1. INTRODUCTION

Apoptosis is a cell death process, which occurs during development and aging of animals and several other processes. Apoptosis (from the Greek words *apo* = from and *ptosis* = falling) is one of the main types of programmed cell death. Apoptosis is carried out in an ordered process that generally confers advantages during an organism's life cycle. But the balance about apoptosis is very important, because too much apoptosis causes cell-loss disorders, whereas too little results in uncontrolled cell proliferation, namely cancer, autoimmune disorders. This balancing process is part of the homeostasis. Between 50 and 70 billion cells die each day due to apoptosis in the average human adult. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight. Homeostasis is achieved when the rate of mitosis (cell proliferation) in the tissue is balanced by cell death. The diseases in which apoptosis has been implicated can be grouped into 2 broad groups: those in which there is increased cell survival (i.e. associated with inhibition of apoptosis) and those in which there is excess cell death (where apoptosis is overactive).

1.1. Signaling background of apoptosis

Signaling for apoptosis occurs through multiple independent pathways that are initiated either from triggering events within the cell or from outside the cell, for instance, by ligation of death receptors.

The mitogen activated protein kinase (MAPK) cascades, which are generally activated in response to mitogenic signals, cytokines and environmental stress, and lead to activation of the members of the MAPK family: three subfamilies have been identified in mammalian cells - extracellular signal-regulated kinase-p44/42 MAPK (ERK1/2), p38 MAP kinase (p38) and c-Jun N-terminal kinase (JNK). These MAPK cascades have been shown to participate in a wide array of cellular functions, such as cell growth, cell differentiation and apoptosis. Although many MAPK activiting stimuli are proapoptotic or antiapoptotic, the biological outcome of MAPK activation is highly divergent and

appears to be largely dependent on the cell type. ERK1/2 pathways are usually linked to growth factor action and are associated with cellular differentiation, proliferation and survival. JNK1/2 and p38 MAPK are serine and threonine protein kinases that are activated by various stress stimuli.

All apoptosis signaling pathways converge on a common machinery of cell destruction that is activated by a family of cysteine proteases (caspases) that are activated by processing from its inactive precursor (zymogen) (Strasser et al., 2000). Thirteen members of the human caspase family have been identified. Some of the family members are involved in apoptosis, and these can be divided into two subgroups. The first group consists of caspase 8, caspase 9, and caspase 10, which contain a long prodomain at the N-terminus, and function as initiators of the cell death process. The second group contains caspase 3, caspase 6, and caspase 7, which have a short prodomain and work as effectors, cleaving various death substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells. The other effector molecule in apoptosis is Apaf-1 (apoptotic protease activating factor), which, together with cytochrome c, recruits pro-caspase 9 in an ATP (or dATP)-dependent manner, and stimulates the processing of pro-caspase 9 to the mature enzyme. The other regulators of apoptosis are the members of the Bcl-2 family. Eighteen members have been identified for the Bcl-2 family, and divided into three subgroups based on their structure. Members of the first subgroup, represented by Bcl-2 and Bcl-xL, have an anti-apoptotic function. Members of the second subgroup, represented by Bax and Bak, as well as members of the third subgroup such as Bid and Bad, are pro-apoptotic molecules. The signal transduction pathway for a death factor (Fas ligand)-induced apoptosis has been well elucidated. Binding of Fas ligand to its receptor results in the formation of a complex (DISC, death-inducing signaling complex) consisting of Fas, FADD, and pro-caspase 8. Pro-caspase 8 is processed to an active enzyme at the disc. There are two pathways downstream of caspase 8. In one type of cells, such as thymocytes and fibroblasts, caspase 8 directly activates caspase 3. In type II cells such as hepatocytes, caspase 8 cleaves Bid, a member of the Bcl-2 family. The truncated Bid then translocates to mitochondria and stimulates release of cytochrome c. Once cytochrome c is released, it binds with Apaf-1 and ATP, which then binds to pro-caspase-9, creating a multi-protein complex known as apoptosome. The apoptosome

cleaves this pro-caspase, rendering the active form of caspase-9, which in turn activates effector caspase-3. (Fig.1).

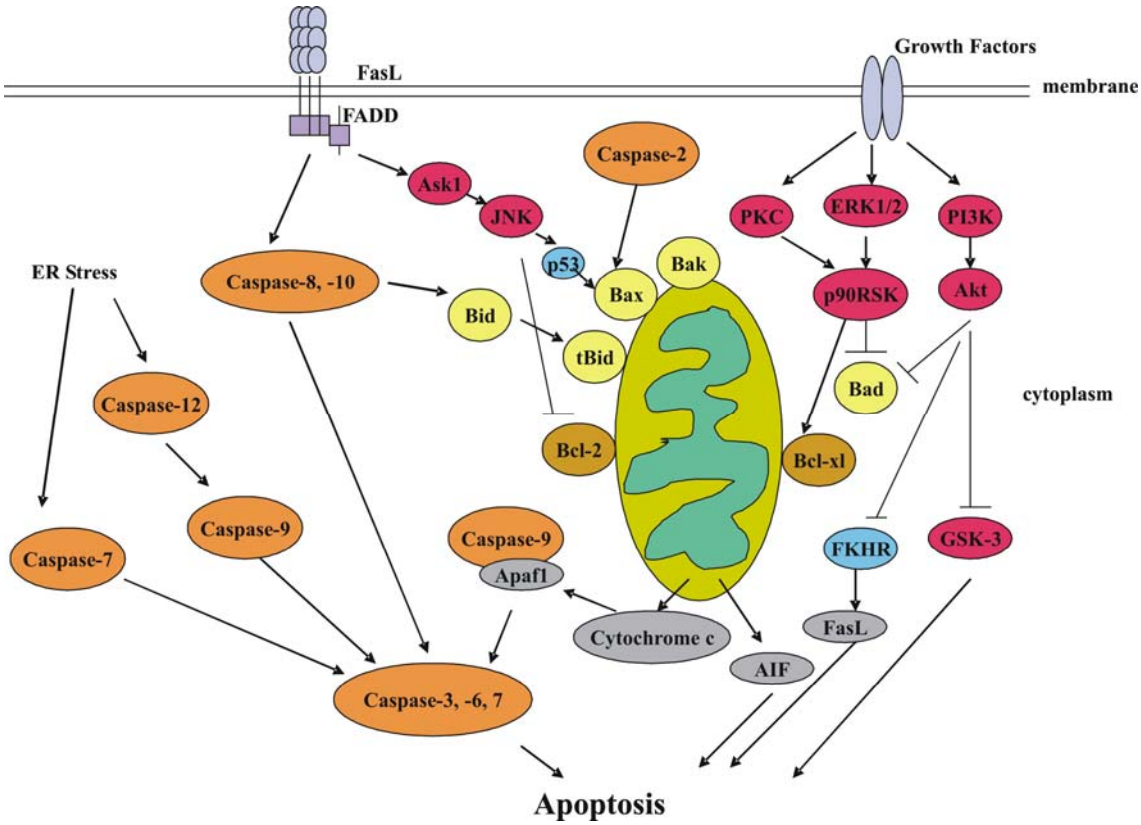

Fig.1. Schematic figure about apoptosis

In our study we focused on the activation of members of mitogen activated protein kinase (MAPK) family: extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2) and p38 MAPK; cAMP-responsive element binding protein (CREB); Bcl-2; phospho-Bad; apoptosis inducing factor (AIF); cytochrome c and caspase-3.

Analysis of these regulatory pathways has led to a better understanding of the etiology and pathogenesis of many human diseases and neurodegenerative/neurodevelopmental diseases. The mechanism by which factors protect the cells from degeneration is unclear and we tried to investigate the challenge of converting that understanding into new therapeutic modalities. Pituitary adenylate cyclase activating polypeptide (PACAP) could be one of the new therapeutic peptides and that is the most important reason for investigating the effects of PACAP in different in vitro and in vivo models.

1.2. Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami on the basis of its ability to stimulate cAMP formation in pituitary cells (Miyata et al., 1989). PACAP exists in two forms, with 27 and 38 amino acid residues (Miyata et al., 1989, 1990). PACAP belongs to the VIP/secretin/glucagon family of peptides and shares 68% identity with vasoactive intestinal peptide (VIP), but its adenylate cyclase stimulating activity has been shown to be 1000-10000 times greater than that of VIP. The primary structure of PACAP-38 is identical among all mammalian species examined, and it also shows marked similarity with lower vertebrates examined, and nonvertebrates, with differences in only 1-4 amino acids (rev: Arimura, 1998). This suggests that the structure of PACAP has remained very conserved throughout phylogenesis and it may reflect its importance in fundamental functions in the nervous system. Despite the high similarity between VIP and PACAP, the distribution of these peptides is quite different. In mammalian tissues, the 38 amino acid form of PACAP is prevalent, constituting approximately 90% of the peptide. The PACAP receptors belong to the family of G protein-coupled receptors with seven transmembrane domains. There are two types of PACAP receptors: PAC1 receptor which bind PACAP with high affinity and VIP with a much lower affinity and VPAC1 and VPAC2 receptors which bind VIP and PACAP with similar affinities (Arimura, 1998, Vaudry et al., 2000). Similar to other “brain-gut peptides” PACAP is localized not only in the central but in the peripheral nervous system and also in non-neural tissues, such as in endocrine glands and the gastrointestinal tract. PAC1 receptor is coupled to adenylate cyclase and phospholipase C (PLC). Through adenylate cyclase activation, it elevates cAMP, and activates protein kinase A (PKA), which can activate the mitogen-activated protein kinase (MAPK) pathways (Vaudry et al., 1998).

PACAP is a potent anti-apoptotic agent (rev.: Somogyvari and Reglodi, 2004). Its anti-apoptotic effects have been extensively studied in various neuronal cell lines. PACAP has been shown to have anti-apoptotic effects also in non-neuronal cells, like mature T cells, thymocytes, ovarian follicular cells, pituitary cells and several cancer cell lines (Delgado et al., 1996, 2000a,b; Gutierrez-Canas et al., 2003; Le et al., 2002; Lee et al., 1999; Oka et al., 1999). However, its effect on the survival of cardiomyocytes is not known. VIP, the closest structural homologue peptide of PACAP, has been

reported to play a cardioprotective role during myocardial ischemia (Gyongyosi et al., 1997; Kalfin et al., 1994; Roudenok et al., 2001). VIP may also play an important role in protection against myocardial fibrosis: endogenous VIP depletion is associated with the degree of fibrosis and increased VIP concentrations have protective effects (Ye et al., 2002, 2003, 2004). A recent paper has reported similar findings with PACAP: it inhibited protein synthesis and DNA synthesis in cardiac fibroblasts, which implies that PACAP may also act as a protective mediator against myocardial fibrosis (Sano et al., 2002). However, the anti-apoptotic effect of PACAP has been shown to be much stronger than that of VIP: VIP is protective against the toxic effects of various agents either at much higher concentrations than that of PACAP or is not protective at all (rev.: Somogyvari-Vigh and Reglodi, 2004). In oxidative stress-induced apoptosis of cerebellar granule cells, VIP had no effect (Vaudry et al., 2002a). Most available data indicate therefore, that the anti-apoptotic effects of PACAP are mediated through the specific PAC1 receptor (rev.: Somogyvari-Vigh and Reglodi, 2004). PACAP is also a highly potent vasorelaxant peptide and causes vasodilation in a wide range of concentrations (Vaudry et al., 2000). The concentration of PACAP is significantly higher in coronary and cerebral arteries compared to other blood vessels, and PACAP causes significant vasodilation in coronary blood vessels, which might be an important caridoprotective factor (Dalsgaard et al., 2003).

The neuroprotective effects of PACAP have been demonstrated against various toxic agents in vitro and in different models of neuronal pathologies in vivo (Somogyvari-Vigh and Reglodi, 2004). VIP, structurally the closest to PACAP, was first shown to protect retinal ganglionic cells against glutamate toxicity in vitro (Shoge et al., 1998). Subsequently, similar neuroprotection has been described for PACAP (Shoge et al., 1999). These pioneer works have been followed by a few other studies showing that PACAP is protective in the retina against various injuries, including excitotoxicity (Silveira et al., 2002; Seki et al., 2003; Rabl et al., 2002). In the past few years, we have provided evidence for the neuroprotective effects of PACAP in various in vivo models such as Parkinson`s disease, Huntington chorea, neurotrauma and focal cerebral ischemia (Somogyvari-Vigh and Reglodi, 2004; Reglodi et al., 2005; Farkas et al., 2004; Tamas et al., 2002). We have conducted several experiments on the effects of

neonatal MSG administration and the protective effects of PACAP in this model (Tamas et al., 2004; Babai et al., 2005, 2006; Racz et al., 2006a,b; Kiss et al., 2006).

In summary, PACAP is a potent anti-apoptotic agent in the neuronal system, but its effect on the survival of cardiomyocytes and endothelial cells is not known. The neuroprotective effects of PACAP have been demonstrated against various toxic agents in vitro, but its effect on the possible protective signaling pathways in the in vivo model of retina degeneration is not known.

2. AIMS AND HYPOTHESIS

The aim of the thesis was to investigate apoptosis and its signaling pathways in different models in vivo and in vitro. Pituitary adenylate cyclise activiting polypeptide (PACAP) could be one of the new therapeutic peptides and that is the most important reason for investigating the effects of PACAP in cardial, endothelial and retinal models.

1. PACAP has well-known neuroprotective effects, and one of the main factors leading to neuroprotection seems to be its anti-apoptotic effects. The peptide and its receptors are present also in the heart, but whether PACAP can be protective in cardiomyocytes, is not known. Therefore, the aim of the study was to investigate the effects of PACAP on oxidative stress-induced apoptosis in cardiomyocytes.

2. In lung cancer and pituitary cells PACAP stimulates production of vascular endothelial growth factor (VEGF). However, no data are presently available on the direct effects of PACAP on endothelial cell survival. Vasoactive intestinal peptide (VIP), the structurally closest related peptide to PACAP, has been reported to promote survival of human corneal endothelial cells. Accordingly, the aim of the study was to investigate the effects of PACAP on endothelial cell survival and on oxidative stress-induced changes in the activation of members of the MAPK family.

3. PACAP and its receptors can be found in the retina and it is an important transmitter in the retinohypothalamic tract. The well-known neuroprotective effects of PACAP have also been shown in vitro in the retina in some pathological conditions such as hypoxia and optic nerve dissection. Elevated glutamate levels lead to retinal damage and PACAP has been shown to be protective against glutamate-induced cell death in the retina in vitro. Recently, we have shown that this protective effect is also present in vivo, in monosodium-glutamate (MSG)-induced retinal degeneration. The underlying molecular mechanism of this protective effect is not yet known. Retinal cell death induced by over-stimulation of glutamate receptors is related to apoptosis. The aim of this part of the study was to further elucidate the possible signaling pathways involved in the protective effects of PACAP against MSG-toxicity in vivo.

3. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE PROTECTS CARDIOMYOCYTES AGAINST OXIDATIVE STRESS-INDUCED APOPTOSIS

A large number of experimental data supports the presence of apoptosis in a variety of cardiovascular diseases and, along with that, numerous chemicals have been reported to be cardioprotective through inhibition of cardiomyocyte apoptosis. Oxidative stress is a major apoptotic stimulus in many cardial diseases. Various growth factors have been shown to protect the heart against oxidative stress, which may involve attenuation of cardiac myocyte apoptosis. PACAP and its receptors are present in the heart, but whether PACAP can be protective in cardiomyocytes, is not known.

Therefore, the aim of the present study was to investigate the effects of PACAP on oxidative stress-induced apoptosis in cardiomyocytes.

3.1. BACKGROUND

Both PACAP and VIP occur in the nerve fibers and cell bodies within the cardiac plexus (Calupca et al., 2000; DeHaven et al., 2002; Richardson et al., 2003). Also, PACAP receptors have been detected in the cardiac neurons. PAC1 receptor, which is specific for PACAP, occurs in multiple isoforms in cardiac neurons, where the common VPAC1 and VPAC2 have also been demonstrated (Bardon et al., 2001; DeHaven et al., 2002; Sano et al., 2002; Wei et al., 1996). Numerous actions on the cardiac myocytes and coronary blood vessels both in vitro and in vivo have been shown: PACAP modulates excitability of intracardiac neurons, affects cardiac rhythm, contractility, output and atrial natriuretic peptide secretion (Basler et al., 1995; Braas et al., 1998, 2004; DeHaven et al., 2004; Hirose et al., 1997, 2001a,b, 2003; Markos et al., 2002; Merriam et al., 2004; Parsons et al., 2000; Seebeck et al., 1996). Due to its inotropic, chronotropic and dromotropic effects, PACAP has been suggested as a candidate for treatment of heart failure (Vaudry et al., 2000). In PAC1 deficient mice, pulmonary hypertension and right heart failure have been demonstrated (Otto et al., 2004).

A large number of experimental data supports the presence of apoptosis in a variety of cardiovascular diseases and, along with that, numerous chemicals have been reported to be cardioprotective through inhibition of cardiomyocyte apoptosis (rev.: Chen and Tu

2002; Kumar et al., 2003). Oxidative stress is a major apoptotic stimulus in many cardiac diseases. Various growth factors have been shown to protect the heart against oxidative stress, which may involve attenuation of cardiac myocyte apoptosis (rev.: Chen and Tu 2002; Kumar et al., 2003; Suzuki, 2003).

There are numerous factors that play a role in both neurodegeneration and cardiomyocyte degeneration, which also implies the possibility of similar protective strategies (Bogoyevitch, 2004; Miller, 2001). The well documented neuroprotective effects of PACAP and the occurrence of PACAP and PACAP receptors in the heart raise the question whether PACAP is able to counteract the deleterious effects of agents leading to cardiomyocyte degeneration. In the present study, we investigated the effects of PACAP on oxidative stress-induced cardiomyocyte death. The main pathways in cardiomyocyte apoptosis are the induction of the pro-apoptotic members of the Bcl-2 family and the activation of the pro-apoptotic terminal caspase-3 (Chen and Tu, 2002; Feng et al., 2004; Kumar et al, 2003; Vaudry et al., 2000). Bcl-2 is an anti-apoptotic protein, while Bad and Bax are pro-apoptotic members of the family. The phosphorylation of Bad inhibits its binding to, and inactivation of anti-apoptotic Bcl-2. Thus, increases in phospho-Bad and expression of Bcl-2 act against apoptosis (Feng et al., 2004). Based on the above-described importance of caspase-3, Bcl-2 and phospho-Bad in cardiomyocyte apoptosis, we investigated the effects of PACAP on the expression of these apoptotic markers in addition to assessing cell viability and apoptosis in a rat cardiomyocyte culture.

3.2. MATERIALS AND METHODS

3.2.1. Cell culture

Primary culture of neonatal rat cardiomyocyte was prepared as described previously (Luodonpa et al., 2001, Tokola et al., 1994). Briefly, cells were obtained from ventricular myocytes of 2–4-day-old Wistar rats, using collagenase (Gibco™ Collagenase Type II, Invitrogen Corp., USA). Isolated cells were plated on collagen I-coated plates (Coll Typ 1 cellcoat, Germany) at the density of 200 000 cm^{-2}. Cells were incubated in DMEM/F12 medium (Sigma–Aldrich, USA) supplemented with 10% of fetal bovine serum (Gibco, USA). The following day, when the cells attached to the plate firmly, the medium was replaced with complete serum free medium (CSFM) containing the following supplements: BSA (2.5%, AlbuMax 1, Invitrogen, USA), insulin (1 μM), transferrin (5.64 μg/ml), selenium (32 nM) (insulin–transferrin–sodium–selenit media supplement, Sigma, Hungary), sodium pyruvate (2.8 mM, Sigma, Hungary), 3,3′,5′-triiodo-l-thyronine sodium salt (1 nM, Sigma, Hungary), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) (PS solution, Sigma, Hungary). Experiments started 48 h after incubation with CSFM and the medium was changed every 24 h.

3.2.2. Experimental protocol

Cultured cardiomyocytes were randomly assigned to different experimental groups:

- control group of cells, incubated in CSFM without treatment;

- cells exposed to 1 mM of H_2O_2;

- cells incubated in medium containing 20 nM PACAP1-38;

- cardiomyocytes subjected to treatment of 250 nM PACAP receptors antagonists PACAP6-38;

- cells treated with 1 mM H_2O_2 together with PACAP1-38;

- to antagonize the effect of PACAP1-38, 250 nM of the PACAP receptors antagonists PACAP6-38 was added simultaneously with 1 mM H_2O_2.

Cells were exposed to the mentioned concentration of chemicals for 4 h. Evaluation of cell survival was performed immediately after termination of treatments. Assessment of apoptotic signaling markers was also started after treatments until permeabilization, and samples were stored at −20 °C until further processing according to the protocols supplied by the manufacturers. Experiments were repeated six times in duplicate wells. The selected dose of H_2O_2 was based on our preliminary studies showing a significant apoptotic effect without considerable necrosis at this concentration in cardiomyocytes, and the dose of PACAP was based on in vitro studies using PACAP as a protective agent against various toxic effects, where the range of 10–100 nM was proven to be the most effective (Ito et al., 1999; Somogyvari-Vigh and Reglodi, 2004; Vaudry et al., 2000, 2002a,b).

3.2.3. Cell viability assay

Viability of cardiomyocytes were determined by colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Hungary). The assay is based on the reduction of MTT into a blue formazan dye by viable mitochondria. At the end of the treatments, the medium was discarded from plates and the cells were subsequently washed twice with phosphate buffered saline (PBS, Sigma, Hungary). Cells were then incubated with PBS containing 0.5 mg/ml of MTT for 3 hours at 37°C in an atmosphere of 5% CO_2. The solution was aspirated carefully and 1 ml of dimethylsulfoxide was added to dissolve the blue-colored formazan particles. Samples from duplicate wells were transferred to a 96-well plate and absorbance was measured by an ELISA reader (Sirio microplate reader, Seac Corp., Italy), at 570 nm representing the values in arbitrary unit (AU). Results are expressed as percentage of control values.

3.2.4. Annexin V and propidium iodide staining of the cardiomyocytes

By conjugating a fluorescent group to annexin V, apoptosis can be quantitatively detected using fluorescence microscopy or flow cytometry, and this assay has been used together with other criteria to detect apoptosis in cardiomyocytes. The ratio of apoptosis was evaluated after double staining with fluorescein isothiocyanate (FITC)-labeled

annexin V and propidium iodide (BD Biosciences) using flow cytometry (Vermes et al., 1995). First, the medium was discarded and wells were washed twice with isotonic sodium chloride solution. Cells were removed from plates using a mixture of 0.25 % trypsin (Sigma), 0.2 % ethylene-diamin tetra-acetate (EDTA; Serva), 0.296 % sodium citrate, 0.6 % sodium chloride in distillated water. This medium was applied for 15 min at 37 °C. Removed cells were washed twice in cold PBS and were resuspended in binding buffer containing 10 mM Hepes NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$. Cell-count was determined in Burker's chamber for achieving a dilution in which 1 ml of solution contains 10^6 cells. One hundred microliters of buffer (10^5 cells) was transferred into 5 ml round-bottom polystyrene tubes. Cells were incubated for 15 min with fluorescein isothiocyanate (FITC) conjugated annexin V molecules and propidium iodide (PI). After this period of incubation, 400 µl of annexin-binding buffer (BD Biosciences) was added to the tubes, as described by the manufacturers. The samples were immediately measured by BD FacsCalibur flow cytometer (BD Biosciences).

Results were analyzed by Cellquest software (BD Biosciences). Quadrant dot plot was introduced to identify living and necrotic cells and cells in early or late phase of apoptosis. Necrotic cells were identified as single PI-positive. Apoptotic cells were branded as annexin V-FITC-positive only and cells in late apoptosis were recognized as double-positive for annexin V-FITC and PI. Cells in each category were expressed as percentage of the total number of stained cells counted.

3.2.5. Assessment of phospho-Bcl-2, phoshpo-Bad and caspase-3 cleavage

Following the 4-h-long treatments in different groups, cells were harvested by trypsin-EDTA according to the protocol described at annexin V-PI staining. Cells were pelleted by centrifugation (175 g, 5 min) and then fixed in 1% formaldehyde in PBS for 10 min at 37 °C. After 1 min of chilling, cell suspensions were centrifuged again followed by permeabilization applying 90% methanol (Sigma, Hungary) for 30 min at 4 °C. Each tube of cells was rinsed twice with 0.5% bovine serum albumin (BSA) (Invitrogen, USA) and finally appropriate amounts of cells ($0.5–5 \times 10^6$) were resuspended in 0.5% BSA. Cells were then incubated for 10 min at room temperature. Subsequent to blocking of cells in BSA, an appropriate dilution of the primary antibody was added to the solution and was incubated for 45 min at room temperature. Polyclonal antibodies against active caspase-3, phospho-Bcl-2 and phospho-Bad were from Cell Signaling

Technology Inc. (USA), and were used at dilutions 1:10 (caspase-3), 1:50 (Bcl-2) and 1:100 (Bad). After centrifugation, the supernatant was carefully aspirated and the cells were resuspended in 100 µl 0.5% BSA containing FITC conjugated secondary antibody (Sheep anti rabbit IgG; Serotec Inc., USA) at a dilution of 1:50, and were incubated for 30 min. Fluorescent staining of samples was quantified by flow cytometric measurement of 10 000 cells. To determine the non-specific marking of cells, the secondary antibody was applied for 30 min without primary antibody following permeabilization. Our results were analyzed by Cellquest software (BD Biosciences, USA) measuring the appearance of caspase-3, phospho-Bcl-2 and phospho-Bad in the cells as mean fluorescence intensity (MFI).

3.2.6. Statistical analysis

Data were presented as mean±S.E.M. from three independent experiments, analyzed with one-way ANOVA followed by Neuman–Keul's *post hoc* analysis, and were considered significant at $P<0.05$.

3.3. RESULT

To establish whether PACAP influences viability of cells exposed to H_2O_2, mitochondrial viability in cardiac cells was measured by MTT assay (Fig.2). Administration of H_2O_2 alone resulted in manifest decrease in cell viability compared to control values (58.2 ± 11.0%). Treatment with PACAP1-38 and PACAP6-38 alone did not alter cell viability. However, when H_2O_2-treated cells were incubated with PACAP1-38, cell viability significantly increased (85.8 ± 15.8% of control) when compared to the viability of H_2O_2-treated cells. Incubation of cultured cells with the PACAP receptors antagonists, PACAP6-38, significantly attenuated the beneficial effect of PACAP1-38 on cell viability (56.1 ± 11.9% of control values) (Fig.2).

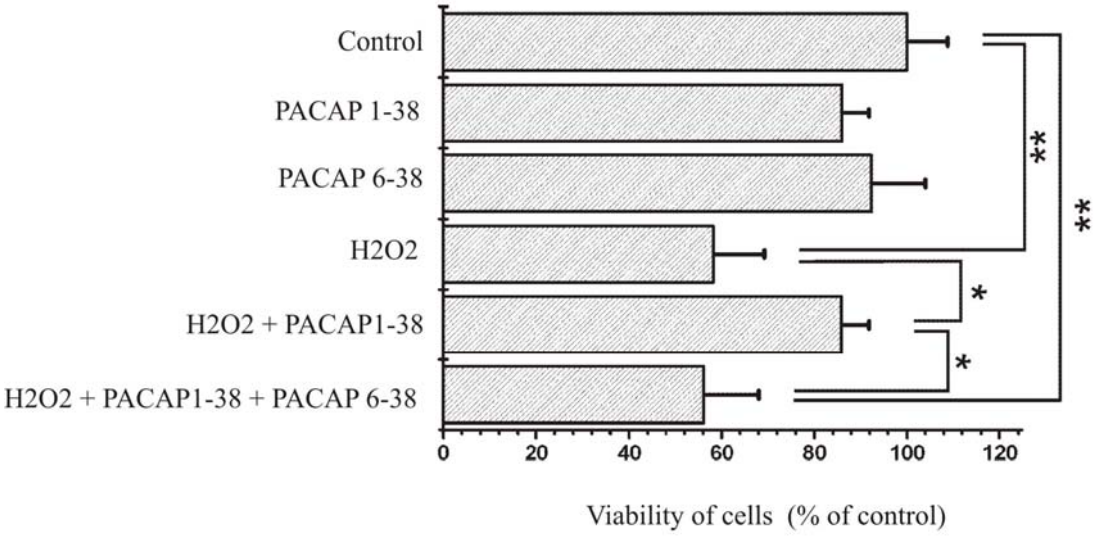

Fig.2. PACAP1-38 counteracts the toxic effect of H_2O_2 in neonatal rat cardiomyocytes as measured by MTT assay. Data are presented as mean ± S.E.M. obtained from six independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

The control group had 90.5 ± 1.3% of intact, living (annexin V and PI negative) cells and 5.4 ± 1.6% of cells in early phase of apoptosis (annexin V positive and PI negative) (Fig.3A,B,C). An increase of apoptotic cells (21.6 ± 3.8%) was observed in the H_2O_2-treated group with a lower number of living cells (76.7 ± 3.6%). PACAP1-38 and PACAP6-38 administration alone caused no changes in the percentage of living and apoptotic cells related to control values. PACAP1-38 administration led to a significant increase in the percentage of living cells (91.1 ± 0.9% of control) and a reproducible decrease in the rate of apoptosis (6.7 ± 1.7%) in cells exposed to H_2O_2 (Fig.3A,B,C). This beneficial effect of PACAP1-38 was diminished by PACAP6-38 (living cells: 81.8 ± 1.2%, apoptotic cells: 14.1 ± 1.8%).

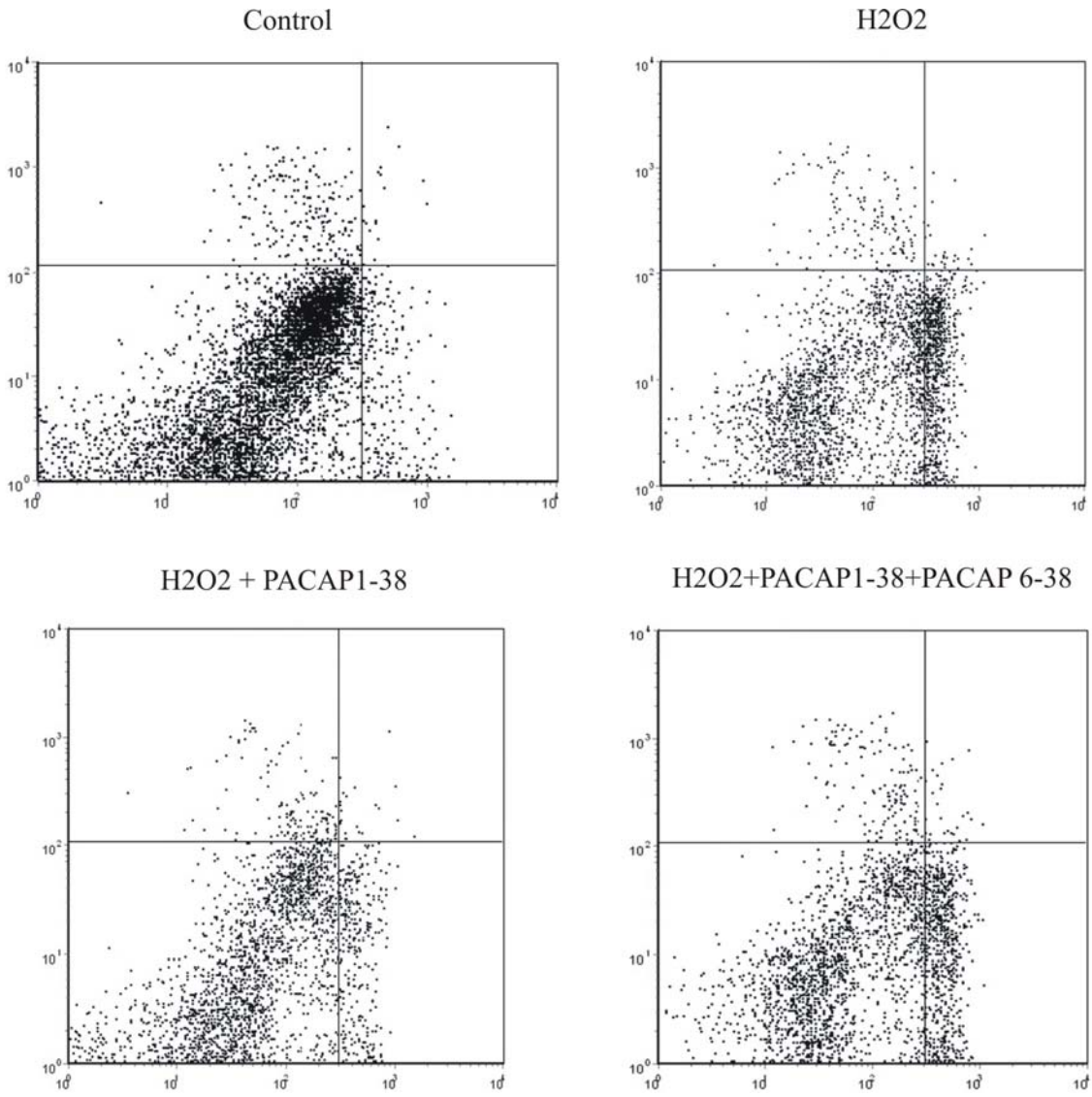

Fig.3A Fig. Distinction between living, necrotic, early and late apoptotic cells. Examples of dot-plots as determined by flow cytometry following annexin V and propidium iodide double-staining. Horizontal axis represents annexin V intensity and vertical axis shows propidium iodide staining. The lines divide each plot into four quadrants: lower left quadrant: living cells, lower right quadrant: early apoptotic cells, upper left quadrant necrotic cells, upper right quadrant: late apoptotic cells.

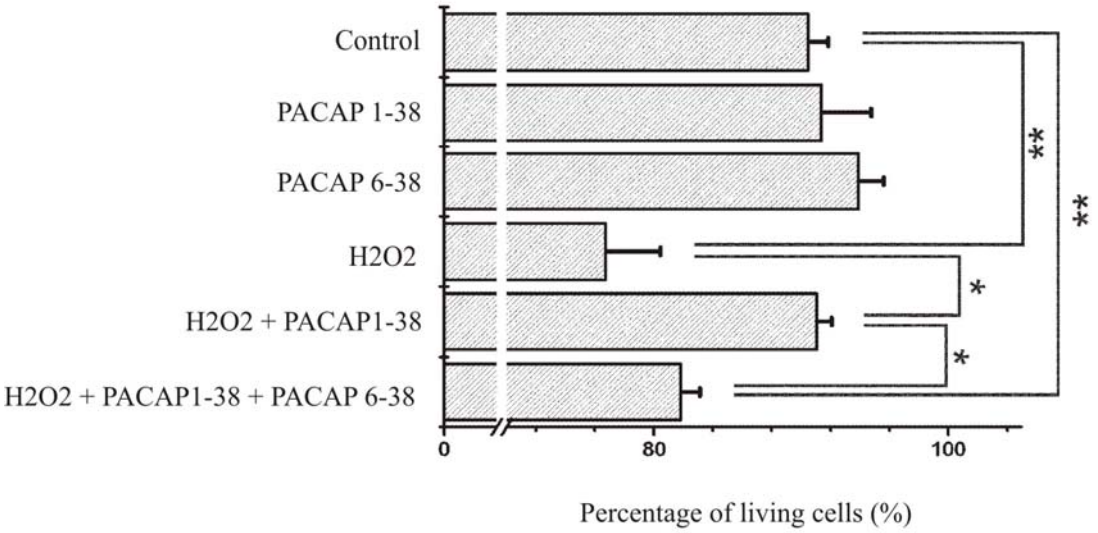

Fig.3B. Graphs demonstrate the mean percentage of living cells. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$.

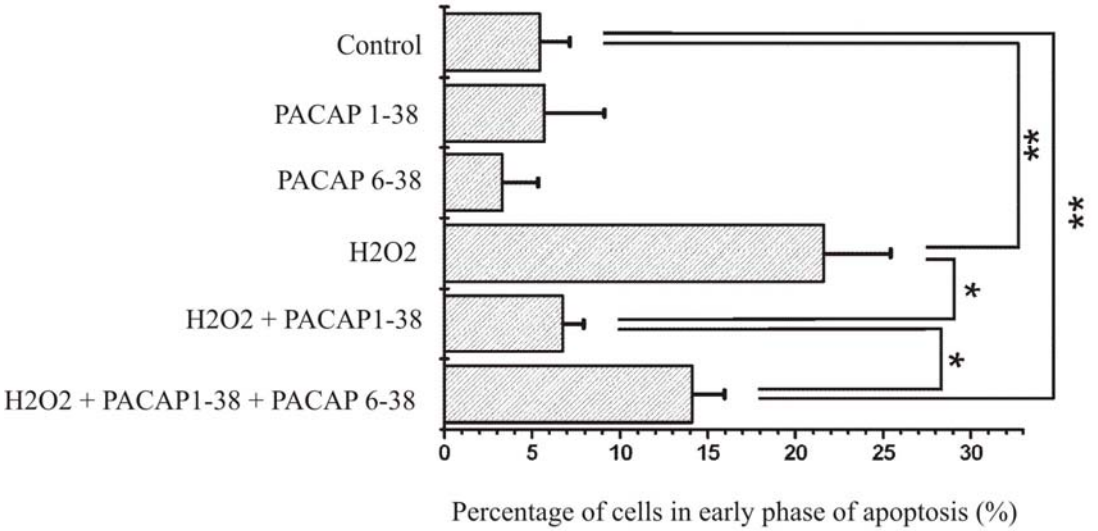

Fig.3C. Graphs demonstrate the ratio of cells in early apoptosis (C). Data are expressed as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$.

The rate of apoptosis was further examined by measuring caspase-3 activity and the level of phospho-Bcl-2 and phospho-Bad using flow cytometry. Neither PACAP1-38 nor PACAP6-38 alone altered the examined apoptotic markers (Fig.4 and Fig.5A,B,C).

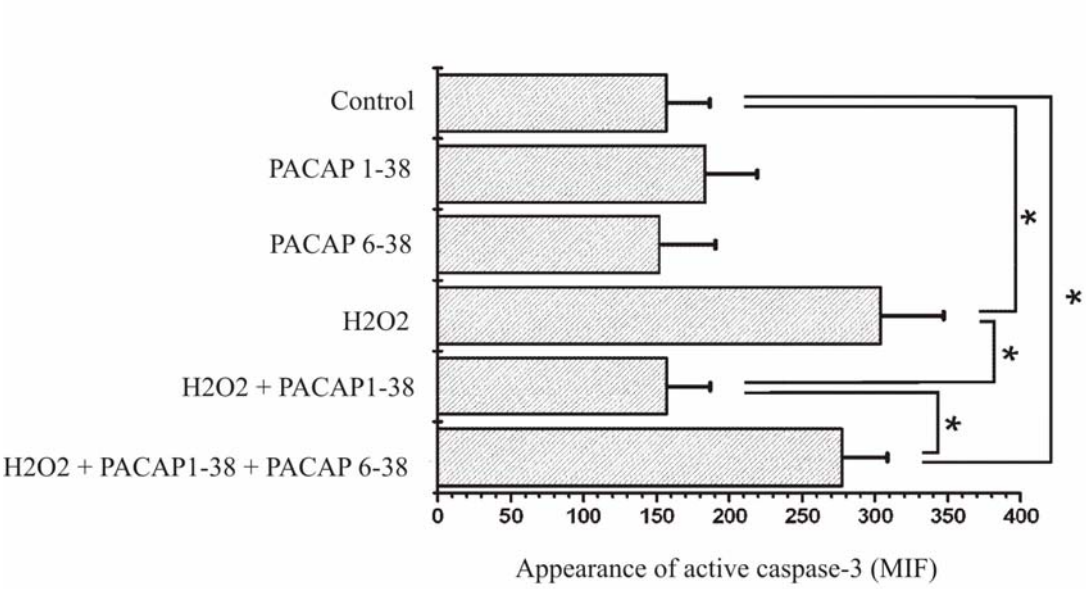

Fig.4. Down-regulation of active caspase-3 by PACAP1-38 in cardiomyocytes exposed to oxidative stress. Data are presented as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$.

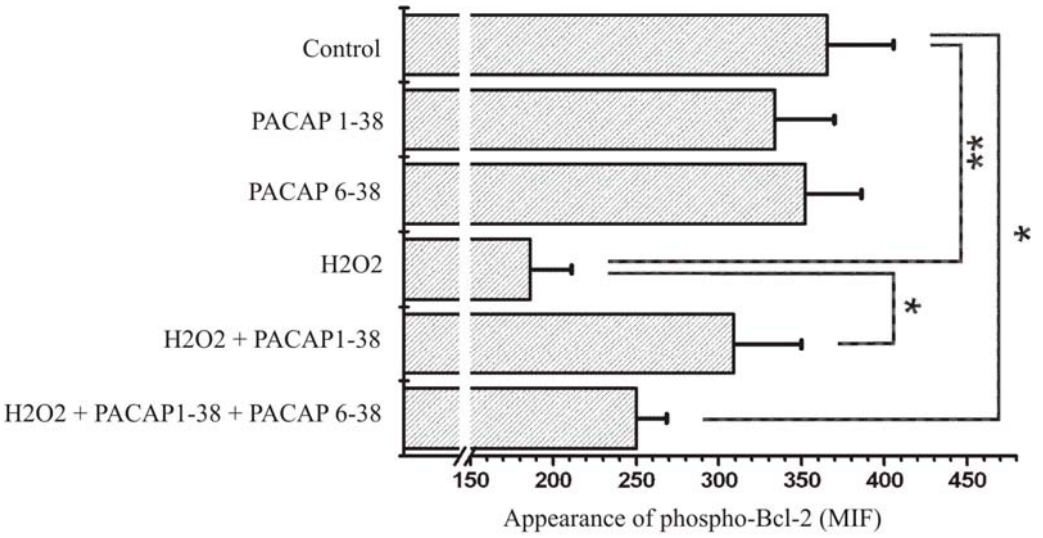

Fig.5A. Effect of PACAP1-38 on appearance of phospho-Bcl-2 in cells submitted to high concentration of H_2O_2. Results are expressed as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$.

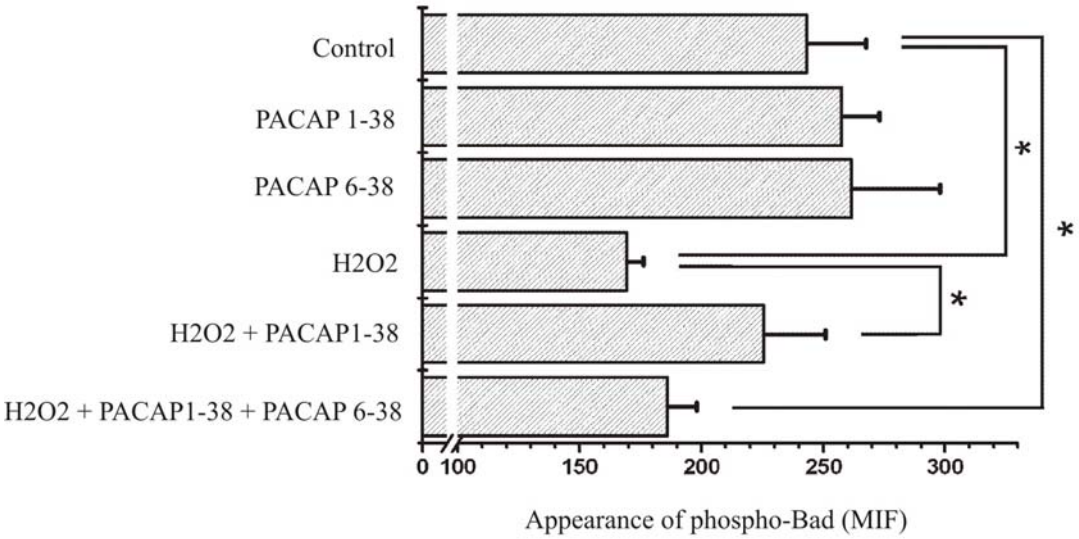

Fig.5B. Effect of PACAP1-38 on appearance of phospho-Bad in cells submitted to high concentration of H_2O_2. Results are expressed as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$.

Regarding the pro-apoptotic marker of caspase cleavage, we found that the applied concentration of H_2O_2 stimulated massive up-regulation of active caspase-3 levels (Fig.4). The amount of active caspase-3 was markedly reduced by PACAP1-38 in cells exposed to H_2O_2. The PACAP antagonist could abolish the advantageous effect of PACAP1-38 on caspase activation as compared to the control group and the group receiving co-treatment with PACAP1-38 and H_2O_2 (Fig.4).

The anti-apoptotic protein Bcl-2 plays an important role in protecting various cell types from injuries and phosphorylation of Bcl-2 at Ser70 may be necessary for its full and potent anti-apoptotic function (Ito et al., 1997). The dramatic reduction in phosphorylated Bcl-2 was induced by H_2O_2 (Fig.5A). PACAP1-38 was able to up-regulate Bcl-2 in cells treated with H_2O_2. This could be inhibited by PACAP6-38 and Bcl-2 was detected around the level of the H_2O_2 treated group (Fig4A).

Bad is a protein with pro-apoptotic effects impairing the function of Bcl-2 and Bcl_{xl}. Its phosphorylation causes binding of Bad to 14-3-3 protein and inhibition of Bad binding to Bcl-2 and Bcl_{xl} leading to cell survival (Feng et al., 2004; Klumpp et al., 2002). It is known that oxidative stress provokes a decrease in the level of phospho-Bad agreeing with our findings which demonstrated significant reduction in phospho-Bad level as a result of H_2O_2 administration (Fig.5B). Co-incubation of H_2O_2 and PACAP1-38 led to the significant up-regulation of phospho-Bad, thus its level reached approximately control values. PACAP receptors antagonists, PACAP6-38, treatment in such a condition was associated with lower phospho-Bad levels (Fig.5B).

3.4 DISCUSSION AND CONCLUSION FROM THIS STUDY

The present study showed that treatment with PACAP could markedly attenuate the oxidative stress-induced apoptosis in cardiomyocytes. Thus, our results show for the first time, that the well known anti-apoptotic effects of PACAP in various neuronal cell lines is also present in heart myocytes.

Accumulating evidence indicates that apoptosis occurs in cardiovascular diseases and may play a significant role in the development of heart failure (Chen and Tu, 2002; Kumar et al., 2003). Oxidative stress seems to be a major factor in the initiation as well as in the regulation of cardiomyocyte apoptosis in different diseases (Kumar et al., 2003). Therefore, it seemed reasonable to study the effect of PACAP in oxidative stress-induced apoptosis of cardiomyocytes. The protective effect of PACAP against oxidative stress-induced apoptosis has been shown in cerebellar granule cells (Ito et al., 1999; Vaudry et al., 2002a,b). Incubation of cerebellar granule cells with H_2O_2 resulted in a dose-dependent decrease of the proportion of surviving cells, which could be counteracted by PACAP via the MAP kinase pathway (Vaudry et al., 2002a). Similar results have been obtained in apoptosis induced by 4-hydroxynonenal, which is a product of membrane lipid peroxidation (Ito et al., 1999). Oxidative stress also leads to inactivation of aconitase, a stress-sensitive enzyme of the tricarboxylic acid cycle in neuronal mitochondria (Tabuchi et al., 2003). In mouse cerebellar granule cells, lowering K^+ levels inactivates this enzyme, accompanying induction of apoptotic cell death. Suppression of apoptosis by PACAP1-38 attenuates the aconitase inactivation, which might be an additional factor in reducing oxidative stress-induced toxicity, since aconitase is a key mitochondrial enzyme influencing the viability of neurons in response to oxidative stress (Tabuchi et al., 2003).

Our results show that PACAP reduced the activation of caspase-3 and increased the activation of Bcl-2 and phospho-Bad following H_2O_2 administration. Caspase activation can be a driving force behind cell death in the myocardium (Chen and Tu, 2002). Both animal studies and human samples show activated caspase-3 in heart failures (Chen and Tu, 2002). The inhibitory effects of PACAP on caspase activation have already been shown in cerebellar granule cells and in chicken sympathetic neurons (Przywara et al., 1998; Vaudry et al., 2000, 2002a,b). In non-neuronal cells, the deactivation of caspase-3

has been demonstrated in rat alveolar cells (Onoue et al., 2004). The Bcl-2 family consists of various members with pro- and anti-apoptotic effects. Bcl-2 is an anti-apoptotic protein, while Bad and Bax are pro-apoptotic. The phosphorylation of Bad inhibits its binding to, and inactivation of, anti-apoptotic Bcl-2. In the heart, increased expression of the anti-apoptotic Bcl-2 correlates with myocardial survival, and increasing phospho-Bad improves myocardial protection (Feng et al., 2004; Kumar et al., 2003). PACAP has been shown to induce Bcl-2 expression in cerebellar granule cells and in a prostate cancer cell line (Falluel-Morel et al., 2004; Gutierrez-Canas et al., 2003), but the effects of PACAP on Bad-phosphorylation has not been reported yet. Our results show that PACAP increased the expression of both Bcl-2 and phospho-Bad (Fig.4).

The exact mechanism of the cardioprotective effects described in the present study is not yet known. Numerous in vitro studies on the neuroprotective mechanism of PACAP have shown that PACAP exerts neuroprotection via PAC1 receptors, through the cAMP/PKA/MAPK pathway (rev.: Somogyvari-Vigh and Reglodi, 2004). The dominance of PAC1 receptors on cardiac myocytes (Sano et al., 2002), and the shown stimulatory effects of PACAP on cAMP production and PKA (Baron et al., 2001; Cui et al., 2000) suggest that cardioprotection may be mediated by pathways similar to those found in neuronal cells. Also, it has recently been shown that PACAP influences the expression of several genes involved in survival in PC12 cells (Grumolato et al., 2003). Among the 75 up- and 70 downregulated genes in PC12 cells, several are involved in controlling apoptosis. For example, PACAP upregulates genes of detoxifying factors that participate in protection against oxidative stress, like peroxiredoxin 5 and thioredoxin reductase (Grumolato et al., 2003). PACAP also has direct antioxidant effects, as shown in a non-cellular in vitro system and in a red blood cell filtration model, although this effect was observed only at concentrations exceeding the usual in vitro neuroprotective range and the concentration used in our present study (Reglodi et al., 2004).

Apart from the direct effects of PACAP on cardiomyocytes, PACAP is also a potent vasodilator peptide (rev.:Vaudy et al., 2000). Intravenous application of the peptide leads to a transient fall in the systemic blood pressure, which, being within the autoregulatory range of the cardiac blood flow, would not influence the coronary blood flow. Furthermore, the vasorelaxant effects of PACAP on coronary blood vessels and thus the increase of coronary blood flow make the peptide a suitable candidate for myocardial preservation (Dalsgaard et al., 2003; Vaudry et al., 2000).

In conclusion, our study shows that PACAP is able to counteract the effects of oxidative stress, probably via reducing the activation of caspase-3 and increasing the activation of Bcl-2 and phospho-Bad following H_2O_2 administration. Our results can be of major clinical importance and serve as a basis for further studies on the protective effects of PACAP in cardiomyocytes.

4. PROTECTIVE EFFECTS OF PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE IN ENDOTHELIAL CELLS AGAINST OXIDATIVE STRESS-INDUCED APOPTOSIS

In the previous study we showed that treatment with PACAP could markedly attenuate the oxidative stress-induced apoptosis in cardiomyocytes. Our results show for the first time, that the well known anti-apoptotic effects of PACAP in various neuronal cell lines is also present in heart myocytes. The survival, growth, and function of endothelial cells are key factors in vascular homeostasis. Oxidative stress is a major cause of several pathological phenomena, including apoptosis in endothelial cells. Stress-induced changes of endothelial cells have been implicated in cardiovascular dysfunctions and several other pathological conditions. The effect of PACAP in oxidative stress-induced apoptosis in endothelial cells is unclear. Therefore, the second part of our study investigated the same model in endothelial cells and the effect of PACAP.

4.1. BACKGROUND

The survival, growth, and function of endothelial cells are key factors in vascular homeostasis (Irani, 2000). Oxidative stress is a major cause of several pathological phenomena, including apoptosis in endothelial cells (Irani, 2000; Cuda et al., 2002; Lee et al., 2004b). Stress-induced changes of endothelial cells have been implicated in cardiovascular dysfunctions and several other pathological conditions (Irani, 2000; Murakami et al., 2005). Oxidative stress leads to activation of numerous signal transduction pathways, including members of the mitogen-activated protein kinase (MAPK) family.

It has been shown that PACAP stimulates production of vascular endothelial growth factor (VEGF), an important factor for development and function of endothelial cells in lung cancer and pituitary cells (Gloddek et al., 1999; Lohrer et al., 2001; Moody et al., 2002). However, no data are presently available on the direct effects of PACAP on endothelial cell survival. Vasoactive intestinal peptide (VIP), the structurally closest related peptide to PACAP, has been reported to promote survival of human corneal endothelial cells (Koh and Waschek, 2000). Therefore the aim of the study was to investigate the effects of PACAP on endothelial cell survival and on oxidative stress-induced changes in the activation of members of the MAPK family.

4.2. MATERIALS AND METHODS

4.2.1. Cell culture

The EOMA CRL-2586 cell line was obtained from mouse hemangioendothelioma (ATCC). EOMA cells were cultured in DMEM/F12 medium (Sigma–Aldrich, USA), supplemented with 10 % of fetal bovine serum (Gibco, USA) and 1 % penicillin-streptomycin (Gibco, USA). Cells were passaged by trypsinization (Trypsin/EDTA; Sigma, USA), followed by dilution in DMEM/F12 medium containing 10 % fetal bovine serum. Experiments started 48 hrs after incubation in humified 95 % air and 5 % CO_2 mixture at 37 °C in the medium.

4.2.2. Experimental protocol

Cultured EOMA cells were randomly assigned to different experimental groups:

- control group of cells, with no treatment;
- cells exposed to 0.5 mM of H_2O_2;
- cells incubated in medium containing 20 nM PACAP1-38;
- EOMA cells subjected to treatment of 250 nM PACAP receptors antagonists, PACAP6-38;
- cells treated with 0.5 mM H_2O_2 together with PACAP1-38;
- to antagonize the effect of PACAP1-38, 250 nM of the PACAP receptors antagonists, PACAP6-38, was added simultaneously with 0.5 mM H_2O_2.

The following experimental protocol is the same, as previously described (3.2.2.).

4.2.3. Cell viability assay

Viability of EOMA cells were determined by colorimetric MTT assay at the end of the 4-hr-treatments, as previously described (3.2.3).

Cell viability was also assessed by live/dead viability/cytotoxicity assay (Invitrogen, Molecular Probes). Cells were incubated for 45 min in the live/dead (L/D) buffer, consisting of calcein-AM (5 µl) and ethidium homodimer-1 (20 µl) in PBS, as outlined by the manufacturer. Ethidium homodimer-1 is a high-affinity, red fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. Calcein-AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product, thus green fluorescence is an indicator of living cells that have esterase activity as well as an intact membrane to retain the esterase products.

4.2.4. Annexin V and propidium iodide staining of the EOMA cells

By conjugating a fluorescent group to annexin V, apoptosis can be quantitatively detected using fluorescence microscopy or flow cytometry, and this assay has been used together with other criteria to detect apoptosis in endothelial cells, as previously described (3.2.4).

4.2.5. Assessment of MAPK : phospho-ERK1/2, phoshpo-p38MAPK and phospho-JNK1/2

Following the 10, 30, 60-min-long treatments in different groups, cells were harvested by trypsin-EDTA according to the protocol described at annexin V-PI staining. The signaling pathways of the MAPK family were assessed by the same protocol, as previously described (3.2.5.)

Polyclonal antibodies against phospho-ERK1/2, phospho-p38 MAPK and phospho-JNK1/2 (Cell Signaling Technology Inc., USA) were used at dilutions 1:1000 (ERK1/2), 1:50 (p38 MAPK) and 1:50 (JNK1/2). After centrifugation, the supernatant

was carefully aspirated and cells were resuspended in 100 µl 0.5 % BSA-containing FITC-conjugated secondary antibody (anti-mouse IgG for ERK1/2, and anti-rabbit IgG for p38 MAPK and JNK1/2, Sigma) at a dilution of 1:50, and were incubated for 30 min. Our results were analyzed by Cellquest software (BD Biosciences, USA) measuring the appearance of phospho-ERK1/2, phospho-p38 MAPK and phospho-JNK1/2 in the cells as mean fluorescence intensity (MFI).

4.2.6. Statistical analysis

All data are presented as mean±S.E.M. Differences between groups were assessed with one-way ANOVA followed by Neuman–Keul's post hoc analysis, and were considered significant when $P<0.05$.

4.3. RESULT

Viability of EOMA cells after H_2O_2 was measured by MTT assay (Fig. 6). H_2O_2 treatment alone led to a manifest decrease of cell viability compared to control values. Treatment with PACAP1-38 and PACAP antagonist, PACAP6-38 alone did not alter cell viability. Cell viability significantly increased when H_2O_2-treated cells were co-incubated with PACAP1-38. Incubation of cultured cells with the PACAP antagonist, PACAP6-38, significantly attenuated the beneficial effect of PACAP1-38 on cell viability (Fig. 6). This effect of PACAP was further verified by qualitative live/dead assay (Fig. 7), which clearly showed that H_2O_2 treatment increased the number of dead cells compared to the control group. PACAP1-38 treatment was able to counteract this effect of H_2O_2 treatment, while PACAP6-38 inhibited the effects of PACAP1-38 (Fig. 7).

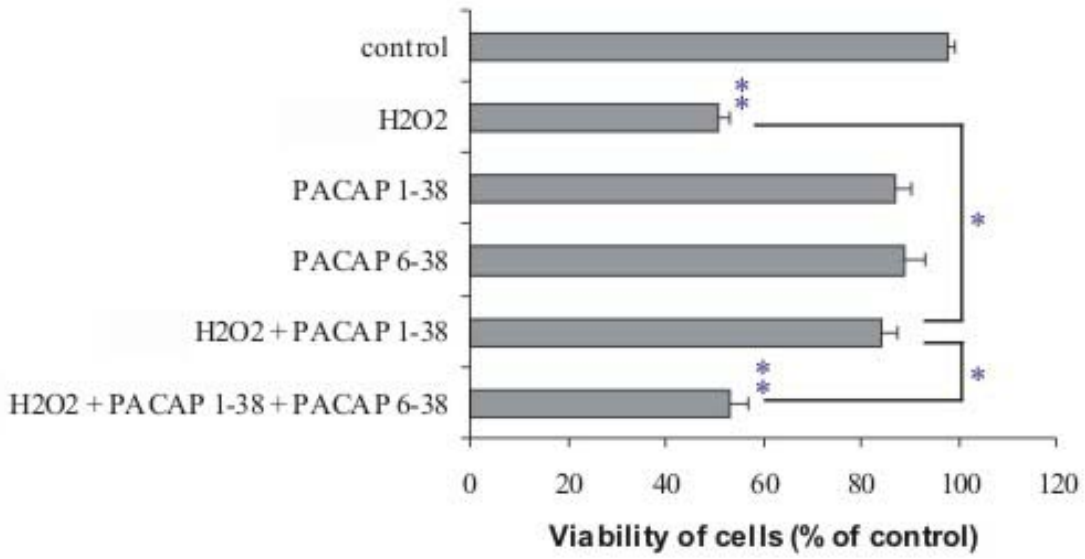

*Fig.6. Viability of endothelial cells as measured by MTT assay. Data are presented as mean ± S.E.M. obtained from six independent experiments. *p<0.05, **p<0.01 compared to control values unless otherwise indicated.*

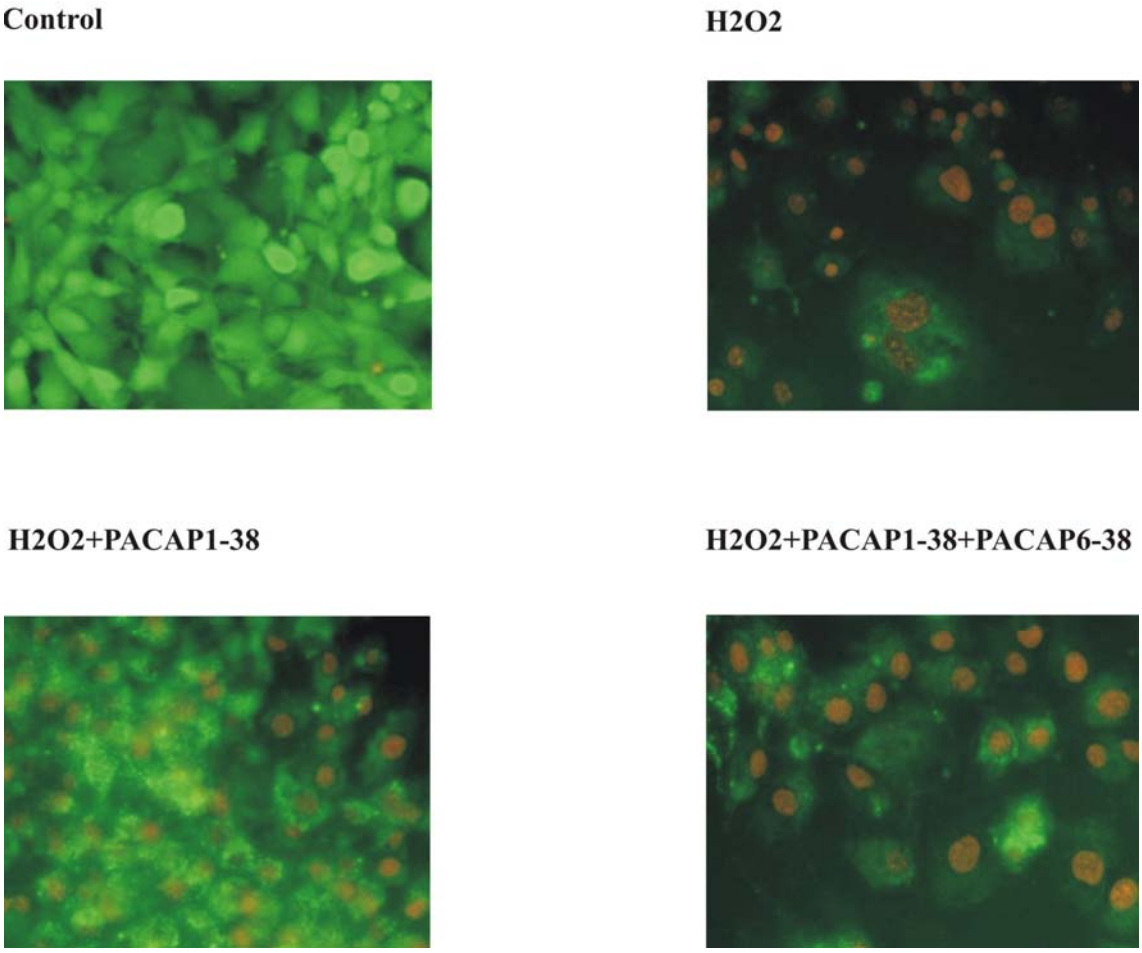

Fig.7. Representative fluorescent images of cell viability in normal control, 0.5 mM H_2O_2-treated, 20nM PACAP1-38 and 250nM PACAP-antagonist-treated EOMA cells. Green fluorescence is an indicator of living cells (Calcein-AM) and red fluorescence (ethidium homodimer-1) indicates dead cells.

Annexin V and propidium iodide staining were used to detect apoptosis in cultured cells. Apoptosis leads to plasma membrane asymmetry and the externalization of phophatidylserine residues, which bind annexin V with high affinity. In the early stages of apoptosis, cells typically have an intact cell membrane. Thus, they are not stained with propidium iodide, whereas externalization of phophatidylserine can be detected by annexin V. In the late phase of apoptosis, cells are stained with both dyes. Using this method, we found that the control group had more than 90% of intact, living cells and only less than 10% of cells in the early and late phases of apoptosis (Fig. 8, 9). An increase of apoptotic cells was observed in the H_2O_2-treated group with a lower number of living cells. PACAP1-38 and PACAP6-38 administration alone caused no changes in

the percentage of living and apoptotic cells compared to control values. PACAP1-38 administration led to a significant increase in the percentage of living cells and a reproducible decrease in the rate of apoptosis in cells exposed to H_2O_2. This beneficial effect of PACAP1-38 was diminished by PACAP6-38 (Fig. 8, 9A,B,C).

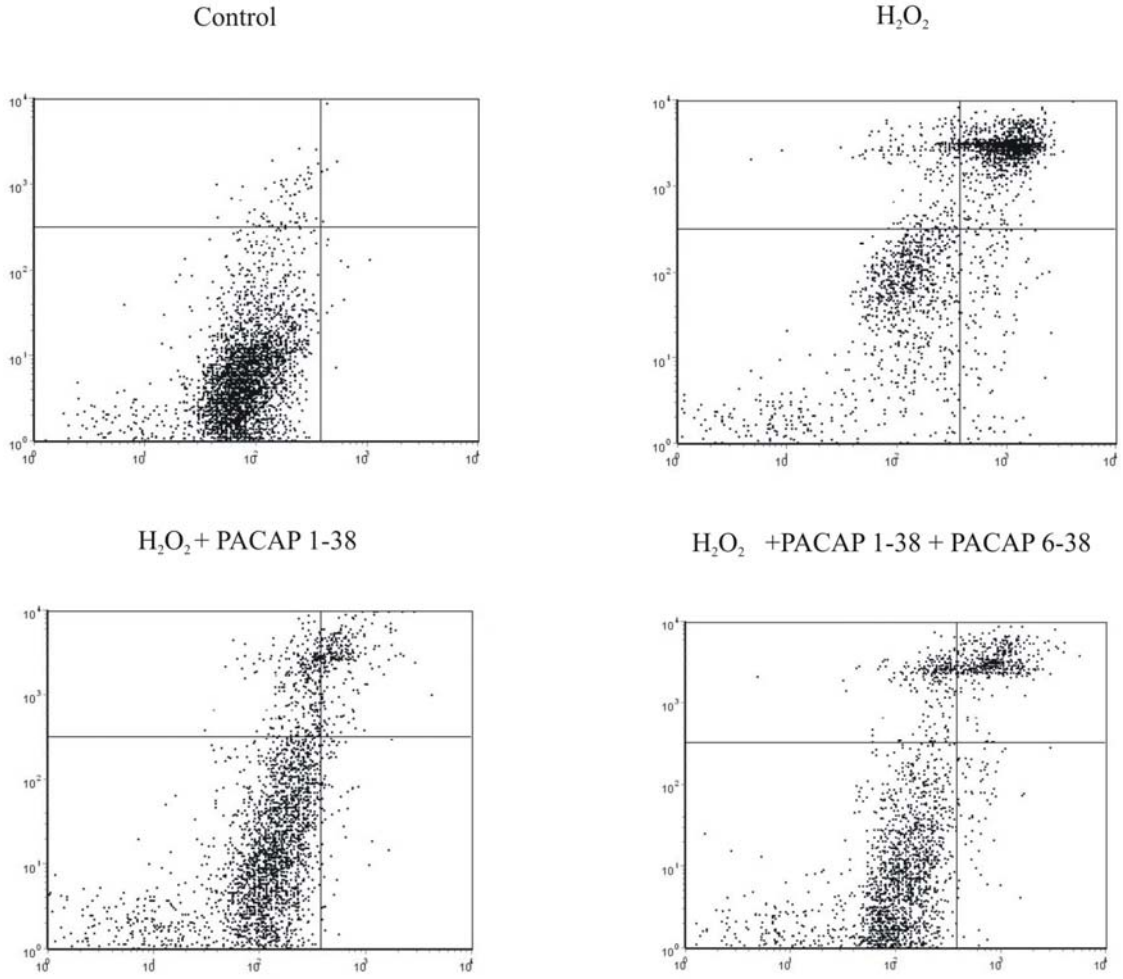

Fig.8. Effect of H_2O_2 and PACAP on apoptosis of EOMA cells. Distinction between living, necrotic, early and late apoptotic cells. Examples of dot-plots as determined by flow cytometry following annexin V and PI double-staining. Horizontal axis represents annexin V intensity and vertical axis shows PI staining. The lines divide each plot into quadrants: lower left quadrant: living cells, lower right quadrant: early apoptotic cells, upper left quadrant: necrotic cells, upper right quadrant: late apoptotic cells.

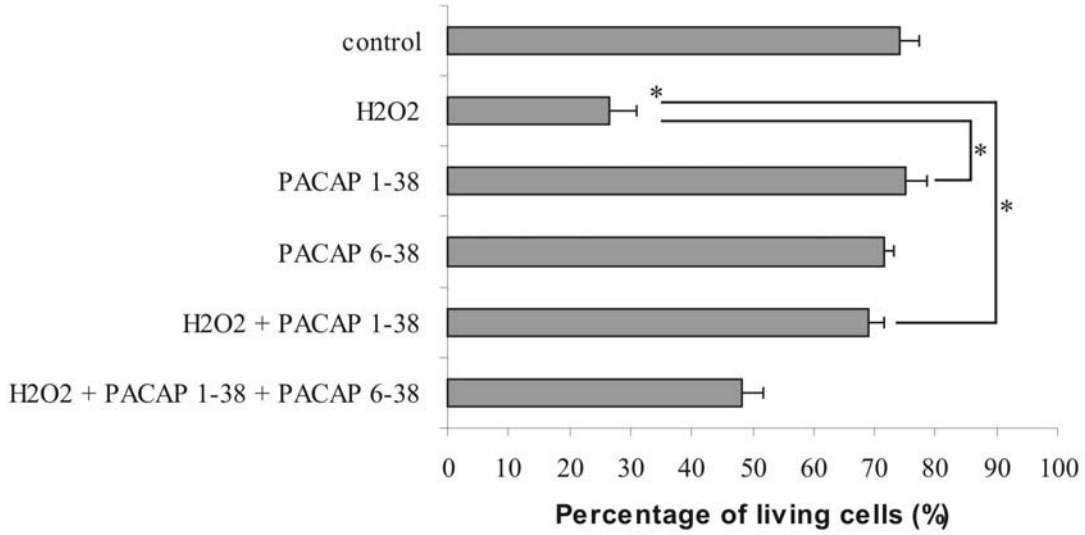

*Fig.9A. Graphs demonstrate the mean percentage of living cells. Data are expressed as mean±S.E.M. *p<0.05, **p<0.01 compared to control values unless otherwise indicated.*

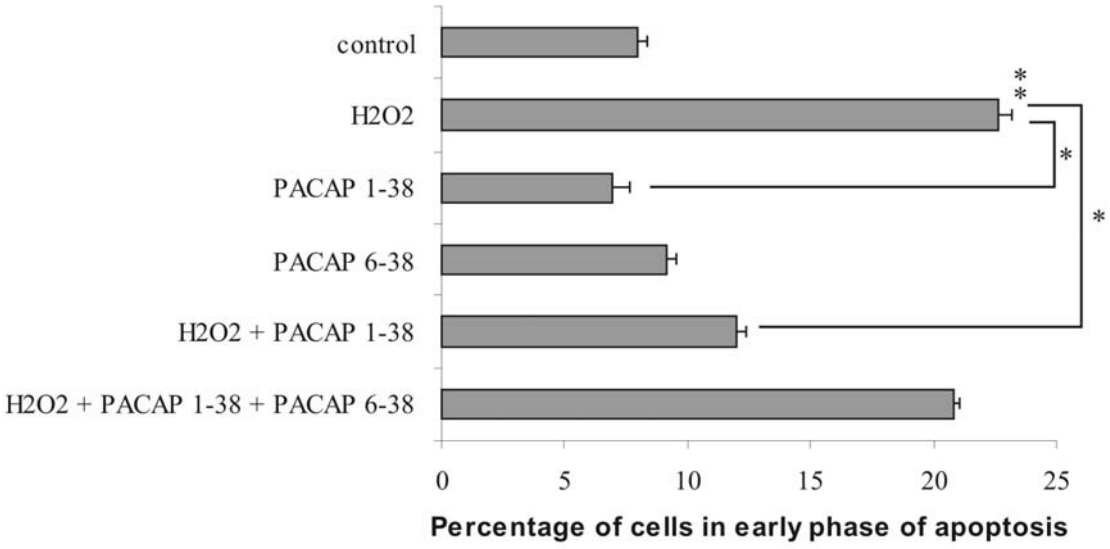

*Fig.9B. Graphs demonstrate the ratio of cells in early apoptosis. Data are expressed as mean±S.E.M. *p<0.05, **p<0.01 compared to control values unless otherwise indicated.*

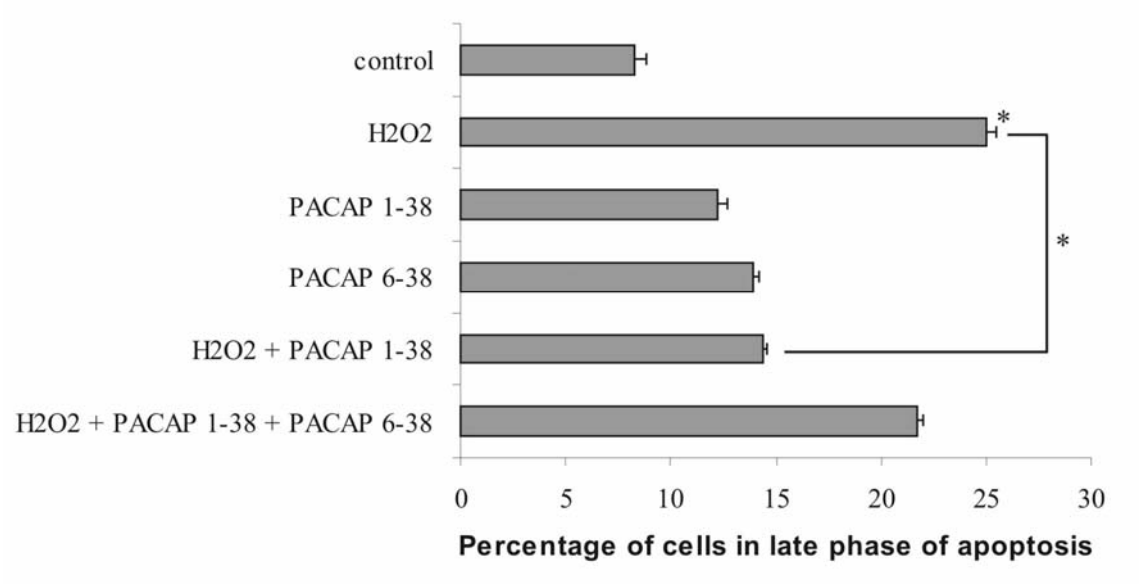

*Fig.9C. Graphs demonstrate the ratio of cells in late apoptosis. Data are expressed as mean±S.E.M. *p<0.05, **p<0.01 compared to control values unless otherwise indicated.*

EOMA cells were incubated with 0.5 mM H_2O_2 for 10, 30, and 60 minutes. ERK1/2, p38 MAPK and JNK1/2 activation appeared at all time-points and maximal peak was reached at the 30-minute time-point (Fig. 10A,B,C). Using flow cytometry, the phosphorylation of ERK1/2, p38 MAPK and JNK1/2 was examined. 0.5 mM H_2O_2 reduced the activation of ERK1/2 and induced that of the pro-apoptotic markers p38 and JNK1/2 (Fig. 10A,B,C). Neither PACAP1-38 nor PACAP6-38 alone significantly altered the phosphorylation of these examined markers. However, the phosphorylation of p38 MAPK and JNK1/2 was markedly reduced by PACAP1-38 in cells exposed to H_2O_2. Also, PACAP1-38 was able to increase the phosphorylation of ERK1/2 in H_2O_2-treated cells. PACAP receptors antagonists, PACAP6-38, decreased the advantageous effect of PACAP1-38 in levels of all three markers (Fig. 10A,B,C).

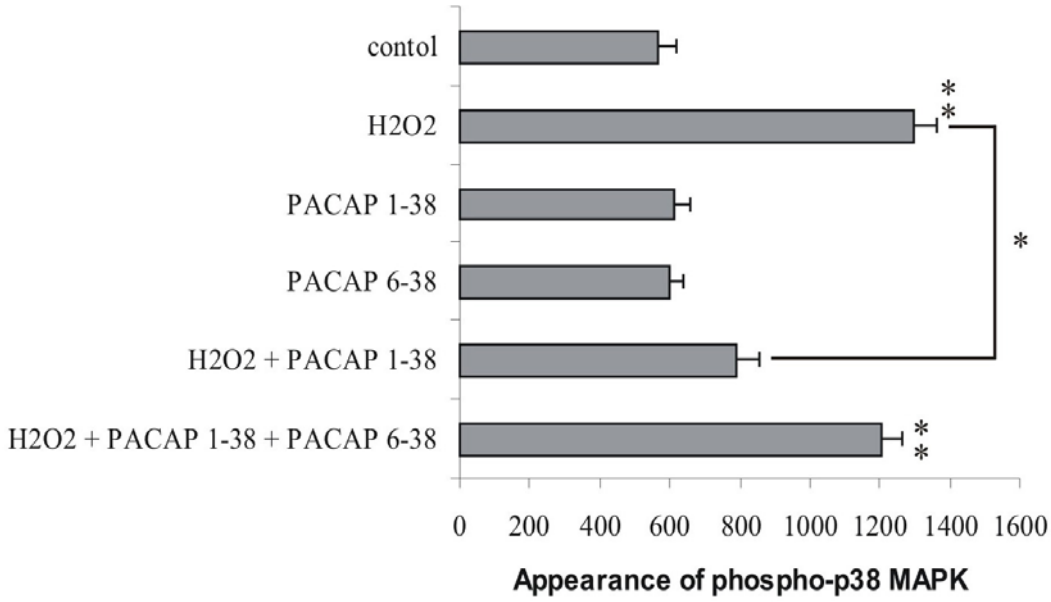

*Fig.10A. Effect of PACAP1-38 on appearance of phosphor-p38MAPK in cells submitted to 0.5 mM concentration of H_2O_2 for 30 min. Results are expressed as mean fluorescent density ±S.E.M. *p<0.05, **p<0.01 compared to control values unless otherwise indicated.*

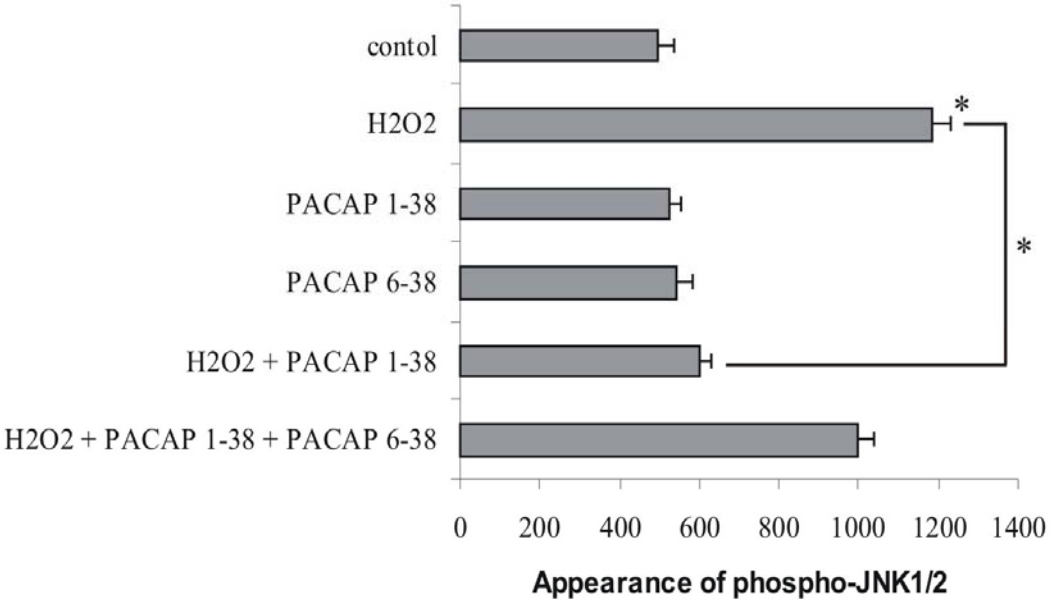

*Fig.10B. Effect of PACAP1-38 on appearance of phospho-JNK1/2 in cells submitted to 0.5 mM concentration of H_2O_2 for 30 min. Results are expressed as mean fluorescent density ±S.E.M. *p<0.05 compared to control values unless otherwise indicated.*

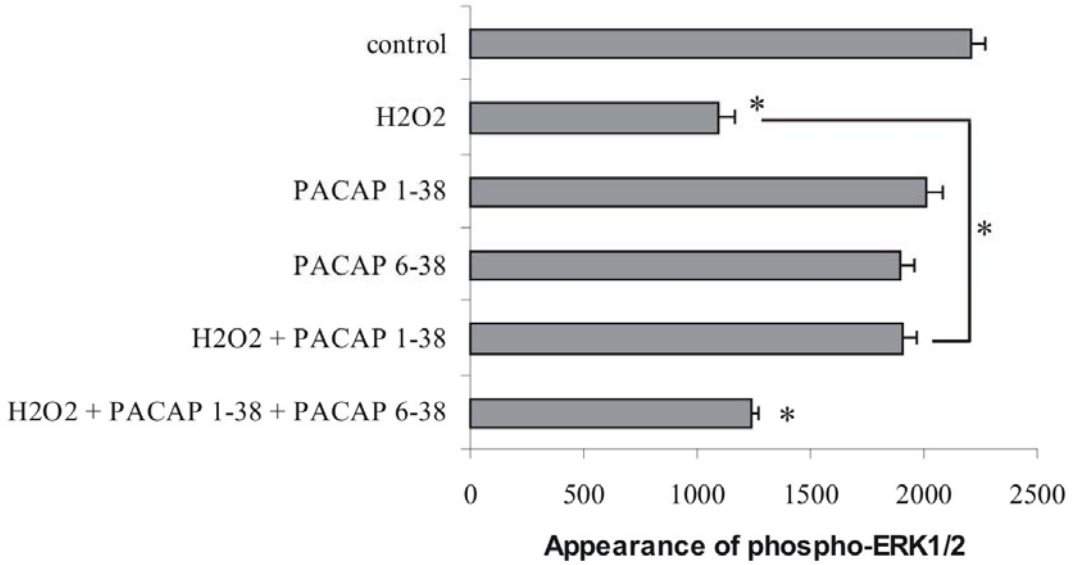

*Fig.10C. Effect of PACAP1-38 on appearance of phospho-ERK1/2 in cells submitted to 0.5 mM concentration of H_2O_2 for 30 min. Results are expressed as mean fluorescent density ±S.E.M. *p<0.05 compared to control values unless otherwise indicated.*

4.4. DISCUSSION AND CONCLUSION FROM THIS STUDY

The present results show that PACAP strongly attenuated the H_2O_2-induced apoptosis and the concomittant increase in the phosphorylation of the pro-apoptotic kinases p38 and JNK1/2, and the decrease in the phosphorylation of the anti-apoptotic ERK1/2. This is the first report on the direct survival promoting effects of PACAP on endothelial cells.

The protective effect of PACAP has been shown against various exogenous toxic agents, including H_2O_2 in cerebellar granule cells and cardiomyocytes (Vaudry et al., 2002a; Somogyvari-Vigh and Reglodi, 2004; Gasz et al., 2006a,b). Cultured cerebellar neurons from PACAP-deficient mice have been reported to be more sensitive to H_2O_2 -toxicity (Vaudry et al., 2005). Numerous studies have demonstrated that the protective effects of PACAP are associated with phosphorylation of the MAP kinases in neuronal cell lines, mainly via cAMP/protein kinase A pathway (Vaudry et al., 1998, 2002a,b; Somogyvari-Vigh and Reglodi, 2004; Falluel-Morel et al., 2004; Girard et al., 2004; Li et al., 2005; Kojro et al., 2006; Pugh and Margiotta, 2006). The protective effect of PACAP in H_2O_2-induced apoptosis of cerebellar granule cells has been shown to be mediated through the MAP kinase pathway (Vaudry et al., 2002a). In this study, the authors found that PACAP produced an elevation of ERK phosphorylation (Vaudry et al., 2002a). The effects of PACAP on ERK phosphorylation have also been described by others (Villalba et al., 1997).

The influence of PACAP on phosphorylation of ERK, JNK and p38 has been reported in several non-neuronal cells: in monocytes (El Zein et al., 2007), neutrophils (Harfi and Sariban, 2006; Kim et al., 2006), microglial cells (Lee and Suk, 2004a), astrocytes (Hashimoto et al., 2003) testicular germ cells (Li et al., 2004), folliculostellate cells (Vlotides et al., 2004) and myeloma cells (Li et al., 2006). In general, most studies show that PACAP increases ERK activity and suppresses JNK and p38 activity, although these effects may be different depending on the cell type and experimental conditions (Sakai et al., 2002).

The effects of PACAP on activation of the MAPK family has been shown to be present also in vivo, in hippocampal neurons under hypoxic conditions: the hypoxia-induced activation of p38 and JNK and the induced suppression of ERK activity were inhibited by PACAP (Shioda et al., 1998, 2006; Dohi et al., 2002). Lower levels of ERK have also been observed in PACAP-deficient mice (Ohtaki et al., 2006). In recent

studies we have found that PACAP is able to attenuate the toxic effects of neonatal monosodium glutamate treatment in the retina, partly via counteracting the monosodium glutamate-induced elevation of JNK- and decrease in ERK-phosphorylation (Racz et al., 2006a,b). Based on the several important actions played by PACAP in the development of the nervous system, PACAP is regarded as a neurotrophic factor (Waschek, 2002; Somogyvari-Vigh and Reglodi, 2004). Other neurotrophic factors have already been shown to enhance survival of endothelial cells: nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factor, and VIP (Donovan et al., 2000; Koh and Waschek, 2000; Kim et al., 2004; Donnini et al., 2006). These data support the role of neurotrophic factors in the maintenance of endothelial functions and survival (Kim et al., 2004). The present results show that PACAP may also be a candidate trophic factor for endothelial functions during the MAPK pathways.

5. IN VIVO NEUROPROTECTION BY PACAP IN EXCITOTOXIC RETINAL INJURY: EFFECTS ON THE APOPTOTIC SIGNAL TRANSDUCTION

In the previous study, we showed that PACAP strongly attenuated the H_2O_2-induced apoptosis and the concomittant increase in the phosphorylation of the pro-apoptotic kinases, and the decrease in the phosphorylation of the anti-apoptotic kinase in cardiomyocite and endothelial cells. This was the first report on the direct survival promoting effects of PACAP on endothelial cells.

The previous chapters dealt with the in vitro antiapoptotic effects of PACAP in cardiovascular system. It is of great importance to study whether the well known in vitro antiapoptotic effects are also present in vivo. Therefore in our further studies we tried to investigate PACAP in the *in vivo* model of retina degeneration.

5.1. BACKGROUND

PACAP and its receptors can be found in the retina and it is an important transmitter in the retinohypothalamic tract. The well-known neuroprotective effects of PACAP have also been shown in vitro in the retina in several pathological conditions such as hypoxia and optic nerve transection. Elevated glutamate levels lead to retinal damage and PACAP has been shown to be protective against glutamate-induced cell death in the retina in vitro. Recently, we have shown that this protective effect is also present in vivo, in monosodium glutamate (MSG)-induced retinal degeneration. The underlying molecular mechanism of this protective effect is not yet known. Retinal cell death induced by over-stimulation of glutamate receptors is related to apoptosis. The aim of the present study was to further elucidate the possible signaling pathways involved in the protective effects of PACAP against MSG-toxicity in vivo.

The abnormal activation of glutamate receptors plays a key role in numerous neuropathological conditions, including diseases of the eye (Danysz et al., 2002; Sucher et al., 1997). It is possible to mimic several pathological conditions in the eye by experimental elevations of glutamate (Vidal-Sanz et al., 2000, Osborne et al., 1999). MSG causes degeneration in diverse brain areas, results in neurobehavioral abnormalities (Kiss et al., 2005) and leads to retinal degeneration when given

systemically in newborn animals or locally in adults (van Rijn et al., 1986; Chambille et al., 1993; Sisk et al., 1985; Ohguro et al., 2002).

Several agents have been shown to alleviate degenerative changes caused by elevation of extracellular glutamate levels in the retina under experimental conditions, such as NMDA-antagonists, Ca^{2+}-channel blockers and fenamates (Sucher et al., 1997; Chen, 1998; Sun, 2001; Toriu, 2000). Trophic factors are also effective in retinal pathologies (Vidal-Sanz et al., 2000).

PACAP is distributed in various peripheral and sensory organs, including the eye (Vaudry et al., 2000; Zhou et al., 2002). It occurs in ocular tissues and exerts several functions in the eyes of various species (Wang et al., 1995; Yamaji et al., 2005, Nakatani et al., 2006). In the retina, PACAP immunoreactivity is present in the amacrine and horizontal cells, in the inner plexiform layer, in the ganglionic cell layer and in the nerve fibre layer (Izumi et al., 2000; Koves, 2000; Seki et al., 2000). PACAP effectively stimulates cAMP in the retina (Onali et al., 1994; D`Agata et al., 1998), where the peptide shows circadian alteration, and is an important transmitter in the retinohypothalamic pathway (Koves et al., 2000; Hannibal et al., 2004; Jozsa et al., 2001). PACAP receptors are also present in the retina with PAC1 receptor as the predominant receptor type in the ganglion cells, amacrine cells, inner nuclear layer and Muller cells (D`Agata et al., 1998; Seki et al., 1997; Nilsson et al., 1994; Kubrusly et al., 2005; Silveira et al., 2002; Seki et al., 2000). Weaker expression of PACAP receptors has been shown in other layers of the retina (Seki et al., 2000).

The present study summarizes the effects of PACAP in MSG-induced retinal degeneration of newborn rats *in vivo*.

5.2. MATERIALS AND METHODS

5.2.1. Animals

Newborn Wistar rat pups were used for the experiments. All procedures were performed in accordance with the ethical guidelines approved by the University of Pécs (No: BA02/2000-31/2001) Laboratories and were maintained at room temperature, in an alternating 12-hr light and 12-hr dark cycle (lighs on at 6 am).

5.2.2. Experimental protocol

Pups were injected s.c. with 4mg/g bodyweight MSG on postnatal days 1 and 5. Preceding each MSG treatment, 100 pmol PACAP1-38 (n=15) or 100 pmol PACAP1-38 together with 1 nmol PACAP6-38 (n=8) in 5 µl saline was injected unilaterally in the vitreous body with a Hamilton syringe. The same volume of saline was injected into the other eye, serving as MSG-treated group. In order to investigate the effects of PACAP1-38 and the PACAP receptors antagonist PACAP6-38, a group of pups (n=4) received only PACAP1-38 (100 pmol) or PACAP6-38 (1 nmol) into one eye, while the other eyes served as saline-treated controls. The used doses of PACAP1-38 and PACAP6-38 were based on our previous studies (Babai et al., 2005, 2006), and the peptides were synthesized as previously described (Gasz et al., 2006a).
Retinas were removed 12 and 24 hours following each treatment and were processed for Western blot analysis.

5.2.3. Western blot analysis

Samples were homogenized in ice-cold isotonic Tris buffer (50mM, pH: 8.0) containing 0.5mM sodium-metavanadate, 1mM EDTA and Protease Inhibitor Coctail (1:1000, Sigma-Aldrich Co, Hungary).
Cytosolic fractions prepared from aliquots of the homogenates by centrifuging them at 15000g for 10min at 4°C were used for demonstrating translocation of cytochrome-c and apoptosis inducing factor (AIF) from mitochondria to the cytosol. Other antigens were determined from the rest of the tissue homogenates following sonication. Proteins were precipitated by TCA, washed 3 times with -20°C acetone, dissolved in Laemli

sample buffer, separated on 12% SDS-polyacrilamide gels and transferred to nitrocellulose membranes. After blocking (2 hours with 3% nonfat milk in Tris-buffered saline), membranes were probed overnight at 4°C with antibodies recognizing the following phospho-specific antigens: Bad (Ser 136) (1:1000), SAPK/JNK (1:2000), ERK (1:2000), CREB (1:1000) and cleaved caspase-3 (1:1000) (Cell Signaling Technology, Beverly, MA, USA), cytochrome c and AIF (Oncogene). Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) before addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution, Bio-Rad, Hungary). Protein bands were visualized with enhanced chemiluminescence labeling using an ECL Western blotting detection system (Amersham Biosciences, Hungary). The developed films were scanned and the pixel volumes of the bands were determined by using NIH's Image J software. Under these conditions the 17kD and the 19kD bands of the cleaved caspase-3 could not be resolved separately. Loading of samples was confirmed even by anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon Int. Inc., USA) immunoblotting (not shown). Pixel volumes of the bands of interest were normalized to that of the appropriate loading control or the respective total protein, such as JNK1/2 (1:1000), caspase 3 (1:1000), Bad (Ser 136) (1:1000) (Cell Signaling Technology, Beverly, MA, USA). Each experiment was repeated minimum three times.

5.2.4. Statistical analysis

All data were expressed as the mean ± S.D. Differences between groups were assessed with one-way ANNOVA. Statistical analysis was performed by analysis of variance and Student's *t* test.

5.3. RESULT

In a previous study, we have shown that the presently applied MSG treatment protocol led to a severe degeneration of the inner retinal layers which could be significantly ameliorated by local pre-MSG PACAP administration (Tamas A. et al., 2004). In our earlier study, the phosphorylation i.e. the activation of ERK1/2 and CREB (p-ERK1/2 and p-CREB) was monitored 12 and 24 h after treatments. There was a gradual increase in both proteins following MSG treatments. PACAP treatments led to further, significant increases in the level of p-ERK1/2 (Fig. 11) and p-CREB (Fig. 12) 12 h after the first and the second PACAP treatments. There was no further elevation after the third treatment in either control or PACAP-treated retinas (data not shown). Interestingly, PACAP increased the ERK 1/2 and CREB phosphorylation 12 h, but not 24 h after the first and second treatments. It suggests that during the first two MSG challenges PACAP induced a transient increase in the activation of ERK 1/2 and of its downstream target CREB, which disappeared by 24 h after the treatment. Then the phosphorylation reached a plateau value, and did not increase any further. These results are in accordance with our earlier histological observations using the same experimental setup (Babai et al., 2005). In that study we showed that in order to achieve significant amelioration in MSG-induced degeneration at least two PACAP treatments are necessary, but there was no further amelioration after the third treatment. Also, our present and earlier observations indicate that repeated application of PACAP may lead to a primed state promoting a long-lasting protection, which has also been observed by others in cerebellar neurons (Vaudry et al., 1998)

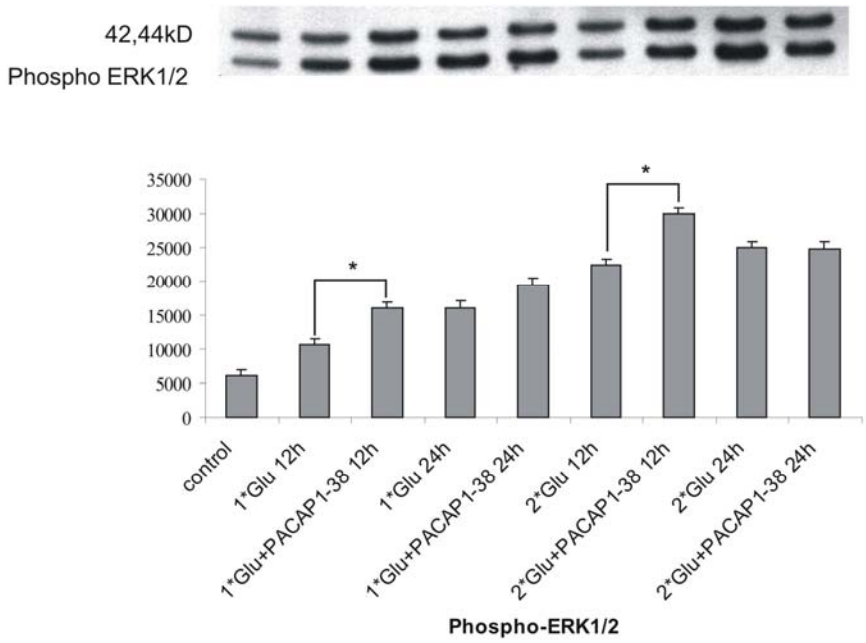

*Fig.11. Effects of PACAP on phosphorylation of ERK1/2 in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. *P<0.05 compared to the Glu-treated control levels. Bar diagrams representing pixel volumes of the phosphorylated ERK1/2 bands from at least 3 independent experiments are presented. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

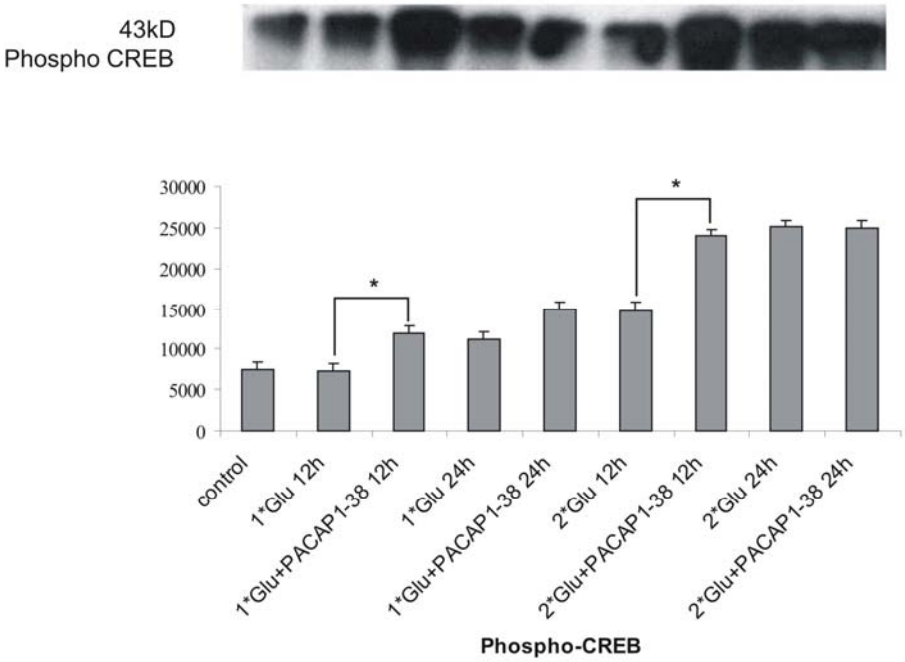

*Fig.12. Effects of PACAP on phosphorylation of CREB in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. *P<0.05 compared to the Glu-treated control levels. Bar diagrams representing pixel volumes of the phosphorylated CREB bands from at least 3 independent experiments are presented. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

Repeated glutamate administration induced apoptotic cell death in the retina that was attenuated by PACAP by a mechanism involving ERK1/2 and CREB. In order to investigate the involvement of another member of the MAP kinase family, we assessed the activation of JNK by using immuno-blotting and a phosphorylation-specific antibody.

JNK activity was slightly, although not significantly decreased by PACAP treatment alone, however, it was increased by the PACAP receptors antagonists, PACAP6-38, 12 hours after administration (Fig. 13A). MSG-treatment elevated JNK activation that was prevented by PACAP, although these changes were not significant during the first 12 hours after MSG injection. However, JNK phosphorylation was significantly elevated 24 hours after the first treatment and 12 hours after the second one. PACAP abolished the MSG-induced elevation of JNK activation, while PACAP6-38 completely counteracted the effect of PACAP (Fig. 13B).

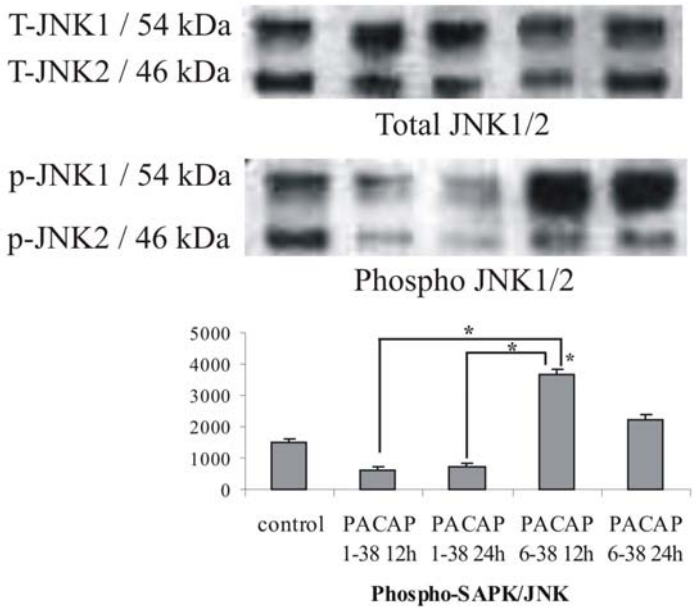

*Fig.13A. Effects of intravitreal PACAP1-38 and PACAP6-38 on phosphorylation and thus activation of JNK in the retina. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) JNK (upper panel) and phosphorylated JNK (lower panel), bar diagrams representing pixel volumes of the phosphorylated JNK bands (normalized to the appropriate total JNK value) from at least 3 independent experiments are presented. *P<0.05 compared to control levels were not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

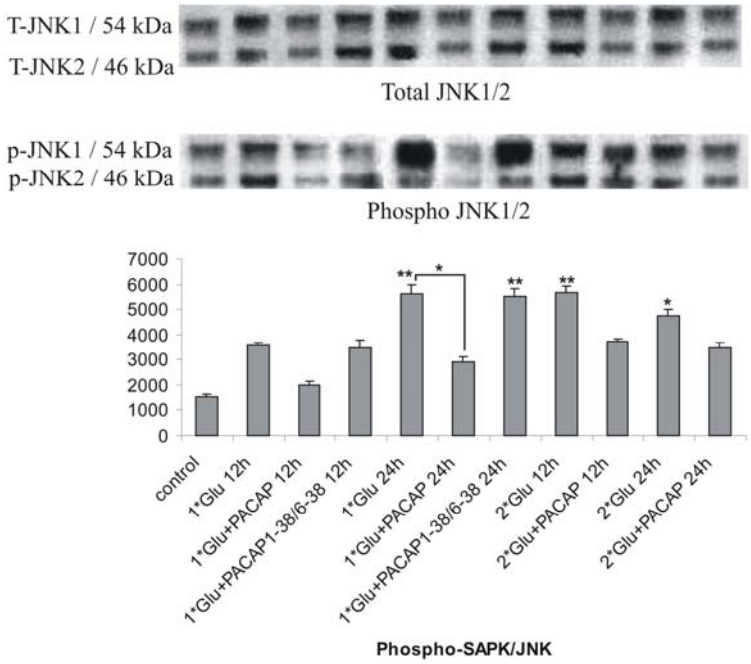

*Fig.13B. Effects of PACAP1-38 on phosphorylation of JNK in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) JNK (upper panel) and phosphorylated JNK (lower panel), bar diagrams representing pixel volumes of the phosphorylated JNK bands (normalized to the appropriate total JNK value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01 compared to control levels were not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

Phosphorylation and thus inactivation of the pro-apoptotic protein, Bad was not altered by PACAP treatment alone, although it was significantly decreased 24 hours after the PACAP receptors antagonists, PACAP6-38, administration (Fig. 14A). MSG induced a significant decrease in Bad phosphorylation, which was abolished by PACAP administration. This effect of PACAP could be blocked by adding PACAP6-38 (Fig. 14B).

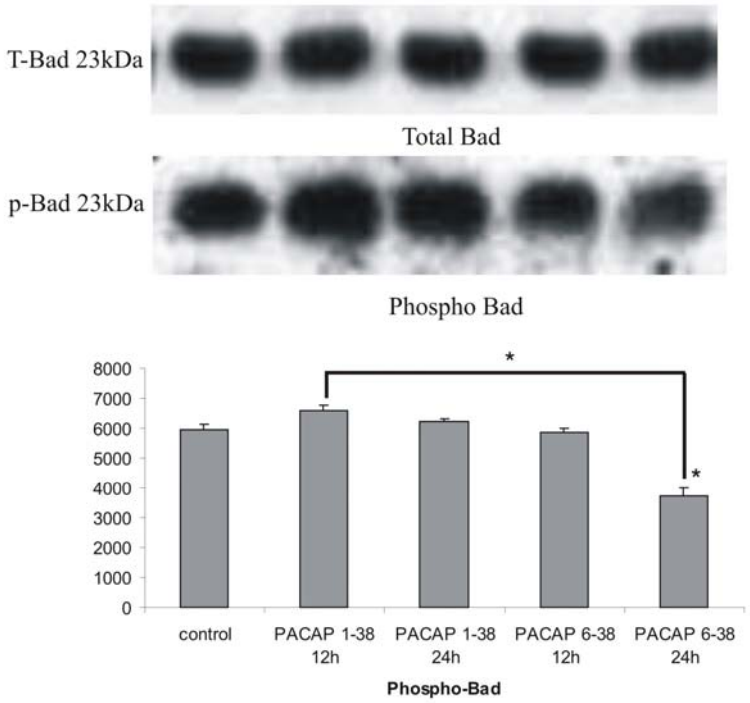

*Fig.14A. Effects of intravitreal PACAP1-38 and PACAP6-38 on phosphorylation of Bad in the retina. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) Bad (upper panel) and phosphorylated Bad (lower panel), bar diagrams representing pixel volumes of the phosphorylated Bad bands (normalized to the appropriate total bad value) from at least 3 independent experiments are presented. *P<0.05 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

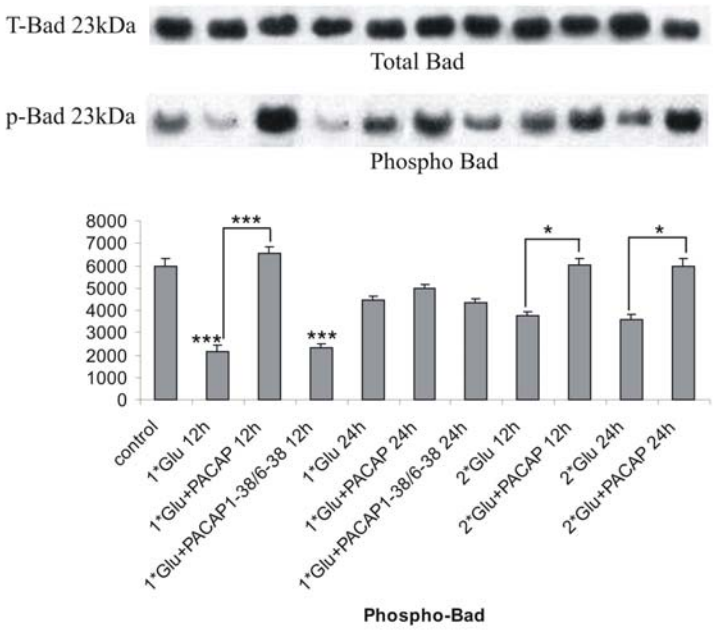

*Fig.14B. Effects of PACAP on phosphorylation of Bad in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) Bad (upper panel) and phosphorylated Bad (lower panel), bar diagrams representing pixel volumes of the phosphorylated Bad bands (normalized to the appropriate total bad value) from at least 3 independent experiments are presented. *P<0.05, ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

Translocation of AIF from the mitochondria to the cytosol signals for the induction of caspase-independent apoptosis mechanism was investigated as well. PACAP1-38 treatment by itself significantly reduced the cytoplasmic translocation of AIF, especially 12 hours after the treatment indicating that by a yet uncharacterized mechanism PACAP1-38 can inhibit even the unstimulated release of apoptotic signal from the mitochondria (Fig. 15A). MSG induced an elevation in AIF translocation 12 and 24 hours after the first treatment (Fig. 15B) This elevation was inhibited by PACAP1-38, and the effect of PACAP1-38 was counteracted by the PACAP antagonist PACAP6-38. The inhibitory effect of PACAP1-38 on the MSG-induced AIF translocation was less pronounced 24 than 12 hours after the first MSG treatment, and AIF levels in the cytoplasm showed a decreasing tendency that was not affected by either MSG or PACAP after the second treatment (Fig. 15B).

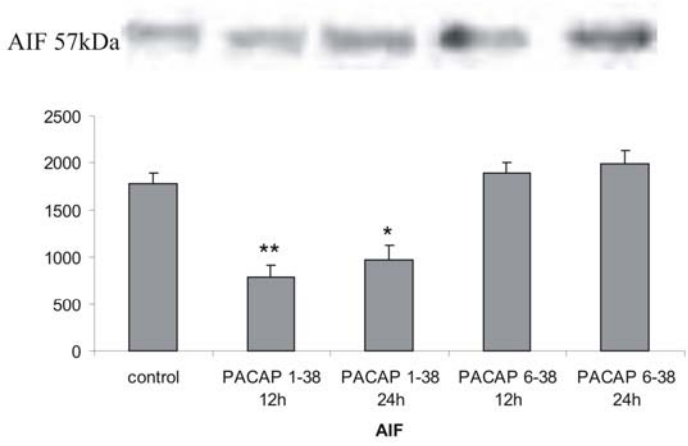

*Fig.15A. Effects of intravitreal PACAP1-38 and PACAP6-38 on phosphorylation of Bad in the retina. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) Bad (upper panel) and phosphorylated Bad (lower panel), bar diagrams representing pixel volumes of the phosphorylated Bad bands (normalized to the appropriate total bad value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

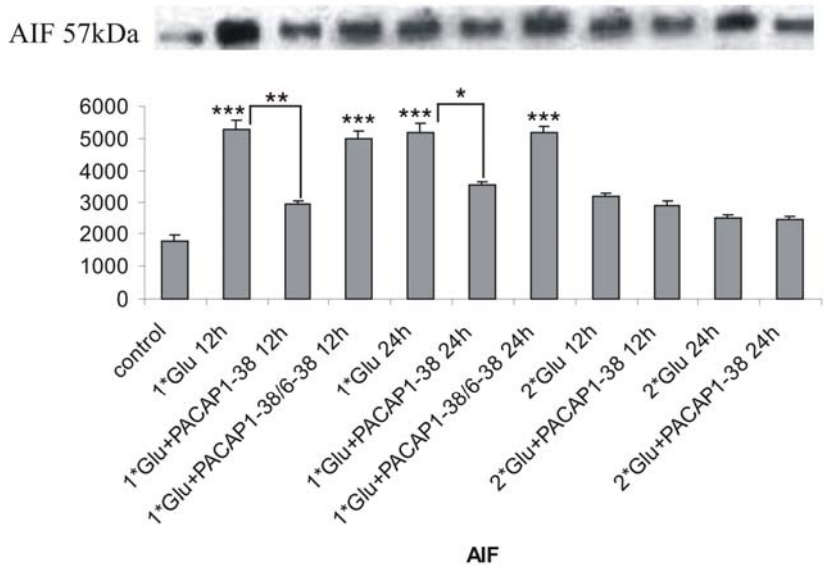

*Fig.15B. Effects of PACAP on phosphorylation of Bad in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) Bad (upper panel) and phosphorylated Bad (lower panel), bar diagrams representing pixel volumes of the phosphorylated Bad bands (normalized to the appropriate total bad value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01, ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

Release of cytochrome c from the mitochondria is an early event in the caspase-mediated apoptotic process, and cytosolic cytochrome c levels resulted from the different treatments showed a pattern somehow different from that of AIF. Namely, PACAP1-38 induced a late decrease (24 hours after the treatment), while PACAP6-38 achieved an early increase (12 hours after the treatment) in cytochrome c release (Fig. 16A). MSG caused a significant elevation of cytoplasmic cytochrome c which could be prevented by PACAP1-38 administration. Again, PACAP6-38 could block the effect of PACAP. Similarly to AIF translocation, MSG-induced cytochrome c release was much more pronounced 12 than 24 hours after the treatment. However, unlike in case of AIF, the second MSG treatment induced an elevation in cytosolic cytochrome c that was higher in extent than in case of the first one. Again, PACAP1-38 attenuated the effect of MSG, and the cytochrome c levels were much decreased 24 hours after the treatment as compared to the 12 h values (Fig. 16B).

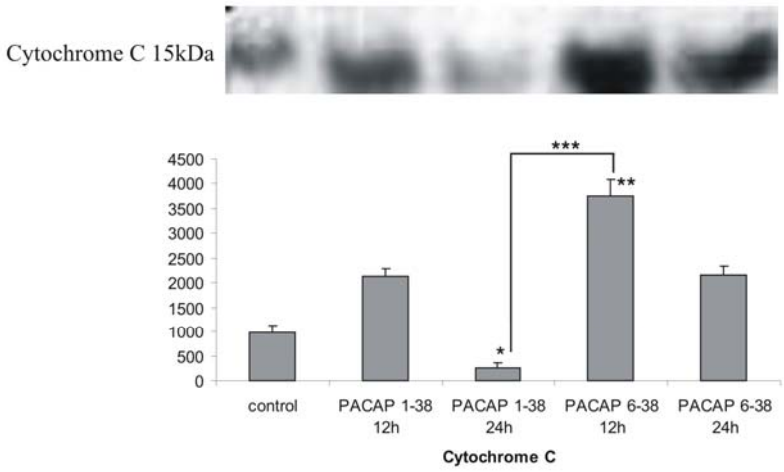

*Fig.16A. Effects of intravitreal PACAP1-38 and PACAP6-38 on levels of cytosolic AIF in the retina. Besides representative immunoblots, bar diagrams representing pixel volumes of the AIF bands (normalized to the appropriate anti-glyceraldehyde-3-phosphate dehydrogenase value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01, ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

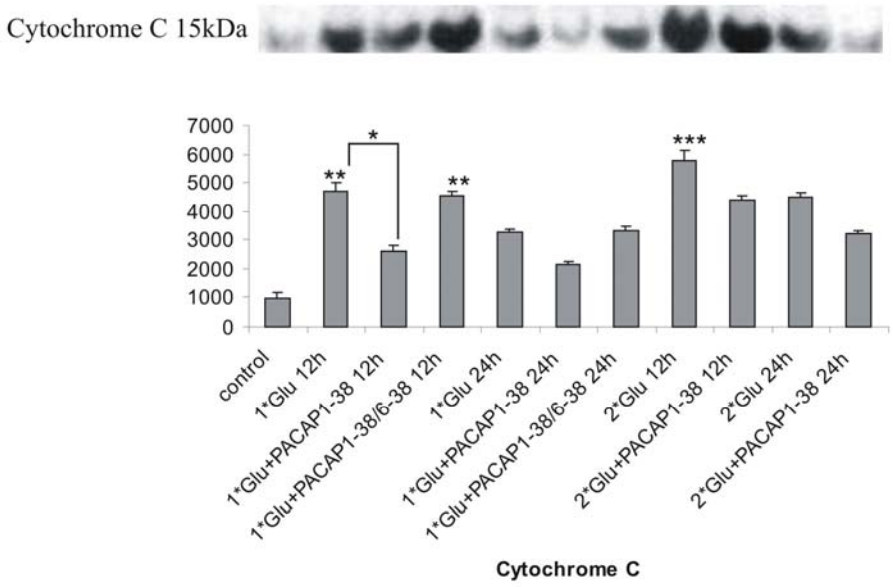

*Fig.16B. Effects of PACAP on AIF translocation in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. Besides representative immunoblots, bar diagrams representing pixel volumes of the AIF bands (normalized to the appropriate anti-glyceraldehyde-3-phosphate dehydrogenase value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01, ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

Cleavage of caspase 3 is a good indicator of caspase activation. PACAP1-38 treatment did not cause significant changes, while PACAP6-38 significantly increased caspase activation 12 and 24 hours after injection (Fig. 17A). MSG administration caused a significant increase in cleaved caspase-3 after the first treatment, with no further elevation after consecutive treatments (Fig. 17B). At these time-points, PACAP-treatment inhibited the MSG-induced caspase activation, and this inhibition could be blocked by PACAP6-38 treatment (Fig. 17B).

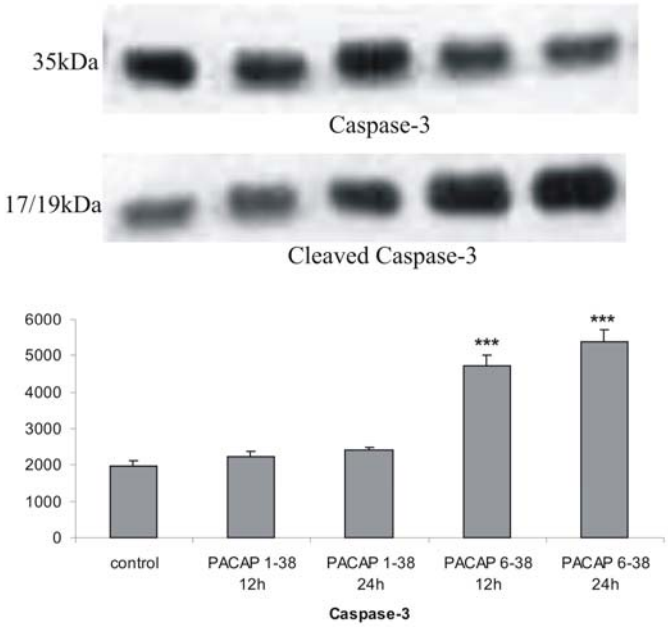

*Fig.17A. Effects of intravitreal PACAP1-38 and PACAP6-38 on the cleavage of caspase-3 in the retina. Besides representative immunoblots of uncleaved (upper panel) and cleaved (lower panel) caspase-3, bar diagrams representing pixel volumes of the cleaved caspase-3 bands (normalized to the appropriate anti-glyceraldehyde-3-phosphate dehydrogenase value) from at least 3 independent experiments are presented. ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

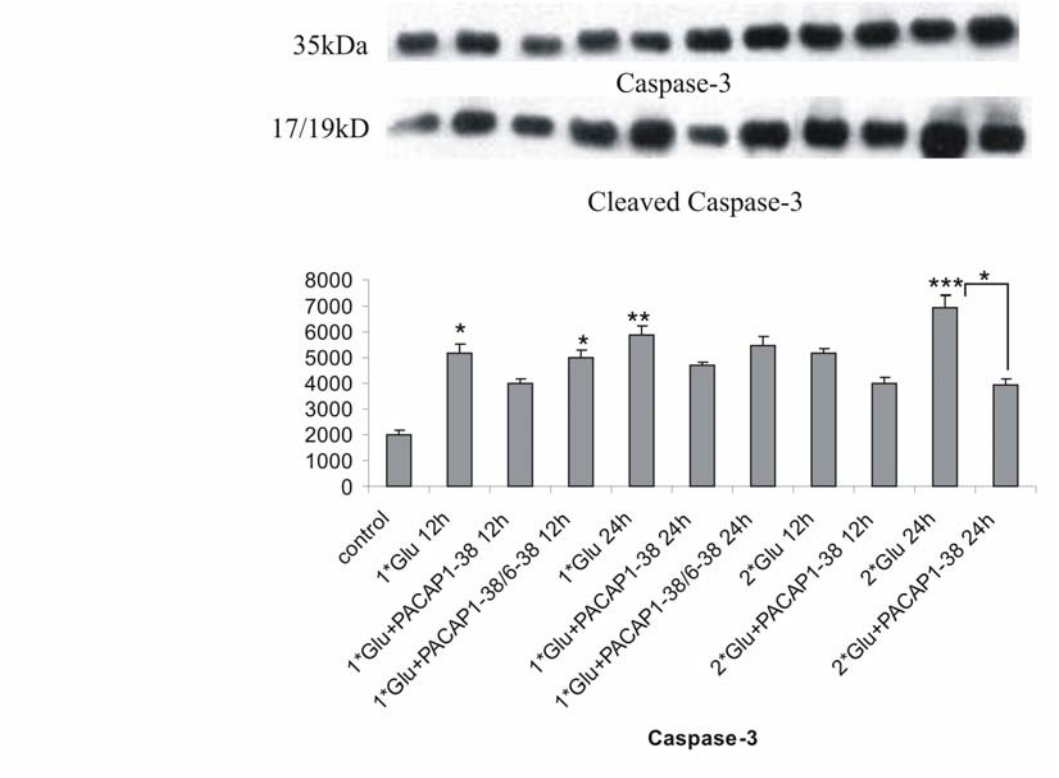

*Fig.17B. Effects of PACAP1-38 on cleaved caspase-3 in retinas treated with MSG on postnatal days 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 h after treatment. Besides representative immunoblots of uncleaved (upper panel) and cleaved (lower panel) caspase-3, bar diagrams representing pixel volumes of the cleaved caspase-3 bands (normalized to the appropriate anti-glyceraldehyde-3-phosphate dehydrogenase value) from at least 3 independent experiments are presented. *P<0.05, **P<0.01, ***P<0.001 compared to control levels where not otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.*

5.4. DISCUSSION AND CONCLUSION FROM THIS STUDY

The present study summarized the data showing that PACAP is neuroprotective in MSG-induced degeneration of the neonatal retina. Intravitreal PACAP administration significantly ameliorated the degeneration of inner retinal layers. According to the changes in signaling molecules it seems that this neuroprotective effect is due, at least partly, to the stimulation of antiapoptotic and the inhibition of proapoptotic signaling pathways.

Glutamate serves as a neurotransmitter in the vertebrate retina, and the majority of inner retinal cells bear ionotropic glutamate receptors making them vulnerable to MSG toxicity (Sucher et al., 1997; Babai et al., 2005). Nearly the same set of neurons has PACAP receptors (Seki et al., 1997, 2000; Babai et al., 2005) which serves as a morphological background for the neuroprotective effect of PACAP in the inner retina.

The neuroprotective effect of PACAP has been demonstrated in several in vitro cell injury models and in various brain pathologies in vivo. In vitro, PACAP enhances neuronal survival under toxic conditions such as oxidative stress, nitric oxide, ethanol, ceramide, 6-hyroxydopamine, beta-amyloid, HIV envelope protein, anisomycin and in vitro hypoxia (Somogyvari –Vigh and Reglodi, 2004; Reglodi et al., 2004). In vivo, the neuroprotective effects of PACAP have been shown in animal models of Parkinson`s disease, cerebral ischemia, Huntington-chorea, nerve transection, diffuse axonal injury and spinal cord injury (Somogyvari –Vigh and Reglodi, 2004). Although most in vivo studies do not provide explanation for the mechanism of neuroprotection, in vitro data indicate that PACAP acts via the specific PAC1 receptors through the cAMP/PKA/MAPK pathway to achieve its effects on neuronal survival (Seki et al., 1997; Waschek et al., 2002; Vaudry et al., 2000). In a model of cerebral ischemia, PACAP has been shown to inhibit the activation of JNK and the ischemia-induced reduction of ERK activity (Dohi et al., 2002; Shioda et al., 1998). Our present results are in accordance with these previous observations: PACAP inhibited the MSG-induced elevation of JNK phosphorylation and induced ERK activity in the retina, in vivo. Furthermore, our results show that the antiapoptotic pathways induced by PACAP in vitro, most probably play a role in its in vivo neuroprotective effects, since PACAP attenuated the MSG-induced activation of not only JNK, but also caspase-3, AIF, cytochrome-c, and elevated the protective phospho-Bad, ERK and CREB.

Cytochrome-c and AIF are both proapoptotic signals released from the mitochondria and play key roles in inducing apoptosis in retinal injuries (Hisatomi et al., 2001, He et al. 2004). The Bcl-2 family consists of both antiapoptotic and proapoptotic proteins involved in cell death/survival. Bad is a proapoptotic member of the Bcl family and serves a crosstalk function between Bcl-2 proteins and several kinase-signaling pathways. Phosphorylated Bad reduces the binding availability of the unphosphorylated form, thus promoting cell survival, which is a part of the retinal intrinsic cell survival program (Kim et al., 2005). In the present study we found that PACAP abolished the MSG-induced reduction of phospho-Bad. It has been shown that increasing cAMP with forskolin leads to phosphorylation of Bad and protects retinal cells from death induced by anisomycin or 2-aminopurine (Campos et al., 2003). Our observations are also in accordance with earlier studies showing that PACAP attenuated the effects of oxidative stress or ceramide on mitochondrial membrane potential (Falluel-Morel et al., 2004, Vaudry et al., 2002a) and inhibited the ceramide-evoked cytochrome-c release in cerebellar granule cells (Falluel-Morel et al., 2004).

A delicate balance between ERK and JNK activation is critical in determining the cell fate and these mediators have been shown to play an important role also in neurotoxicity in the retina (Munemasa et al., 2005). PACAP induces phosphorylation of ERK in cerebellar cultures and in hippocampal ischemia-reperfusion injury where it also inhibits JNK activation (Dohi et al., 2002, Shioda et al., 1998, Vaudry et al., 2003). Our present and earlier observations are in accordance with these reports: PACAP increased ERK activation and counteracted the JNK increasing effect of MSG treatment. Caspase-3 is considered as an executor of the apoptotic program, which also plays a role in retinal cell death (Nickells, 2004). In vitro studies have shown that caspase-3 activation becomes intense 12 hours after glutamate exposure of the retina in the ganglionic cell layer and inner nuclear layer and then persist till the end of the 24-hour-observation period (Chen et al., 2001). Similarly to these immunohistochemical observations, we found that caspase activation increased 12 and 24 hours after MSG exposure. Compounds that block caspase activation have been shown to be protective against glutamate-mediated cell death in the retina (Chen et al., 2001). We found that PACAP was able to attenuate the MSG-induced elevation of caspase-3 activity that conformed with data on the effect of PACAP in other neuronal cultures (Vaudry et al., 2000,2002a,b).

6. SUMMARY OF NEW FINDINGS

1. Treatment with PACAP markedly attenuates oxidative stress-induced apoptosis in cardiomyocytes via reducing the activation of caspase-3 and increasing the activation of Bcl-2 and phospho-Bad. Our results show, for the first time, that the well known anti-apoptotic effects of PACAP in various neuronal cell lines is also present in heart myocytes (Fig.18).

2. PACAP enhances endothelial cell survival, thus the cytoprotective, anti-apoptotic effect of PACAP is also present in these cells. Furthermore, we showed, for the first time, that PACAP influences the MAPK pathway, including ERK-, JNK-, and p38-signaling pathways to promote survival of endothelial cells (Fig.18).

3. The MSG-induced degeneration of the retina is partly due to the decrease of antiapoptotic, and increase of proapoptotic molecules. Local PACAP treatment attenuates these MSG-induced changes in apoptotic signaling pathways: PACAP increased the levels of the antiapoptotic phospho-ERK and CREB and phospho-Bad, and reduced the proapoptotic signaling molecules such as JNK, AIF, cytochrome-c and caspase-3 in the retina. These results may serve as a background for further studies attempting to reduce retinal degeneration induced by various agents (Fig.18).

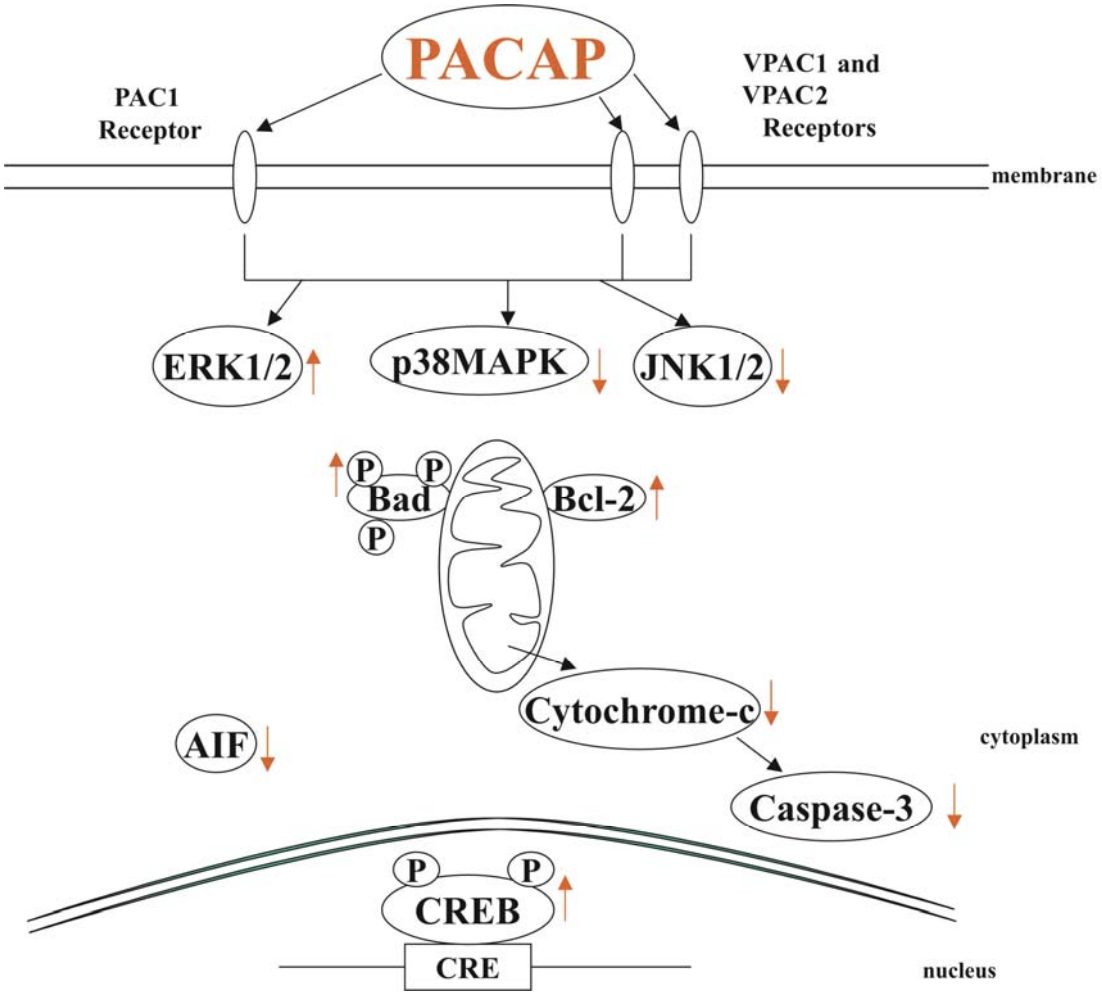

Fig.18 . Schematic figure about signaling pathways of PACAP. Effects of PACAP on the investigated signaling pathways on cardiomyocyte, endothelial cells and in the retina. The upper red arrows indicate increased or decreased levels of investigated proteins.

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8. ABBREVIATIONS

Akt	protein kinase B
AIF	apoptosis inducing factor
APAF	apoptosis activating factor
ASK1	apoptosis signal-regulating kinase 1
CREB	cAMP-responsive element binding protein
DMEM/F-12	Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12
ERK	extracellular signal-regulated kinase
FADD	FAS associated death domain
FASL	FAS ligand
GSK-3	glycogen synthase kinase 3
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MSG	monosodium-glutamate
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
PACAP	pituitary adenylate cyclase activating polypeptide
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
p38	p38 mitogen activating protein kinase
SAPK	stress activated protein kinase
VIP	vasoactive intestinal peptide

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10. PRESENTATIONS AND PUBLICATIONS

Publications related to the thesis

1. **Rácz B**, Tamás A, Kiss P, Tóth G, Gasz B, Borsiczky B, Ferencz A, Gallyas F Jr, Rőth E, Reglődi. Involvment of ERK and CREB signalling pathways in the protective effect of PACAP on monosodium glutamate-induced retinal lesion.
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2. **Rácz B**, Gallyas F Jr, Kiss P, Tóth G, Hegyi O, Gasz B, Borsiczky B, Ferencz A, Rőth E, Tamás A, Lengvári I, Lubics A, Reglődi D. The neuroprotective effects of PACAP in monosodium glutamate-induced retinal lesion involves inhibition of proapoptotic signaling pathways.
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3. **Rácz B**, Reglődi D, Kiss P, Babai N, Atlasz T, Gábriel R, Lubics A, Gallyas F Jr, Gasz B, Tóth G, Rőth E, Hegyi O, Lengvári I, Tamás A. In vivo neuroprotection by PACAP in excitotoxic retinal injury: review of effects on retinal morphology and apoptotic signal transduction.
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4. **Rácz B**, Gasz B, Borsiczky B, Gallyas F Jr, Tamás A, Józsa R, Lubics A, Kiss P, Rőth E, Ferencz A, Tóth G, Hegyi O, Wittmann I, Lengvári I, Somogyvári-Vigh A, Reglődi D. Protective effects of pituitary adenylate cyclase activating polypeptide in endothelial cells against oxidative stress-induced apoptosis
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Presentations

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