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Effects of pituitary adenylate cyclase activating polypeptide (PACAP) in corneal epithelial regeneration and signal transduction in rats

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Abstract
Corneal epithelium responds to insults with a rapid wound healing, which is essential for maintaining vision. The proper balance of apoptotic and proliferation-stimulating pathways is critical for normal regeneration. Pituitary adenylate cyclase activating polypeptide (PACAP) is an important growth factor during the development of the nervous system and exerts cytoprotective effects in injuries. The aim of the present study was to investigate the effects of PACAP on corneal epithelial wound healing in rats and on two important protective signaling molecules, Akt and ERK1/2, both of which have been reported to play important roles during cell survival and regeneration, including corneal wound healing. Wistar rats received PACAP treatment in form of eyedrops, containing 1, 5 and 10 µg PACAP27, immediately and every two hours after corneal abrasion. Corneas were stained with fluorescein dye and further processed for histological staining or Western blot analysis for Akt and ERK1/2 expression. Our results showed that topical PACAP application enhanced corneal wound healing, as the area of injury was significantly less in PACAP-treated groups. Furthermore, both ERK1/2 and Akt signaling was induced upon PACAP administration in both injured and intact corneas. In summary, the present results show that PACAP enhances corneal wound healing in a rat model of corneal abrasion.

Keywords PACAP · Cornea · Akt · ERK · Repair

Introduction

The main refractive structure of the eye is the cornea, the transparency of which is a prerequisite for the normal vision. The corneal epithelium, a thin stratified squamous non-keratinizing epithelium, is continuously subjected to physical, chemical and biological insults (Yu et al. 2010). Corneal epithelium responds to insults with a rapid wound healing, which is essential for maintaining vision. In the area of injury, keratinocytes undergo apoptosis, and proliferation of new cells occurs from the border of the wound. The proper balance of apoptotic and proliferation-stimulating pathways is critical for normal wound healing (Ljubimov and Saghizadeh 2015; Lu 2006; Netto et al. 2005). Several growth factors, transcription factors and cytokines have been identified in this process (Baldwin and Marshall 2002; Lyu and Joo 2005; Saika et al. 2004; Yu et al. 2010). On the other hand, impaired corneal wound healing and/or excessive apoptosis are known in numerous pathological conditions, including diabetes, contact lens-induced injuries and complications of refractive surgery (Netto et al. 2005; Wilson et al. 2007; Zagon et al. 2006).

Pituitary adenylate cyclase activating polypeptide (PACAP), originally isolated from the hypothalamus, has a diverse array of effects in various organs (May et al. 2021;

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Vaudry et al. 2009). PACAP is an important growth factor during the development of the nervous system and has also cytoprotective effects in injuries (Cherait et al. 2021; Li et al. 2021; Martinez-Rojas et al. 2021; Reglodi et al. 2018; Toth et al. 2020; Van et al. 2021; Vaudry et al. 2009; Waschek 2002). PACAP has two biologically active forms: PACAP27 and PACAP38, with 27 and 38 amino acid residues, respectively. PACAP and its receptors occur in ocular tissues, and the peptide has several biological effects in the eye (Nilsson et al. 1994; Seki et al. 2000; Shioda et al. 2016; Wang et al. 1995). The most intensively studied effects of PACAP in the eye are its retinal effects: PACAP is a well-established retinoprotective peptide and is an important modulator in the retinohypothalamic pathway (Atlasz et al. 2010, 2016; D'Amico et al. 2021; Fahrenkrug et al. 2005; Hannibal and Fahrenkrug 2004; Shioda et al. 2016). However, PACAP has several other functions in the non-retinal parts of the eye. For example, PACAP enhances the sphincter muscle response to stimulation and relaxes the dilator muscle (Yamaji et al. 2005; Yoshitomi et al. 2002). PACAP receptors have been described in the non-retinal ocular elements and PACAP decreases uveal vascular resistance and increases choroideal blood flow (Nilsson 1994; Nilsson et al. 1994). PACAP is also suggested as a sensory and inflammatory neuropeptide in the eye (Wang et al. 1995). Systemic injection of PACAP has been shown to modify certain protein components of the rat tear film (Gaal et al. 2008). Subsequently, PACAP was shown to stimulate tear secretion and suppress corneal keratinization (Nakamachi et al. 2016). Mice lacking endogenous PACAP, on the other hand, display reduced tear secretion and dry eye symptoms that can be attenuated by PACAP eyedrops (Hirabayashi et al. 2022; Nakamachi et al. 2016). Not only the water secretion was induced involving aquaporin 5 channel (Nakamachi et al. 2016) but the secretion of lactoferrin, an important tear protein, was also stimulated by PACAP (Nakajima et al. 2013).

Less is known about the effects of PACAP in the cornea. The presence of PACAP and its receptors have been shown in the cornea (Maugeri et al. 2022a; Wang et al. 1995). In an earlier study, PACAP eyedrops induced growth of neuronal processes and accelerated recovery of corneal sensitivity in rabbits (Fukiage et al. 2007). Although focusing only on the neuronal recovery, this study has drawn the attention to the possibility that PACAP, in form of eyedrops, could enhance corneal recovery. Subsequently, a series of studies have provided evidence for the protective effects of PACAP in corneal epithelial and endothelial cells (Ma et al. 2015; Maugeri et al. 2018, 2019, 2020; Wang et al. 2019; Wu et al. 2015) demonstrated that PACAP27, and more potently, a recombinant PACAP-derived peptide, MPAPO, facilitated corneal wound closure in mice and synapse growth in trigeminal ganglion cells. Similar results have been obtained with a recombinant PACAP-N terminal agrin domain protein (Wu et al. 2015). The aim of the present study was to investigate the effects of PACAP on epithelial wound healing in another species, in rats, and on two important protective signaling molecules, Akt and ERK1/2, both of which have been reported to play important roles during cell survival and regeneration, including corneal wound healing (He et al. 2006; Mester et al. 2009; Yin and Yu 2009).

Materials and methods

Corneal abrasion

Male Wistar rats (weighing 250–300 gr, n = 36) were used in the experiments. Animals were housed under standard laboratory conditions, under approved protocols (No: BA02/2000-20/2006). Animals were anesthetized with 50 mg/kg pentobarbital and eyes were examined under dissecting microscope. A corneal trepan was used to mark a 2-mm diameter circular area in the center of the cornea (Zagon et al. 2006), and the encircled corneal epithelium was then removed using microsurgical forceps on both eyes of the animals. Care was taken not to injure the underlying corneal stroma. The whole procedure was performed under the dissecting microscope.

PACAP treatment

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 eyedrops, with each drop containing 1, 5 or 10 µg PACAP27 in 40 µl vehicle (n = 7 for each group). 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 and other descriptions, eyes were examined 6 h after injury, when significant wound healing was already present (Nakamura et al. 2003).

Fluorescein and histological staining

Rats were sacrificed under anesthesia and eyes were stained with fluorescein dye (Haag-Streit, Switzerland). Eyes were removed and placed in a cup 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 then calculated using Spot advance software. Statistical analysis was performed using ANOVA test, and differences were

considered significant when $p < 0.05$ between control and PACAP-treated corneas. After the fluorescein-stained 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 (Sigma, Hungary). Photographs were taken using a Nikon FXA photomicroscope attached to a digital camera (Spot RT Color camera). Routine histology was performed in order to demonstrate the injured cornea.

Western blotting

For Western blot studies, corneal abrasion was performed as detailed above. PACAP treatment was performed immediately after the lesion and after two hours. Eyedrops contained 10 µg PACAP27 in 40 µl vehicle. 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 as described earlier (Racz et al. 2007a), using 15 µg/ml protein/sample. Membranes were probed overnight at 4 °C with the following primary antibodies: phospho-specific anti-Akt-1 Ser473 (1:1000 dilution; R&D Systems, Budapest, Hungary), phospho-specific anti-ERK1/2 Thr202/Tyr204 (1:1000 dilution; R&D Systems, Budapest, Hungary) and anti-aktin (1:5000 dilution; Sigma-Aldrich Chemical Co., Budapest, Hungary). Membranes were washed six times for 5 min in Tris buffered saline (pH = 7.5) containing 0.2% Tween prior to addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000; BioRad, Budapest, Hungary). The antibody-antigen complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by means of NIH ImageJ program. All experiments were performed at least four times. All data were expressed as mean ± SEM. 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. Experimental design is summarized in Fig. 1.

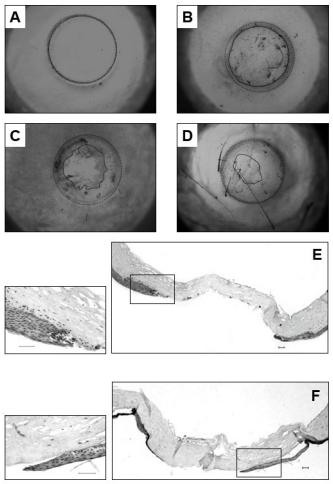

Fig. 2 (A-D): Representative photographs of fluorescein-stained corneas. Picture of intact eye, showing only the site of the abrasion (A), 6 h after injury in control, vehicle-treated eye (B), 6 h after injury in an animal treated with 5 µg PACAP27 (C) and 6 h after injury in an animal treated with 10 µg PACAP27 (D). Dotted lines mark the original site of abrasion and filled lines outline remaining unhealed corneal areas (E,F): Representative microphotographs of haematoxylin-eosin stained corneas showing the epithelial injury 6 h after abrasion in control (E) and 5 µg PACAP27-treated (F) corneas. The outlined areas are shown with higher magnification in the inlets. Scale bars: 50 µm

Results

Corneal wound healing

Six hours after corneal abrasion, the healing process was clearly visible in fluorescein-stained eyes in all animals. In control, vehicle-treated corneas, wound healing occurred in a concentric manner, from the edges of the original injury site (Fig. 2. A, B). The area of injury, as calculated with Spot advance program, was significantly smaller in corneas treated with 5 or 10 µg PACAP27 than in control, vehicle-treated eyes (Fig. 2. C, D and Fig. 3.) indicating an increased epithelial recolonisation of the injured area in PACAP

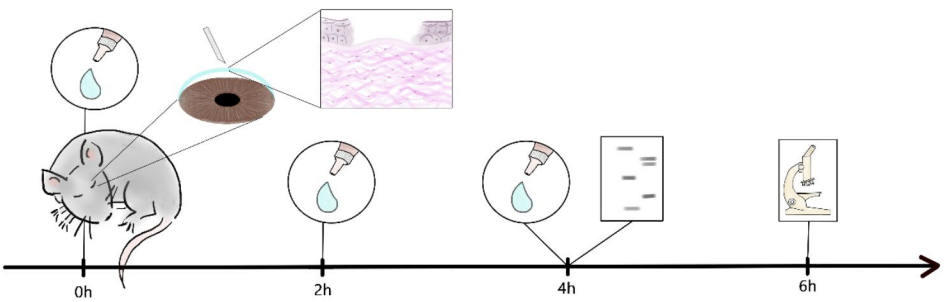

Fig. 1 Schematic representation of the experimental design

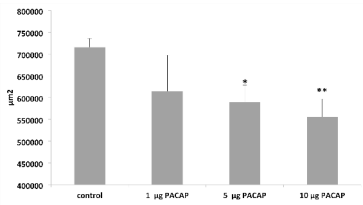

Fig. 3 Graphs showing the area of corneal epithelial injury 6 h after abrasion in control, vehicle-treated corneas and in corneas treated with different concentrations of PACAP27. Data are given as mean $\mu m^2 \pm$ SEM. *P < 0.05, **P < 0.01 compared to control, vehicle-treated corneas

treated corneas. This difference was approximately 20% ($p < 0.05$) and 25% ($p < 0.01$) in the corneas treated with 5 or 10 µg PACAP27, respectively. The lowest dose of PACAP (1 µg) also led to a faster epithelial regrowth (approximately 15% smaller injured area compared to vehicle treated), however, difference between PACAP- and vehicle-treated corneas was not statistically significant. These results were confirmed by routine histological staining, where the differences between PACAP- and vehicle-treated corneas, although not quantified, were clearly visible (Fig. 2. E, F).

Phosphorylation of ERK1/2 and Akt

Even loading was confirmed by actin expression (Fig. 4. A). Both Akt and ERK1/2 phosphorylation was detected at low levels in normal corneas (Fig. 4. B, C). ERK1/2 phosphorylation was significantly induced after the corneal abrasion. Phosphorylation was significantly stimulated by PACAP27 in both uninjured corneas and after abrasion (Fig. 4. B). Akt phosphorylation was not induced by the injury alone. However, PACAP27 stimulated Akt phosphorylation in both intact and in injured corneas, and it was significant after the injury (Fig. 4. B).

Discussion

The present study showed that topical PACAP application enhanced corneal wound healing in rats and 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 (Dejda et al. 2008; Nonaka et al. 2020; Shioda et al. 2019; Somogyvari-Vigh and Reglodi 2004; Toth et al. 2020; Vaudry et al. 2009). In the eye, the retinoprotective effects of PACAP are well-established and have been reviewed several times (Atlasz et al. 2010, 2016; Gabriel et al. 2019; Postyeni et al. 2021). PACAP is protective against glutamate toxicity in retinal neurons (Gabriel et al. 2019; Shoge et al. 1999). In retinal explants, PACAP has been shown to be protective against thapsigargin-induced photoreceptor cell death and anisomycin-induced cell death in the neuroblastic layer (Silveira et al. 2002). PACAP-treated turtle eyecup preparations survive and show electrical activity for a significantly longer time (Rabl et al. 2002). In vivo, PACAP has been shown to be protective against optic nerve transection, glutamate- and kainate-induced excitotoxic injury and ischemic degeneration (Atlasz et al. 2007, 2008, 2009, 2010; Babai et al. 2005; Racz et al. 2007a, b; Seki et al. 2006, 2008). Recent studies have proven the protective effects in models of glaucoma, diabetic retinopathy and retinopathy of prematurity (Kvarik et al. 2016, 2021; Szabo et al. 2021). In the background of this retinoprotective effect several metabolomics changes have been identified in addition to the antiapoptotic and antioxidant effects (D'Alessandro et al. 2014). Our research group has shown that PACAP, given in form of eyedrops, can pass the ocular barriers and reach the retina, where it can exert protective effects (Kovacs et al. 2021; Werling et al. 2016). This opens ways for novel therapeutic strategies to avoid invasive intraocular treatments and have retinoprotective effects in form of eyedrops. PACAP administered in eyedrops can have local effects on the cornea, which has drawn less attention, but recent results point to the possible therapeutic potential of PACAP on corneal injuries.

Corneal epithelial cells respond rapidly to environmental stressors, and the balance of regulating pathways leading to fast wound healing is essential to visual acuity. A previous study has described that application of PACAP solution on the surface of the cornea induced trigeminal nerve regeneration important for corneal sensitivity (Fukiage et al. 2007). The main finding of the present study is that topical application of PACAP27 in form of eyedrops enhanced corneal regeneration in rats similarly to earlier findings in mice (Ma et al. 2015; Wang et al. 2019; Wu et al. 2015).

A series of recent studies have proven that PACAP is also protective on corneal endothelial cells, which face the anterior chamber. Maugeri et al. showed that PACAP and all of its 3 receptors (PAC1, VPAC1, VPAC2) can be found in the human corneal endothelial cells (Maugeri et al. 2018, 2019) and PACAP increased cellular viability in growth factor-deprived cells (Maugeri et al. 2018). PACAP also increased the endothelial barrier functions shown by the increased electrical resistance, the restored expression of tight junction-related proteins (ZO-1 and claudin-1) and the expression of integrin alpha3 and Na/K ATPase (Maugeri et al. 2018, 2019). The authors performed a wound healing assay on endothelial cells and found that PACAP was able to restore the migration of endothelial cells (Maugeri et al. 2018, 2019). A subsequent study has demonstrated that this protective effect is via the PAC1 receptor and transactivation of the epidermal growth factor receptor through which it stimulates MAPK/ERK1/2 signaling (Maugeri et

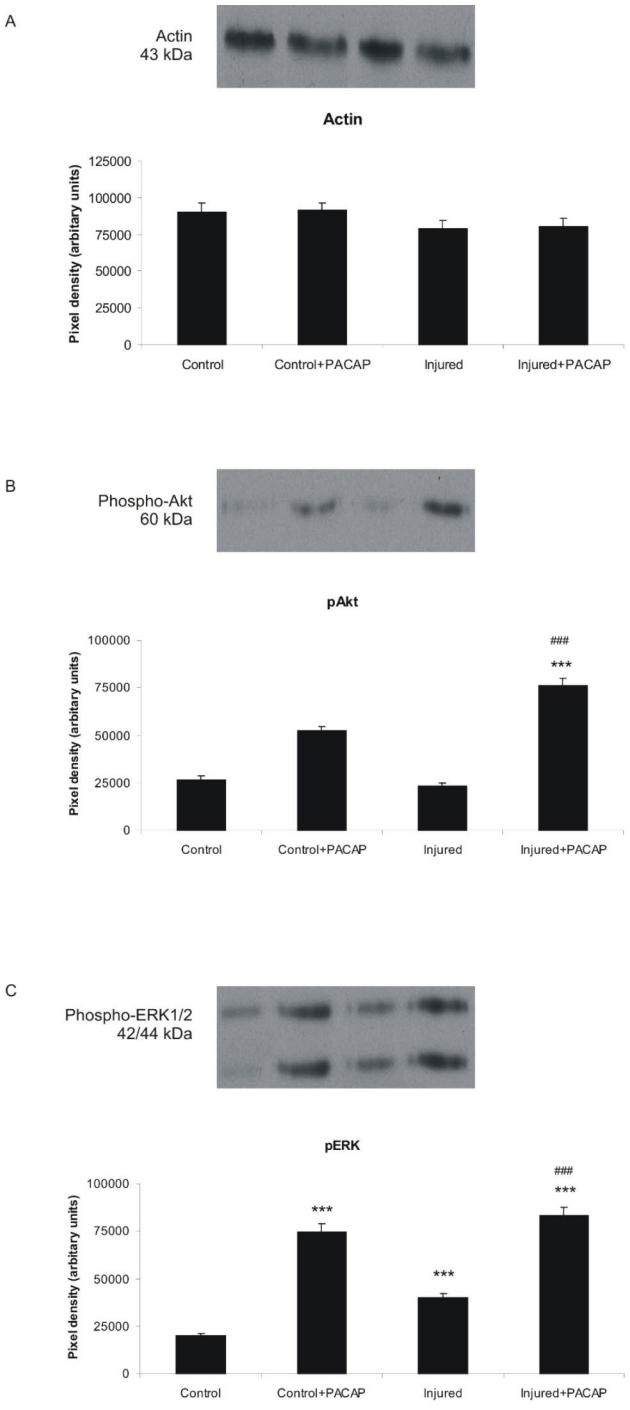

Fig. 4 Effect of corneal injury and PACAP treatment on Akt and ERK activation in the cornea. Activation of Akt and ERK was demonstrated by their phosphorylation detected by immunoblotting utilizing phosphorylation-specific primary antibodies. Representative blots of three experiments as well as quantitative evaluation of the pixel densities are shown. Values are given as mean ± SEM. Actin was used as a loading control. $^{***}P < 0.001$ versus control corneas, $^{###}P < 0.001$ versus injured corneas

al. 2019). A recent study from the same research group has confirmed similar effects in corneal endothelial cells against UV light-induced damage (Maugeri et al. 2020), similarly to activity-dependent protein, a protective peptide stimulated by PACAP (Maugeri et al. 2022b). PACAP treatment counteracted the UV-induced apoptotic death of the endothelial cells and restored the barrier functions, similarly to the effects against growth factor deprivation-induced damage (Maugeri et al. 2020).

The cAMP-induced pathways are important in corneal functions such as wound healing and homeostasis (Grueb et al. 2008; Nakamura and Nishida 2003). cAMP can also potentiate the effects of growth factors, such as it has been described for epidermal growth factor during corneal epithelial migration (Nakamura and Nishida 2003). Several growth factors have been shown to play important roles during corneal wound healing (Baldwin and Marshall 2002; Kamil and Mohan 2021; Lyu and Joo 2005; Saika et al. 2004; Yu et al. 2010). Phosphatidyl-inositol-3-kinase (PI3K)-Akt pathways and the mitogen activated protein kinase (MAPK) family are major pathways governing corneal epithelial healing (He et al. 2006). 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 (Kakazu et al. 2004; Yanai et al. 2006). 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 (You et al. 2001). The effects of PACAP, a strong stimulator of cAMP, have been reported earlier on these signaling molecules in other cells/tissues. For example, the stimulating effect of PACAP on ERK phosphorylation has been described in the retina (Racz et al. 2006), in endothelial cells (Racz et al. 2007b), in astrocytes (Hashimoto et al. 2003), cortical neurons (Stumm et al. 2007) and in cerebellar granule cells (Vaudry et al. 2002). Similarly, the effects of PACAP on Akt phosphorylation have been reported in cardiomyocytes (Racz et al. 2008), monocytes (El Zein et al. 2007), Schwann cells (Castorina et al. 2015) and in sympathetic neuronal cells (May et al. 2010). Both Akt and ERK activation have been shown in the background of PACAP-induced neurite outgrowth (Shibato et al. 2021). 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 in rats, similarly to earlier findings in mice (Ma et al. 2015; Wang et al. 2019).

Several other factors have been shown to be involved in PACAP-induced wound healing. Ma et al. (2015) showed that expression of nerve growth factor, transforming growth factor beta and fibronectin were higher in PACAP27- and MPAPO-treated corneas (Ma et al. 2015). In contrast, factors

involved in pathological inflammation-related angiogenesis (vascular endothelial growth factor and intercellular cell adhesion molecule 1) were decreased after these treatments. The involvement of cyclin D1 has also been shown in the PACAP-induced corneal epithelial cell proliferation (Wang et al. 2019). Recently, we have demonstrated the presence of PACAP and its specific PAC1 receptor in the human eye (Patko et al. 2022). As results obtained in rats are similar to those previously shown in mice, it seems that these protective effects are not species-specific, but more general. The occurrence of PACAP and its receptors in the human eye therefore imply that the results from these animal studies have translational value and most probably are also present in the human eye that could have a therapeutic potential.

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