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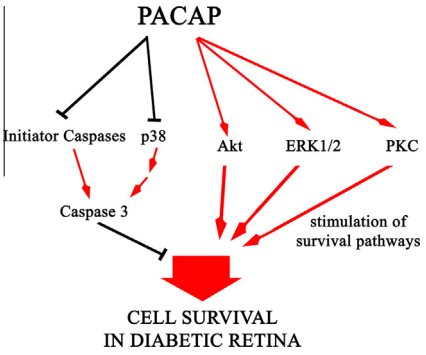
Graphical abstract

PACAP promotes neuron survival in early experimental diabetic retinopathy pp xxx–xxx

Krisztina Szabadfi *, Aliz Szabo, Peter Kiss, Dora Reglodi, Gyorgy Setalo Jr., Krisztina Kovacs, Andrea Tamas, Gabor Toth, Robert Gabriel



Highlights

• PACAP attenuates the degenerative neurochemical changes in diabetic retina. • PACAP decreased TUNEL-positive, including dopaminergic amacrine cells. • PACAP treatment could activate Akt/ERK/PKC pathways in diabetic retina. • PACAP reduced the activate forms of initiator and executioner caspases. • PACAP may have therapeutic potential in early diabetic retinopathy.

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PACAP promotes neuron survival in early experimental diabetic retinopathy

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ABSTRACT

Metabolic changes induced by diabetes lead to a multifactorial progressive disease of the retina with an extremely complex pathogenesis. One of the mechanisms of retinal cell death in diabetes is via apoptosis. Our previous results show that pituitary adenylate cyclase activating polypeptide (PACAP) attenuates the morphological and neurochemical changes in a rat model of diabetic retinopathy. The aim of this study was to investigate the mechanisms of this protective effect.

Retinas of streptozotocin-induced diabetic rats were analyzed using apoptosis detection combined with immunolabeling. Western blot was used to measure levels of pro- and anti-apoptotic pathways.

Intraocular PACAP injection markedly attenuated diabetic retinal injury: increased levels of the anti-apoptotic p-Akt, p-ERK1, p-ERK2, PKC, Bcl-2, while decreased levels of the pro-apoptotic p-p38MAPK and activated caspases (8, 3, 12) were detected. The number of apoptotic cells increased in all nuclear layers of diabetic retinas, but significantly decreased after PACAP treatment. Our results clearly demonstrate that the protective effects of PACAP are mediated, at least partly, by attenuating apoptosis, including also that of the dopaminergic amacrine cells. Inhibition of apoptosis is one of the PACAP-induced pathways with therapeutic potential in early experimental diabetic retinopathy.

1. Introduction

Diabetic retinopathy is the most common ocular complication of the systemic disease. The metabolic changes induced by diabetes lead to a multifactorial progressive disease of the retina with an extremely complex pathogenesis. All major cell types of the retina are affected: neuronal as well as the Muller glial cells and pigment epithelial cells. The retinal neurodegeneration involving ganglion and bipolar cells is caused by the activation of different metabolic pathways (Ola et al., 2012). Diabetes-associated hyperglycemia is generally considered as the key initiator of retinal damage, by activation and dysregulation of several metabolic and signaling pathways, such as protein kinase C (PKC), polyol pathway, and/or poly-ADP-ribose polymerase activation. These cascades lead to increased oxidative stress, apoptosis, inflammatory response, and angiogenesis. The activation of these complex pathways is closely linked to the degeneration of a variety of cell types in the retina (Abu El-Asrar et al., 2007).

Before severe vascular complications emerge in the retina, two early pathological processes are already in progression. One is neuronal damage indicated by alterations in the electroretinogram (Gastinger et al., 2006). Second, cell death occurs both in the vasculature and among neuronal cells of the retina. In this process the pigmented epithelium may also be affected (Tang and Kern, 2011). One of the potential mechanisms of retinal cell death in diabetes is via apoptosis. A ten-fold increase in the frequency of apoptosis has been observed after 1 month of streptozotocin-induced

Abbreviations: ER, endoplasmatic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCL, ganglion cell layer; GSK3β, glycogen synthase kinase 3 beta; ERKs, extracellular signal-regulated kinases; INL, inner nuclear layer; IPL, inner plexiform layer; MAPKs, mitogen activated protein kinases; ONL, outer nuclear layer; OPL, outer plexiform layer; PACAP, pituitary adenylate cyclase activating polypeptide; PAC1-R, PACAP type 1 receptor; PKA, protein kinase A; PKC, protein kinase C; TH, tyrosine hydroxylase; TUNEL, terminal transferase dUTP nick end labeling; VIP, vasoactive intestinal peptide.

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diabetes (Barber et al., 1998; Hammes et al., 1995; Kern and Barber, 2008; Ola et al., 2012). The molecular events regulating apoptosis in the retina are complex and involve several pro- and anti-apoptotic factors (Barber et al., 1998). In the early phases of the apoptotic process, mitochondria release several apoptogenic proteins, such as cytochrome-c and apoptosis-inducing factor into the cytosol. Level and activity of initiator and effector caspases have also been described to increase in early diabetic retinopathy (Kannan and Jain, 2000).

Pituitary adenylate cyclase activating polypeptide (PACAP), a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide superfamily, is a neuropeptide with highly potent neuroprotective and general cytoprotective effects. PACAP and its receptors occur in the retina (Izumi et al., 2000; Seki et al., 2000). PACAP receptors can be divided into two main groups: PAC1 (PAC1-R), which binds PACAP with much higher affinity than VIP, and VPAC receptors (VPAC1-R and VPAC2-R), which bind PACAP and VIP with similar affinities (Vaudry et al., 2009). Various pathways regulated by PAC1-Rs are different in distinct cell types depending on the expressed splice variant, the PACAP concentration and other factors present. PACAP has been shown to protect neurons *in vitro* and *in vivo* mainly through the PAC1-R, involving various downstream mechanisms of the protein kinase A (PKA) and PKC pathways (D'Agata and Cavallaro, 1998; Ohtaki et al., 2008; Shioda et al., 2006; Somogyvari-Vigh and Reglodi, 2004; Vaudry et al., 2009; Waschek, 2002). PACAP has strong anti-apoptotic effects in various different neuronal and non-neuronal cell types, exerted by acting at different levels of the apoptotic cascade (Seaborn et al., 2011; Somogyvari-Vigh et al., 1998; Somogyvari-Vigh and Reglodi, 2004).

There is increasing evidence that PACAP is protective in retinal pathologies. It has been shown that PACAP attenuates retinal damage in excitotoxic, ischemic and UV light-induced retinal degeneration (Atlasz et al., 2010; Nakamachi et al., 2012). We have recently described that PACAP is protective in diabetic retinopathy. This protection was manifested in the elevated level of tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis measured by qRT-PCR and Western blot methods. Also the morphological characteristics of the dopaminergic cells resembled those in the healthy retina, which resulted in a morphologically more retained retinal structure (Szabadfi et al., 2012a). The degeneration of the dopaminergic amacrine cells is one of the early events in the course of diabetic retinopathy. In addition, we found an upregulation of PAC1-R in PACAP-treated diabetic retinas, suggesting an autoregulatory induction role of PACAP, further augmenting its own action. A recent study has shown that the initial upregulation of PACAP, VIP, and related receptors and subsequent downregulation in retina of diabetic rats along with the protective effects of PACAP treatment, suggest a role for both peptides in the pathogenesis of diabetic retinopathy by different mechanisms (Giunta et al., 2012). In the light of the above results the aims of the present study were (i) to provide evidence that PACAP-treatment attenuates apoptosis in certain cell types of the retina, particularly in the TH-containing dopaminergic amacrine cells, (ii) to identify PACAP-induced pathways involved in the structural rescue of the diabetic retina, with special attention to clarify the molecular background of the PACAP-induced protection in early diabetic retinopathy.

2. Materials and methods

2.1. Animals

Adult male Wistar rats ($n = 36$, weight: 300 g; source: Animal House of the University of Pecs, Medical School) were housed under light/dark cycles of 12:12 h; all experimental procedures were in accordance with approved protocols (University of Pecs, Hungary, BA02/2000-24/2011). For induction of diabetes, 70 mg/kg streptozotocin (Sigma, Hungary) was intravenously injected ($n = 20$). Diabetes induction and PACAP treatment was performed according to our previous descriptions (Szabadfi et al., 2012a). Blood glucose concentration was measured before the induction of diabetes and weekly thereafter (Glucotrend Accu-Check, Roche, Hungary). Rats with glucose levels higher than 11 mmol/l were classified as diabetic and were included in further experiments. PACAP (100 pmol/5 µl saline solution; 20 µM) was injected three times during the last week of survival: 7, 4, and 1 day before sacrifice into the vitreous body of the right eye ($n = 20$) with a Hamilton syringe under isoflurane anesthesia. The same volume of saline was injected into the other eye to serve as untreated diabetic control. The dose of PACAP was based on previous observations where this dose was effective (Szabadfi et al., 2012a; Tamas et al., 2004). A separate group of animals without induced diabetes served as control injected with saline ($n = 16$). The vitreous of the right eyes of these rats were injected with PACAP ($n = 16$). One day after the last PACAP treatment, animals were sacrificed with an overdose of anesthetic and eyes were further processed for examination.

2.2. TUNEL protocol and immunohistochemistry

Terminal transferase dUTP nick end labeling (TUNEL) was performed with fluorescein detection (TUNEL Kit, Roche, Hungary) in 10 µm thick cryostat sections after 4% PFA fixation, according to the manufacturer's protocol ($n = 8$/control; $n = 12$/diabetes, and $n = 12$/diabetes + PACAP).

Each slide was stained with 50 µl of the TUNEL reaction mixture at 37 °C for 1 h. The TUNEL reaction mixture contained 45 µl Label solution and 5 µl Enzyme solution. For positive control, retinas were incubated with recombinant DNase I (Roche, Hungary) for 10 min at 37 °C to induce DNA strand breaks prior to labeling procedures. For negative control, slides were incubated with only the Label solution (without terminal transferase) instead of the TUNEL reaction mixture. Finally, slides were rinsed in phosphate buffer with saline and coverslipped in Fluoromount-G (Southern Biotech, USA). The number of TUNEL-positive cells ± SEM was measured in 1000 µm section length of the retina. We have also determined the percentage of TUNEL-positive cells in each cellular layer of the neuroretina, values are given in percentages.

For the colocalization study, TUNEL staining was used to stain 10 µm cryostat sections simultaneously with antibodies to TH and PAC1-R. These were detected with corresponding Cy5, Alexa Fluor "568", and Alexa Fluor "405" in the dark (Table 1), then coverslipped using Fluoromount-G (Southern Biotech, USA). For control experiments, primary antibodies were omitted, and cross-reactivity of the non-corresponding secondary antibodies with the primaries was also checked. Photographs were taken with Fluoview FV-1000 Laser Confocal Scanning Microscope (Olympus, Japan) and further processed with Adobe Photoshop 7.0 program. Images were adjusted for contrast only, aligned, arranged, and labeled using the functions of the above program. Images were evaluated by an examiner blinded to the treatment.

2.3. Western Blot analysis

Retinas ($n = 8$ in control groups; $n = 8$ in diabetes, and $n = 8$ in PACAP-treated diabetic groups) were removed 24 h after the last PACAP treatment. Samples were processed for Western blot analysis as described earlier (Racz et al., 2006). Membranes were probed overnight at 4 °C with anti-caspase 8, anti-caspase 3, anti-caspase 12, anti-p38 mitogen activated protein kinases (MAPK), phospho-specific anti-p38MAPK (Thr180/Tyr182), anti-Akt,

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Table 1
Antibodies used in immunohistochemical and western blot experiments.

Primary antibodies	Company	Raised in	Dilution	Secondary antibodies	Company	Dilution	Methods
Anti-TH	Millipore, USA	Mouse	1:1000	Cy5	Invitrogen, USA	1:500	Immunhisto-chemistry (combined with TUNEL)
				Alexa Fluor "405"			
Anti-PAC1-R	Kind gift of Prof. Seiji Shioda	Rabbit	1:100	Alexa Fluor "568"			
Anti-caspase 3	Santa Cruz, Hungary	Rabbit	1:200	Horseradish-peroxidase conjugated secondary antibody	BioRad, Hungary	1:3000	Western blot
Anti-caspase 8	Cell Signaling Technology, USA	Rabbit	1:500				
Anti-caspase 12		Rabbit	1:1000				
Anti-p38MAPK		Rabbit	1:500				
Phospho-specific anti-p38 MAPK (Thr180/Tyr182)		Rabbit	1:500				
Anti-Akt		Rabbit	1:500				
Phospho-specific anti-GSK3β Ser9		Rabbit	1:500				
Phospho-specific anti-Akt-1 Ser473	R&D Systems, Hungary	Rabbit	1:1000				
Anti-ERK1, anti-ERK2	Santa Cruz. Hungary	Rabbit	1:2000				
Phospho-specific anti-ERK1/2 Thr202/Tyr204	Cell Signaling Technology, USA	Rabbit	1:1000				
Anti-Bcl-2		Rabbit	1:500				
Anti-PKC	Sigma–Aldrich, Hungary	Mouse	1:500				
Anti-GAPDH		Mouse	1:5000				

Abbreviations: ERKs: extracellular signal-regulated kinases; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GSK3β: glycogen synthase kinase 3 beta; PKC: protein kinase C; TH: tyrosine hydroxylase.

phospho-specific anti-Akt-1 Ser473, phospho-specific anti-GSK3β Ser9, anti-PKC, anti-ERK, phospho-specific anti-ERK1/2 Thr202/Tyr204, anti-Bcl-2, and anti-GAPDH antibodies (Table 1). Membranes were washed 6 times for 5 min in Tris buffered saline (pH 7.5) containing 0.2% Tween prior to addition of goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. The antibody-antigen complexes were visualized by chemiluminescence. After scanning, results were quantified by the NIH ImageJ program. The retina from each rat was analyzed twice in two separate experiments. The band intensities were normalized to GAPDH levels. Data are presented by pixel density in arbitrary unit ± SEM.

2.4. Statistical analysis

Statistical comparisons were made using one-way ANOVA test followed by Tukey-B posthoc analysis ($^{*}p < 0.05$; $^{**}p < 0.001$ vs. control group; $^{\#\#}p < 0.001$ vs. diabetic group). Data are presented as mean ± SEM.

3. Results

3.1. Apoptosis in retinal neurons

We have previously described that cones and dopaminergic amacrine cells suffer damage and Müller glial cells increase their glial fibrillary acidic protein expression in early diabetic retinopathy (Szabadfi et al., 2012a). Based on these results we examined the cell death processes in diabetic retinopathy. In contrast to the positive control (Fig. 1A) apoptotic cells were not observed in non-diabetic (Fig. 1B) and PACAP-treated non-diabetic retinas (**not shown**). However, TUNEL-positive cells were observed in all nuclear layers of the diabetic retinas (outer nuclear layer – ONL, inner nuclear layer – INL, and ganglion cell layer – GCL). Apoptotic cells included photoreceptors, bipolar, amacrine, horizontal, Müller glial, and ganglion cells (Fig. 1C). Comparison of the retinal tissues from diabetic and PACAP-treated diabetic rats revealed a different level of TUNEL-labeling. The number of TUNEL-positive cells was significantly higher in diabetic retinas (42.00 ± 11.53 cells/1000 μm retinal length) than after PACAP treatment (23.00 ± 3.63 cells/1000 μm section length) ($p < 0.05$; Fig. 1E). Distribution of TUNEL-labeled cells was almost similar in the diabetic and the PACAP-treated diabetic retina (29.38% – 12.33 ± 3.76 cells vs. 29.00% – 6.67 ± 1.45 cells in the ONL; 46.05% – 19.33 ± 5.61 cells vs. 43.48% – 10.00 ± 4.16 cells in the INL, and 24.62% – 10.33 ± 3.93 cells vs. 27.57% – 6.33 ± 3.38 cells in the GCL in the whole retinal section).

Double and triple labeling was used to confirm that dopaminergic amacrine cells were among the degenerating apoptotic cells. Therefore immunocytochemistry was combined with TUNEL. The rationale of this experiment was that we observed decreased dopaminergic amacrine cell density in diabetic retinopathy (Szabadfi et al., 2012a). These cells are known to be among the first degenerating cells in diabetic retinopathy (Seki et al., 2004). Dopamine is a trophic factor for maintaining retinal integrity and it is also known to contribute to several important physiological processes (e.g. light adaptation). TUNEL positive dopaminergic cells were consistently found in diabetic retinas (Fig. 1D), but not in vehicle-treated control (not shown) and PACAP-treated diabetic retinas (Fig. 1F). These data confirm that, among others, dopaminergic amacrine cells become apoptotic in the diabetic retina. After PACAP treatment PAC1-R containing cells and dopaminergic amacrine cells were found without TUNEL labeling, in contrast to the diabetic retinas (Figs. 1D,F).

3.2. Examination of pro- and anti-apoptotic factors by Western blot

Diabetes lasting for 3 weeks influenced the expression of both the pro- and anti-apoptotic factors. Among the pro-apoptotic factors, levels of caspase 8, caspase 3, caspase 12 (Fig. 2), p38MAPK, and p-p38MAPK (Fig. 3) were determined. Among the anti-apoptotic factors, the levels of the total protein (Akt and ERK) and phosphorylated survival kinases (p-ERK1, p-ERK2, p-Akt) and other

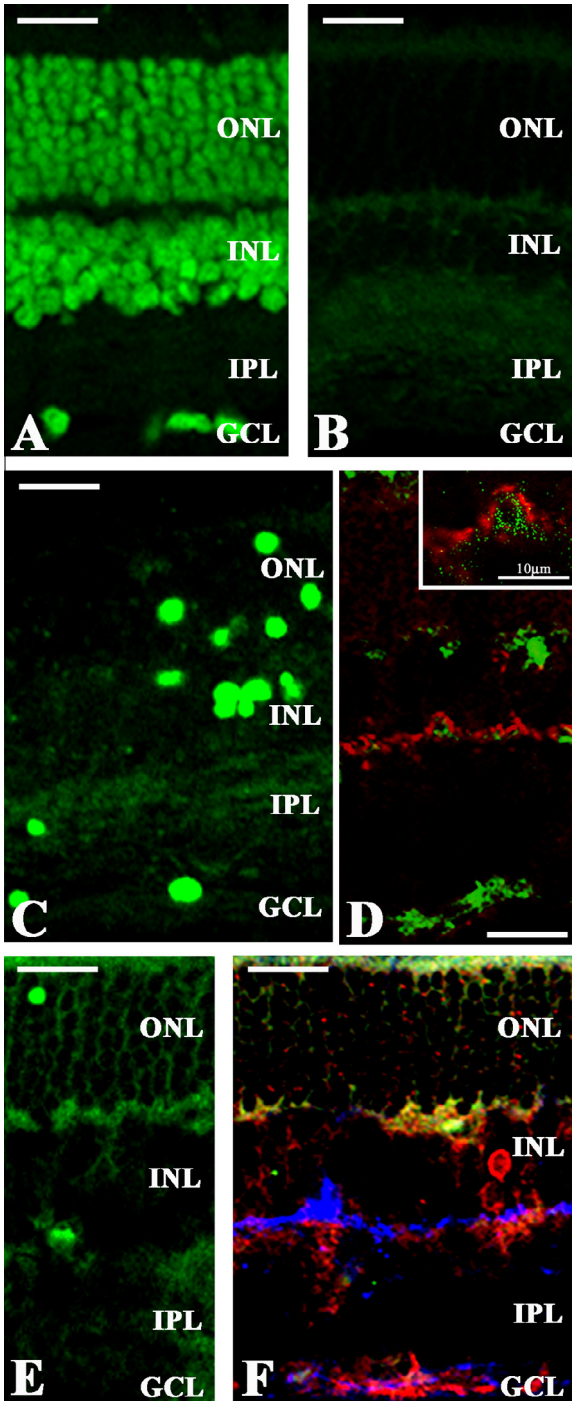

Fig. 1. TUNEL labeling method and its combination with immunohistochemistry: TUNEL positive control (A), non-diabetic (B), diabetic (C,D) and PACAP-treated diabetic (E,F) retinas. In control retina preparation we could not observe TUNEL-positive cells (B), but in diabetic retina all cellular layers (ONL, INL, GCL) contained apoptotic cells (green, C). Dopaminergic amacrine cells die by apoptosis in diabetic retina (green – TUNEL; red – dopaminergic amacrine cell; inset: TUNEL-positive dopaminergic cell with high magnification (D). Decreased number of TUNEL-positive cells could be detected in PACAP-treated diabetic retinas, only a few green cells could be found in ONL and INL (E). In diabetes + 3 × PACAP-treated group we could observe retained dopaminergic amacrine (blue) and PAC1-R containing (red) cells; a few TUNEL-positive cells are seen (green; F). Scale bars in all pictures: 20 μm. Abbreviations: ONL – outer nuclear layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

survival pathway constituents such as PKC and Bcl-2 (Figs. 4A,B) was also determined in the retinas.

Level of all examined caspases in both inactive (caspase 8–55 kDa, caspase 3–32 kDa, and caspase 12–55 kDa) and active (caspase 8–31 kDa and 26 kDa, caspase 3–12 kDa, and caspase 12–36 kDa) forms were significantly increased in diabetic retinal lysates indicating the involvement of these caspases in diabetic retinopathy-induced cell death. Administration of PACAP in diabetic rats significantly reduced the cleaved forms of the three above mentioned caspases (Fig. 2A,B,C). The activated (cleaved) form of both caspase 3 and caspase 12 could be detected only in the diabetic retinas (Figs. 2B,C). In addition, diabetes increased the total protein level of the p38MAPK and the phosphorylation of the pro-apoptotic p38MAPK, which was significantly attenuated by PACAP treatment (Fig. 3).

Total ERK1 levels increased in diabetes compared to control, control + PACAP and diabetes + PACAP conditions, while ERK2 showed an opposite pattern (Fig. 4A). PACAP treatment alone slightly increased the amount of phosphorylated form of both ERKs, while diabetes induced similar changes in phosphorylation of ERK1 and ERK2 than it was observed in the case of total ERK proteins (increased p-ERK1 and decreased p-ERK2, respectively). PACAP administration in the diabetic retina increased the phosphorylation of both ERK1 and ERK2 (Fig. 4A).

Total Akt level were identical under all examined conditions (Fig. 4B). PACAP treatment alone did not change the amount of phosphorylated form of Akt, but diabetes led to a significant decrease in the levels of p-Akt, and its' downstream target p-GSK3β, which followed the pattern of p-Akt level. PACAP administration in the diabetic retina increased the phosphorylation of the cytoprotective kinases Akt (Fig. 4B). Changes in the anti-apoptotic PKC and Bcl-2 protein showed similar pattern to that of p-Akt (Fig. 4A,B). Our results indicate that retinal PACAP treatment attenuated the diabetes-induced pro-apoptotic pathways, while it elevated the proteins of the anti-apoptotic signaling.

4. Discussion

This study reports that PACAP-induced pathways may attenuate apoptosis in diabetic retinopathy. Previous studies have shown that PACAP protects the retina from excitotoxic, ischemic, and UV-A-induced degeneration (Atlasz et al., 2010; Nakamachi et al., 2012; Racz et al., 2006; Szabo et al., 2012). PACAP exerts its protective effects by increasing anti- and decreasing pro-apoptotic factors.

Apoptosis is an early and persistent event in the diabetic retina. It can be observed even after termination of hyperglycaemia and as early as 1 month after the induction of diabetes in rats (Gao et al., 2009). Apoptosis of retinal neurons has been recognized as a critical event and a prominent pathological feature of diabetic retinopathy. The increased retinal apoptosis mainly affects the ganglion cells (Abu El-Asrar et al., 2004; Barber et al., 1998; Hammes et al., 1995; Kern and Barber, 2008). In our previous study, we showed that PACAP protected the cells in ganglion cell layer in experimental diabetes (Szabadfi et al., 2012a). In addition to the cell loss of ganglion cell layer, the degeneration of dopaminergic amacrine cells is another early neuronal event in diabetic retinopathy (Seki et al., 2004). In this study we found significantly more TUNEL-positive cells in all nuclear layers of diabetic retinas and we confirmed that dopaminergic amacrine cells died by apoptosis. Our present results provide evidence that ganglion cells, photoreceptors, bipolar, horizontal, Müller glial, and amacrine cells underwent apoptosis and that PACAP treatment could attenuate this degeneration. Among amacrine cells, dopaminergic cells are certainly affected. We suggest that PACAP has an ameliorating effect on dopaminergic cell degeneration. It has recently been suggested in an avian model of retinal development that PACAP may support

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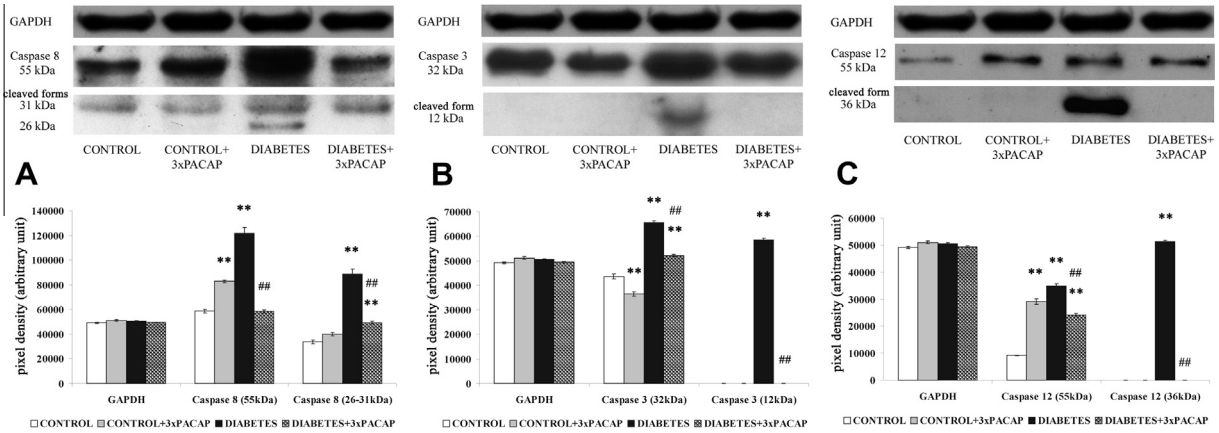

Fig. 2. Analysis of caspases in control, control + PACAP, diabetic, and PACAP-treated diabetic retinas. GAPDH served as normalization control. Blots and relative quantities (arbitrary unit) are presented of caspase 8 in (A), caspase 3 in (B), and caspase 12 in (C). Retinal caspase 8, caspase 3, and caspase 12 levels in diabetic eyes were significantly decreased in PACAP-treated diabetic eyes. Diabetes significantly increased both the non-activated (caspase 8–55 kDa, caspase 3–32 kDa, and caspase 12–55 kDa) and the cleaved (caspase 8–31 kDa and 26 kDa, caspase 3–12 kDa, and caspase 12–36 kDa) form of caspase 8, caspase 3, and caspase 12, whereas treatment with PACAP reduced both the active and the cleaved forms of the examined caspases. Data are presented as mean ± SEM. $^{**}p < 0.001$ compared to control; $^{\#\#}p < 0.001$ compared to diabetic retinas (one-way ANOVA with Tukey-B posthoc analysis).

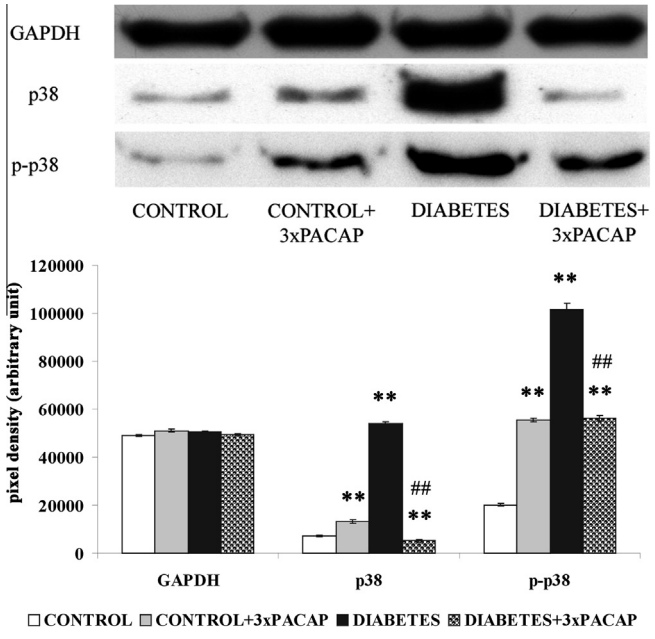

Fig. 3. Analysis of p38MAPK and p-p38MAPK in control, control + PACAP, diabetic, and PACAP-treated diabetic retinas. GAPDH served as normalization control. Results are presented in blots and relative quantities (arbitrary unit).After 3 weeks of diabetes, expression of the pro-apoptotic factor p38MAPK and p-p38MAPK was significantly higher in the retinas from control animals, while significantly decreased in the PACAP-treated diabetic retinas. Data are presented as mean ± SEM. $^{**}p < 0.001$ compared to control; $^{\#\#}p < 0.001$ compared to diabetic retinas (one-way ANOVA with Tukey-B posthoc analysis).

the appearance of cells with newly acquired dopaminergic phenotype (Fleming et al., 2013). Although this possibility cannot be discounted in our case, it is less likely than protection mediated by PACAP in case of the original dopaminergic cells.

Our further aim was to understand more details about the protective mechanisms of PACAP in diabetes. The effects of PACAP on apoptosis have been studied in several *in vitro* and *in vivo* models. PACAP influences apoptotic signaling at various levels, from initiation to downstream cytosolic and mitochondrial pathways and finally affecting executor caspases (Somogyvari-Vigh and Reglodi, 2004; Vaudry et al., 2009). A metabolic abnormality characteristic of diabetes is sufficient to upregulate the expression of the apoptosis-promoting factors and it stimulates the death pathways in retinal cells (Martin et al., 2004). Our previous observations have shown that the protective effect of PACAP involves complex pathways *in vivo*, downregulating pro-apoptotic while upregulating anti-apoptotic signaling molecules in excitotoxic and ischemic retinal injury (Racz et al., 2006; Szabo et al., 2012). Our present results further suggest that the protective effects of PACAP involve several mechanisms.

One element in this complex process is the downregulation of the precursor and active forms of caspase 8, caspase 3, and caspase 12 by PACAP in diabetic retinas. The last stage of apoptosis is marked by the activation of caspases. Activation of initiator caspases, such as caspase 8, by death receptors activates in turn the executioner caspases, such as caspase 6, 7, and 3. Endoplasmatic reticulum (ER) stress activates caspase 12, another initiator caspase, which also activate the downstream caspases, resulting in apoptosis (Degterev et al., 2003; Nakagawa et al., 2000). Our results correlate with those by Martin et al. (2004), who found strong activation of caspases in diabetic retinas. The reduced caspase activity after PACAP treatment found in the present study is in agreement with the results of several previous studies, reporting on the cytoprotective effects of PACAP. PACAP, in various concentrations, has been described to act on caspases in several neuronal and non-neuronal cell types, such as cerebellar granule cells, endothelial cells, and thymocytes (Racz et al., 2007; Vaudry et al., 2000, 2002; Zhang et al., 2012). There are less data available on the *in vivo* effects of PACAP treatment on caspases. Our present results confirm that the well-known caspase-inhibiting effect of PACAP is also present *in vivo*, in a model of diabetic retinopathy.

The upstream signaling leading to caspase activation can be very divergent. However, in the retinal degeneration models studied so far, there were standard pathways established. In these experiments p-Akt and p-ERK1/2 were always anti-apoptotic while p-p38MAPK was pro-apoptotic (Racz et al., 2006; Seaborn et al., 2011). PACAP has been previously shown to activate several anti-apoptotic factors and inhibit pro-apoptotic signaling molecules. MAPKs seem to play an important role in the PACAP-induced cellular protection in several retinal injuries *in vitro* and *in vivo* (Dziema and Obrietan, 2002; Mester et al., 2011; Racz et al., 2006; Szabo et al., 2012). We found that PACAP-treatment suppressed the expression and the phosphorylation of p38MAPK in diabetes. It has been shown that p38MAPK inhibitor SB202190 decreased

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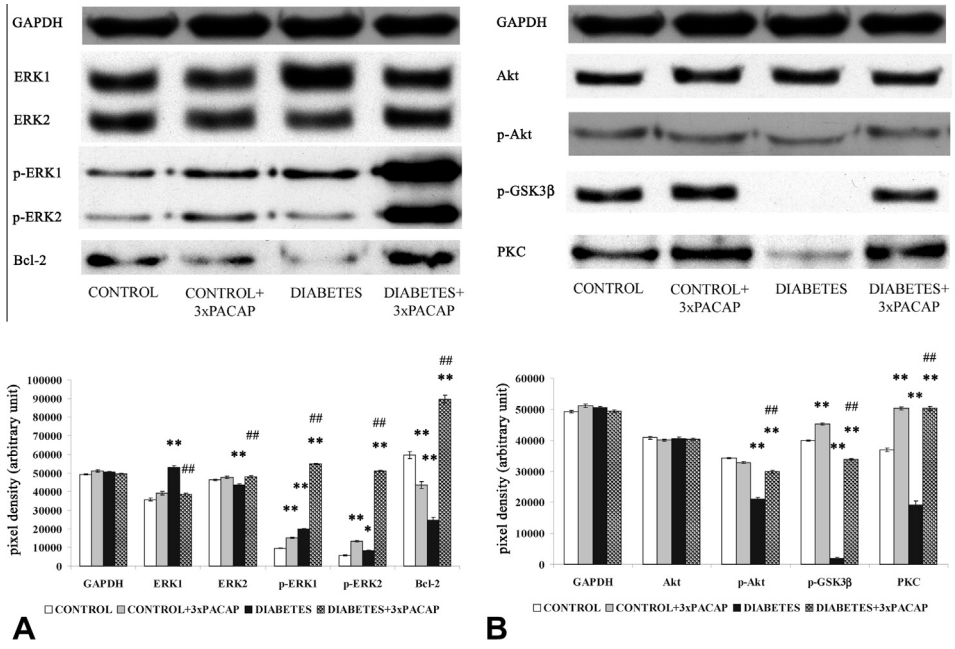

Fig. 4. Western blots of the protective factors protein levels: (A) ERK1/2, p-ERK1/2, Bcl-2; (B) Akt, p-Akt, p-GSK3β, PKC in retinas from different groups. GAPDH served as normalization control. In PACAP-treated diabetic retinas p-ERK1, p-ERK2, PKC, and Bcl-2 protein levels were significantly increased compared to diabetic retinas. p-Akt level decreased, no p-GSK3β could be observed in diabetes and increased in PACAP-treated retinas. Data are presented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.001$ compared to control; $^{\#\#}p < 0.001$ compared to diabetic retinas with one-way ANOVA with Tukey-B posthoc analysis.

the p38MAPK in the response of IL-1β (Frost et al., 2000). This process could be attenuated by PACAP treatment in early diabetic retinopathy: PACAP decreased the IL-1 level in ischemic retinal degeneration (Szabo et al., 2012) and under hyperglycemic conditions it decreased the degenerative effects on ARPE 19 cells *in vitro* (Scuderi et al., 2013). The activated ERK1/2 in PACAP-treated diabetic retinas after 3 weeks of diabetes suggests that the activity of MAPKs pathways may account, in part, for the relative protection of the retinal cells. PKC comprises a superfamily of isoenzymes that is activated in response to various stimuli, and which can take part in the delay of the onset or stop the progression of diabetic complications such as diabetic retinopathy (Pathak et al., 2012). PACAP treatment could potentiate these effects in early diabetic retinopathy. Bcl-2 family proteins are central coordinators of mitochondria-mediated apoptotic pathways. This family consists of anti-apoptotic members, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bax. It has been previously described that Bcl-2 levels decrease early in diabetic retinopathy, possibly leading to apoptosis of retinal cells (Gao et al., 2009). In agreement with this observation, the expression of the anti-apoptotic Bcl-2 was decreased in diabetic retinas as early as 3 weeks after induction of diabetes. In PACAP-treated retinas, this reduction was not so marked, possibly accounting for the observed protection by the neuropeptide. In the present study we also demonstrated that PACAP reduces apoptosis via elevated level of p-Akt protein and its downstream target GSK3β phosphorylation. Our result are in agreement with former studies on mesangial cells in induced type I diabetes (Landau et al., 2009; Lin et al., 2006), where these groups reported that increased levels of p-GSK3 were associated with suppressed apoptotic signals. In contrast to Abu El-Asrar et al. (2007) we found that Akt phosphorylation was reduced in diabetes, which was prevented by PACAP-treatment. As previously described, Akt signaling seems to play an important role in the neuroprotective effects of PACAP in different retinal and other injuries (Lazarovici et al., 2012; Li et al., 2005; Racz et al., 2006; Szabo et al., 2012).

Most of the cytoprotective effects of PACAP are mediated through activation of PAC1-R, which can induce a signaling cascade to stimulate protective factors and block caspase activation (Seaborn et al., 2011). Endogenous PACAP has also been found to have protective effects. We have shown that mice lacking endogenous PACAP are more vulnerable to injuries, including retinal ischemia (Reglodi et al., 2012; Szabadfi et al., 2012b). Furthermore, PACAP and its specific PAC1-R have been reported to be upregulated after various types of injuries (Somogyvari-Vigh and Reglodi, 2004). These data imply that cells expressing higher levels of PACAP and/or PAC1-R are more resistant to harmful stimuli. Indeed, we did not observe TUNEL positive PAC1-R containing cells any of the diabetic retinas, suggesting that PAC1-R containing cells are more resistant. In addition, we have described in our previous study that mRNA and protein levels for PAC1-R are higher in diabetic retinas after PACAP-treatment (Szabadfi et al., 2012a).

Tsutsumi et al. (2002) described that activation of VPAC1-R has been implicated in elevating glucose output, whereas activation of VPAC2-R may be involved in insulin secretion. PACAP exerts an inhibitory activity on hyperglycemia-induced endothelial cell proliferation, thus suggesting that the effect might be mediated by PAC1 and VPAC2 receptors (Castorina et al., 2010). We have also found unusual cells, like pericytes, granulocytes, and macrophages in PACAP-treated diabetic retina (Szabadfi et al., 2012a). According to our preliminary data (Szabadfi et al., 2013) this can be correlated with the changing mRNA and protein levels of VPAC1-R and VPAC2-R, through which receptors PACAP and VIP may have an action in inflammation. Thus all three PACAP receptors may have positive contribution to fighting diabetes and its consequences.

Agents which elevate the anti-apoptotic and decrease pro-apoptotic pathways can be used at formulating neuroprotective strategies. Based on our results, intravitreal PACAP-treatment acts directly along with its receptors by regulating the levels of both anti- (ERK1/2; Bcl-2; Akt; PKC) and pro-apoptotic (caspase 8; caspase 3; caspase 12; p38) proteins to which may lead to protection. The alterations in the TUNEL-labeled cells and levels of pro- and anti-apoptotic factors suggested that apoptosis would be reduced by PACAP administration in diabetic retinopathy.

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Thus, as described above, four pathways influenced by PACAP (MAPKs, PI3 K/Akt, PKC, and inhibiting ER stress by reducing active caspase 12 release) converge to minimize apoptotic damage of retinal neurons in PACAP-treated diabetic retinas. These lines of evidence suggest that PACAP might have therapeutic potential in the treatment of diabetes. Besides the effects in pancreas islets, only a few studies have shown that PACAP may also attenuate the diabetes-related pathologies. Systemic PACAP treatment decreases the streptozotocin-induced nephropathy in rats (Banki et al., 2013; Li et al., 2008), and attenuates experimental neuropathy (Dickinson et al., 1999), as well as diabetic retinopathy. In this latter respect, our findings in recent and the present studies have revealed that diabetes-induced pro-apoptotic pathways can be inhibited, while anti-apoptotic survival pathways can be stimulated by PACAP treatment *in vivo*. This series of events, leading to sufficient protection is required to maintain the structural and functional integrity of the retina during diabetic challenge. Further work is necessary to clearly distinguish what is cause or consequence in PACAP-mediated signaling in order to reduce apoptotic activity under pathologic conditions.

5. Conclusions

Our results clearly demonstrate that the protective effects of PACAP may be mediated through attenuating apoptosis. We conclude that there is a therapeutic potential of PACAP through rescuing neurons from apoptosis in diabetic retinopathy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuint.2013.11.005.

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