levels of sodium and lower levels of potassium in the above-described reflectory tear production. Other components can also be changed depending on the provoking stimuli. Certain proteins remain the same, while others change, like the antibacterial lyzozyme and lactoferrin. PACAP immunoreactive fibers have already been shown in the lacrimal gland, originating from the sphenopalatine ganglion, where 10% of the neurons contain PACAP. The effects of PACAP in the salivary and other exocrine glands make it probable that PACAP exerts comparable effects in the morphologically similar lacrimal gland. For example, PACAP increases saliva production, with increased protein secretion, while it inhibits Ca^{2+} channels. Substances increasing cAMP or cGMP levels, influence lacrimal production. Therefore, it is hypothesized that PACAP affects the functioning of the lacrimal gland.

The aim of the first experimental part was to investigate whether systemic PACAP administration influences the protein composition of rat tear and whether PACAP occurs in the tear. For comparison, we also investigated the presence of PACAP in the aqeous humor.

I.2. The sensitivity of the cornea plays a very important role in the protection of the eye bulb. It isd associated with its rich sensory innervation, reacting to different stimuli in varying pattern. The corneal epithelium is constantly exposed to physical, chemical and biological stimuli. It reacts to injuries with fast recovery. In the area of injury, keratocytes suffer apoptosis, but new cell proliferation starts from the periphery. The balance between apoptotic and proliferation pathways is very important in reepithelization. Several growth factors, transcription factors and cytokines play a role in these processes. Inappropriate corneal healing can be observed in several diseases, such as diabetes, contact lense wearing and refractive eye surgery complications.

PACAP is a trophic factor during the development of the nervous system, while it plays a role during regeneration. The corneal effects of PACAP are less known, in spite of the fact that PACAP and its receptor have already been shown in the cornea. In a previous study, PACAP27 was given in form of eye drops, and enhanced nerve growth was observed after corneal injury in rabbits. Although this study focused only on neuronal regeneration, it drew the attention to the possibility of giving PACAP in form of eye drops. We hypothesize that PACAP can facilitate corneal wound healing. The aim of the second part of the study was to investigate the effects of PACAP on corneal reepithelization and in addition, the expression of two protective molecules (Akt and ERK1/2).

I.3. The retina is an extended part of the central nervous system. In our experiments, we used rat retina, which is similar to the human retina in its vascularization and in the organization of the layers (3 main nuclear and 2 fibrous layers). The first report on the retinoprotective effects of PACAP was a study showing that PACAP protected cultured retinal neurons against glutamate toxicity. Elevated glutamate concentrations lead to excitotoxic cell death in the nervous system, including apoptotic cell death in the retina. 10nmol/L-1 µmol/L PACAP27 and -38 attenuated the 1 mmol/L glutamate-induced cell death in a dose-dependent manner. This was antagonized by cotreatment with the PACAP antagonist PACAP6-38, and the PKA inhibitor H-89. Anisomycin induces cell death within the neuroblastic layer of retinal explants from newborn rats. A dose-dependent prevention of cell death was found by PACAP38 treatment.

Glutamate, as the main excitatory transmitter in the central nervous system, is able to exert toxic effects when present at high concentrations. The excitotoxic injury of the retina is a major factor in several retinal diseases, such as glaucoma and ischemic retinopathy. Monosodium glutamate (MSG), when given systemically to newborn rats, passes the blood-retina barrier and leads to severe retinal degeneration. The internal to outer limiting membrane distance is approximately half of the normal retinas, mesaured 3 weeks after birth. With the almost entire disappearance of the IPL, the fusion of the INL and GCL can be observed. The number of cells/100 µm in the GCL is also approximately half of normal retinas, as measured in light microscopical sections from central retina areas of the same eccentricities, where GCL appears in one row.

Although PACAP has been shown to cross the blood-brain barrier, systemic PACAP treatment leads only to a slight amelioration of the retinal morphology following MSG-induced degeneration. However, local PACAP38 treatment, reached by intravitreal injections of 1-100 pmol PACAP, results in a significant attenuation of this degeneration. While 1 pmol

PACAP leads to a slight improvement, treatment with 100 pmol PACAP results in an almost intact appearance of the retina. Similar protection can be achieved by PACAP27 treatment. The PACAP antagonists, PACAP6-38 and PACAP6-27 leads to a further aggravation of the MSG-induced degeneration, indicating that endogenous PACAP plays an important protective role in the natural defense of the retina against damage. PACAP is protective against excitotoxic retinal lesion both in newborn and in adult rats. PACAP has also been shown to be protective in several other models of retinal injuries. It has protective effects in retinal ischemia, induced by high intraocular pressure or bilateral carotid artery occlusion. Similarly, PACAP protects against optic nerve transection, kainic acid-induced degeneration and diabetic retinopathy. Given that the retinoprotective effects of PACAP are well established, we tested whether it could protect against UV light-induced retinal degeneration.

Global atmospheric changes may increase the level of ultraviolet radiation on earth. This would contribute to enhanced chronic exposure of the eye to UV light. Long wavelength UV-A (315-440 nm) can pass through all optic media and cause photochemical damage to the retina. Exposure to sun-light, particularly UV-A, along with use of certain photoactive pharmaceuticals, has been associated with phototoxicity in humans. Phototoxicity involves generation of reactive oxygen species such as H_2O_2, singlet oxygen and hydroxyl radicals after interaction of light, especially UV-A, with photosensitive pharmaceuticals. The reactive oxygen species can attack protein and DNA to form oxidative adducts. Free radical scavengers such as glutathione and vitamins C and E are thought to serve as components of an endogenous defense system that helps to limit light-induced retinal damage. Another potential source of endogenous neuroprotection is the presence of neurotrophic factors in the retina.
The aim of the third experimental part was to investigate the effects of PACAP in UV-A-induced retinal lesion.

Detailed research description

II. Investigation of the effects of PACAP on the tear composition

II.1. Materials and Methods

Rat tear specimens were collected by application of sterile filter paper strips before saline or PACAP injection 1, 6 and 24h later (n=5 at each time point). Wistar rats (200-250 gr) were injected intravenously with 20 µg PACAP38 dissolved in 100 µl physiological saline. The experiment was repeated three times. All procedures were performed in accordance with the

ethical guidelines approved by the University of Pécs (No: BA02/2000-31/2001). Paper strips were placed directly into 0.5 ml sterile Eppendorf tubes and stored at -20°C until examination. Proteins absorbed in the paper strips were eluted by 30 µl of sample buffer (0.125 M Tris/HCl, pH=6.8, 4% sodium dodecyl sulfate, and 10% ß-mercaptoethanol). Samples were prepared for microchip electrophoresis (Agilent 2100 Bioanalyzer System). Protein 230 Plus LabChip Kits were applied in the study. Microcapillaries of the protein chips were filled up with the gel-dye mix of the kit. The linear polymer of the gel ensured the separation of the protein-SDS complexes on the basis of their molecular mass, within the 14 and 230 kDa range. The molecular weight markers (ladder) of the LabChip Kit were applied for the molecular weight determination of the unknown proteins. Evalution of the results was performed by the Protein 230 assay software. All samples were electrophoretized at least three times.

The spots of interest were excised from the gel, placed in Eppendorf tube, and destained by washing three times for 10 min in 200µl of 50% (v/v) acetonitrile, 50 mM NH_4CO_3 solution. The gel pieces were dehydrated at room temperature and covered with 10 µl of trypsin (0.04 mg ml^{-1}) in Tris buffer (2.0 mM, pH 8.5) and left at 37°C overnight. The spots were crushed, and peptides were extracted in an ultrasonic bath (15 min) with a 15-µl aqueous solution of acetonitrile and formic acid (49:50:1 v/v/v). After extraction, the solution of the peptides was lyophilized and redissolved in distilled water. The aqueous solutions of the lyophilized protein tryptic digests were loaded onto the target plate by mixing 1.0 µl of each solution with the same volume of a saturated matrix solution, prepared by dissolving α-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% trifluoracetic acid (1:2, v/v). The mass spectrometer used in this work was an Autoflex II time-of-flight (TOF/TOF) operated in the reflector for matrix-assisted laser desorption/ionization (MALDI) TOF peptide mass fingerprint or LIFT mode for MALDI TOF/TOF with an automated mode using the FlexControl software. The instrument uses a 337-nm pulsed nitrogen laser. External calibration was performed in each case using Bruker Peptide Calibration Standard. The sequence homology of the identified keratins was analyzed by Clustal W II programme.

II.2. Results

The quantity of the proteins eluted from the filter paper strips was sufficient for the analysis of the protein composition by microchip technology. Protein content of the rat tear showed a complex pattern; several protein peaks could be detected within the 14 to 80 kDa molecular mass region. Characteristic proteins of the tear, as lysozyme, albumin, immunoglobulin A,

and lactoferrin could be detected in the rat samples. There were no significant alterations observed in the quantity of the characteristic proteins after PACAP administration. However, as early as 1 h after PACAP injection, an extra peak appeared in the protein profile, in the high molecular weight range (60-70 kDa), in all rat tear samples. This alteration was detected in all samples after 6 and 24 h in higher amounts. Marked differences could be detected on SDS gel between the control and PACAP-treated samples, including the 50-70 kDa range that we found altered with electrophoretic microchip. Therefore, in the present study, only the spots of 50-70 kDa were excised and further analyzed by MALDI TOF mass spectrometry. It was found that several proteins were altered in the examined molecular range. A few of these proteins identified in the given molecular range were repressed by PACAP, while others were stimulated. Among both the repressed and stimulated proteins, different types of rat keratin were identified. The keratin types expressed in samples from PACAP-treated rats show a great homology in the C-terminal amino acid sequence, and they are different from those found in control samples. The keratin homologues stimulated by PACAP were keratin 1; keratin 10; keratin type I cytoskeletal 13, keratin type I, KA10. Those repressed by PACAP treatment were keratin complex 2, basis, gene 5, and keratin, type II cytoskeletal 5. Keratin complex 2, basis, gene 6a isoform I was found in both samples, but its expression was reduced by PACAP. Furthermore, PACAP treatment suppressed the expression of actins and aldehyde dehydrogenase class 3.

II.3. Discussion

This part of the study showed that PACAP treatment caused alterations in the protein composition of tear film. Analysis of body fluid proteome has become one of the most promising approaches to discovery of biomarkers for human diseases. investigating the tear proteome is especially advantageous because of the noninvasive sample collection. Several neuropeptides have already been shown to influence tear secretion and composition, such as substance P and somatostatin. MALDI TOF mass spectrometry is a sensitive and high-throughtput technique. The effects of PACAP on a complex protein map using mass spectrometry has only been demonstrated in PC12 cells, which showed more than a hundred proteins altered by PACAP. Our results show that, in the 50-70 kDa molecular weight range, PACAP induced expression of certain keratins, while other keratins, along with beta and gamma actins, aldehyde dehydrogenase, showed a decrease after PACAP treatment. Keratins 1 and 10 are associated in pairs and are characteristic for suprabasal terminally differentiating epithelial cells, similarly to keratin 13, all of which were induced by PACAP

treatment. Different corneal keratins are suggested to play roles in corneal integrity, renewal, and re-epithelialization. The class 3 aldehyde dehydrogenase showed a marked decrease after PACAP treatment. Aldehyde dehydrogenase has also been described to be responsible for preventing accumulation of cytotoxic products in the cornea and in protecting against ultraviolet radiation.

We also showed that PACAP is present in the human tear, but not in the aqeuos humor. The presence of PACAP in human tear suggests that PACAP plays an important role in the superficial tissues of the eye. As PACAP is a generally accepted cytoprotective peptide, it is conceivable that it plays a role in the regeneration of the cornea, the possibility of which is studied in the second part of the dissertation.

III. Effects of PACAP in corneal epithelial regeneration

III.1. Materials and methods

Male Wistar rats (250-300 gr, n=20) were used. Animals were anesthetized with 50 mg/kg pentobarbital and eyes were examined under dissecting microscope. Corneal abrasion was performed with corneal trepan, causing a 2-mm diameter circular injury in the center of the cornea, and the encircled corneal epithelium was removed using microsurgical forceps under the dissecting microscope.

PACAP27 (20, 100 and 200 µg) was dissolved in 800 µl distilled water. Eyes were treated immediately after surgery and every two hours with these drops, with each drop containing 1, 5 and 10 µg PACAP27 in 40 µl vehicle. Only one eye was treated with PACAP27 in each animal, the other eye received distilled water treatment at the same time intervals, serving as control injured eyes. Normal, intact corneas were removed from 2 animals. According to preliminary studies eyes were examined 6 hours after injury, when significant wound healing was already present.

Rats were sacrificed under anesthesia and eyes were stained with fluorescein dye. Eyes were removed and placed in cup of filled with soft modeling clay, ensuring a central positioning. Photographs were taken using a Nikon FXA photomicroscope attached to a digital camera (Spot RT Color camera). The injured area was calculated using Spot advance software. Statistical analysis was performed using Student's t-test, and differences were considered significant when $p<0.05$ betwen control and PACAP-treated corneas. After the fluorescein-staned photographs were taken, corneas were also further processed for routine histological staining. Following fixation in 4% paraformaldehyde, serial, 10 µm thick sections were made and stained with haematoxylin-eosin.

For Western blot studies, corneal abrasion was performed as above, and corneas were removed after 4 h in order to investigate protective signaling pathways during wound healing in corneal injury (n=7). Normal, intact corneas were also removed from 4 animals in order to investigate the baseline phosphorylation of Akt and ERK1/2. Samples were processed for Western blot analysis. Membranes were probed overnight at 4 °C with the following primary antibodies: phospho-specific anti-Akt-1 Ser473, phosphospecific anti-ERK1/2 Thr202/Thr204 and anti-aktin. Membranes were washed 6x5 min in Tris buffered saline (pH=7.5) containing goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. The antibody-antigen complexes were visualized by means of enhanced chemiluminescence. Results were quantified by means if NIH ImageJ program. All experiments were performed at least four times. Statistical comparisons were made using the ANOVA test followed by Bonferroni's post hoc analysis. Differences with P values below 0.05 were considered as significant.

III.2. Results

Six hours after corneal abrasion, the healing process was clearly visible in fluorescein-stained eyes in all animals. The area of injury, as calculated with Spot advance program, was signaficantly smaller in corneas treated with 5 or 10 µg PACAP27 than in control, vehicle-treated eyes. The decrease was approximately 20% and 25% in the corneas treated with 5 or 10 µg PACAP27, respectively. The smallest dose of PACAP (1 µg) also led to a decreased injury size, however, difference between PACAP- and vehicle-treated corneas was not statistically significant. These results were confirmed by routine histological staining. Both Akt and ERK1/2 phosphorylation was detected at low levels in normal corneas. ERK1/2 phosphorylation was significantly induced after the corneal abrasion. Phosphorylation was significantly stimulated by PACAP27 in both uninjured corneas and after abrasion. Akt phosphorylation was not induced by the injury alone. PACAP27 significantly stimulated Akt phosphorylation in both intact and in injured corneas.

III.3. Discussion

In this part of the study, we provided evidence that topical PACAP application enhances corneal regeneration. This effect of PACAP was not known before. These results are in accordance with the generally accepted cytoprotective and regenerative functions of PACAP. Our study also showed that topical PACAP application induced ERK1/2 and Akt signaling in the injured cornea. Based on numerous studies, PACAP, mainly via its PAC1 receptor, exerts cytoprotective effects in a number of cells/tissues. The cAMP-induced pathways are

important in corneal functions such as wound healing and homeostasis. cAMP can also potentiate the effects of growth factors, such as it has been described for epidermal growth factor during corneal epithelial migration. Several growth factors have been shown to play important roles during corneal wound healing. Phosphatidyl-inositol-3-kinase (PI3K)-Akt pathways and the mitogen activated protein kinase (MAPK) family are major pathways governing corneal epithelial healing. The involvement of Akt activity has been described in the action of several growth factors, such as insulin-like growth factor 1 and 2, epidermal growth factor and hepatocyte growth factor, during corneal mitosis, migration and wound healing. Similarly, MAPKs, including ERK1/2, play important roles in these processes. It has been described that glial cell-derived neurotrophic factor induces ERK1/2 in corneal epithelial cells. The effects of PACAP, a strong stimulator of cAMP, have been reported earlier on these signaling molecules in other cells/tissues, for example in the retina, in endothelial cells, in astrocytes, cortical neurons and in cerebellar granule cells. Similarly, the effects of PACAP on Akt phosphorylation have been reported in cardiomyocytes, monocytes and in sympatic neuronal cells. Our present results show that these pathways are stimulated by PACAP in the cornea and they may play important roles during corneal epithelial wound healing.

IV. Effects of PACAP in UV-A radiation-induced retinal degeneration models in rats

IV.1. Materials and metods

Adult Wistar rats (250-300 gr, n=22) were used. Animals were anesthetized with 50 mg/kg pentobarbital and pupils were dilated with one drop of phenylephrine 5% and one drop of cyclopentolate (10 mg/ml). A slow flow NaCl 0.9% prevented drying of the cornea. Before exposure to UV-A light, retinal image position and focus were established with 570 nm light. Retinal irradiance was calculated by the method described by Calkins and Hochheimer. The UV-A radiation source was 45 minutes (315-400 nm, 1.5 mW/cm^2) with a XLPS-10 Xenon lamp. PACAP38 (100 pmol, was dissolved in 5 µl saline) injection was given immediately following UV-A radiation with a Hamilton syringe into the treated (right) eye. The same volume of vehicle was injected into the left eye, serving as a control, non-treated eye.

One day, 2 days and 1 week after irradiation the animals were sacrificed with an overdose of pentobarbital and were enucleated. The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. Tissues were embedded in Durcupam ACM resin, cut at 2 µm and stained with toluidine blue. Photographs were taken with a digital CCD camera using the Spot program, from central retina areas of nearly same eccentricities. Measurements were taken

from digital photographs with the NIH Image 1.55 program. Samples for measurements derived from at least six tissue blocks prepared from at least three animals (n=2-5 measurements from one tissue block). The following membrane were measured: 1. cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM); 2. the width of the outer and inner nuclear and and outer and inner plexiform layers (ONL, INL, OPL, IPL); 3. the number of cells in the ONL/500 μm^2; 4. the number of cells in the INL/500 μm^2; 5. the number of cells/100 µm section length in the ganglion cells layer (GCL). Statistical comparisons were made using the ANOVA test followed by Tukey-B' post hoc anlysis.

IV.2. Results

In control preparations all characteristic layers of the rat retina were well visible. Under the pigment epithelium, the photoreceptor layer (PL) is followed by several rows of photoreceptor cell bodies, forming the outer nuclear layer (ONL). A thin outer plexiform layer (OPL) is followed by 4-5 rows of the cell bodies of bipolar, amacrine and horizontal cells (INL). Finally, the inner plexiform layer (IPL) is followed by cells in the ganglion cell layer (GCL).

The retinal structure, especially the ONL, was slightly damaged after 1 day of the diffuse UV-A exposure. During this short period, a number of cells were damaged, supported by the detection of the empty cell body shapes in the ONL and the INL. As a consequence, the number of cells in the ONL and INL significantly decreased (42% and 27% of controls, respectively). The thickness of retinal layers was also reduced. However, intravitreal PACAP treatment retained the structure of ONL and the number of GCL was unchanged compared to the control retina.

Two days after the radiatino, degenerative alteration in the ONL and INL could be detected. The number of cells in both layers was markedly reduced compared to control retinas. Pycnotic cells and empty cell body-shapes spaces could be observed in the nuclear layers. Intravitreal PACAP administration caused a significant improvement in the retinal cell numbers. The number of cells/100 µm in the GCL was also approximately 50% of the normal retinas. In retinas with intravitreal PACAP treatment, the number of cells in the GCL was significantly higher than in control eyes.

Severe degeneration could be observed 1 week after diffuse UV-A exposure. Several neurodegenerative structures were detected in the ONL and INL. The outer segments of the photoreceptors also showed signs of degeneration. The IPL was markedly swollen and in this

layer scattered dense dots were seen representing degenerating bipolar cells terminals. The cell number in the ONL, INL and also in the GCL was significantly diminished. Significant protection could be noted following intravitreal PACAP treatment also in this case. The retinal layers were distinct, the cells were intact in the nuclear layers and the swelling of the IPL was reduced. The cell number in the ONL, INL and GCL was increased and an improvement could be found in the whole retinal thickness.

IV.3. Discussion

In the present study we showed that PACAP is protective against UV-A-induced retinal injury. The major signs of damage were the degeneration of photoreceptors, as shown by the decrease of their cell number. At later time points, the number of bipolar cells and of ganglion cells was also markedly decreased. All these changes could be, at least partially, counteracted by PACAP administration. In addition to the neuroprotection, in the present study we also observed that PACAP decreased the significant edema in the retina 7 days after irradiation, a similar observation has been made in ischemic brain edema.

The PACAP-induced retinoprotection rather reflects a general neuroprotective mechanism than a phenotype-specific cellular protection. Using several different cell markers we could not find a correlation between cell type specificity and the protective effects of PACAP. The efficacy of PACAP against a diverse array of noxious stimuli in the retina also supports this hypothesis. Our study shows, for the first time, that PACAP has neuroprotective effects in the retina against diffuse UV-A-induced degeneration, a finding that adds to the numerous other observations on the retinoprotective effects of the peptide.

V. Summary of new results

1. We showed the presence of PACAP in human tear. PACAP, injected systemically, alters the composition of tear proteins.
2. We provided evidence that local administration of PACAP enhances corneal wound healing. In addition, PACAP stimulates the activation of two protective factors, ERK and Akt.
3. We proved that intravitreal PACAP treatment is protective in UVA light-induced retinal degeneration.

VI. Publications related to the thesis

1. Brubel R., Reglodi D., Jambor E., Koppan M., Varnagy A., Biro Zs., Kiss P., Gaal V., Matkovits A., Farkas J., Lubics A., Bodis J., Bay Cs., Veszpremi B., Tamas A., Nemeth J., Mark L.: Investigation of pituitary adenylate cyclase activating polypeptide in human gynecological and other biological fluids by using MALDI TOF mass spectrometry. J Mass Spectr 2011; 46: 189-194. (IF: 3.289)
2. Atlasz T., Szabadfi K., Kiss P., Marton Zs., Griecs M., Hamza L., Gaal V., Biro Zs., Tamas A., Hild G., Nyitrai M., Toth G., Reglodi D., Gabriel R.: Effects of PACAP in UV-A radiation-induced retinal degeneration models in rats. J Mol Neurosci 2011; 43: 51-57. (IF. 2.992)
3. Atlasz T., Szabadfi K., Kiss P., Racz B., Gallyas F., Tamas A., Gaal V., Marton Zs., Gabriel R., Reglodi D.: Pituitary adenylate cyclase activating polypeptide in the retina: focus on the retinoprotective effects. Ann NY Acad Sci 2010; 1200: 128-139. (IF: 2.847)
4. Gaal V., Mark L., Kiss P., Kustos I., Tamas A., Kocsis B., Lubics A., Nemeth V., Nemeth A., Lujber L., Pytel J., Toth G., Reglodi D.: Investigation of the effects of PACAP on the composition of tear and endolymph proteins. J Mol Neurosci 2008; 36: 321-329. (IF: 2.061)

Total impact factor of publications related to the thesis: 11.189

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