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Effects of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) on the PKA-Bad-14-3-3 Signaling Pathway in Glutamate-induced Retinal Injury in Neonatal Rats

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The neuropeptide PACAP (pituitary adenylate cyclase activating polypeptide) and its receptors are widely expressed in the nervous system including the retina. PACAP has well-known neuroprotective effects in neuronal cultures *in vitro* and against different insults *in vivo*. Recently, we have shown that PACAP1-38 is neuroprotective against monosodium glutamate (MSG)-induced retinal degeneration. Studying the molecular mechanisms of this protection has revealed that PACAP1-38 stimulates anti-apoptotic mechanisms such as phosphorylation of ERK1/2 and inhibits pro-apoptotic signaling molecules such as JNK1/2, p38MAPK, caspase-3 and the translocation of mitochondrial cytochrome *c* and apoptosis inducing factor in glutamate-treated retinas *in vivo*. In the present study we investigated the effects of PACAP1-38 on a further signal transduction pathway possibly involved in the protective effect of intravitreal PACAP1-38 administration against apoptotic retinal degeneration induced by neonatal MSG treatment. The focus of the present study was the protein kinase A (PKA)-Bad-14-3-3 transduction pathway. *In vivo* MSG treatment led to a reduction in the levels of anti-apoptotic molecules (phospho-PKA phospho-Bad, Bcl-xL and 14-3-3 proteins) in the retina. Co-treatment with PACAP1-38 counteracted these effects: the level of phospho-PKA, phospho-Bad, Bcl-xL and 14-3-3 were increased. All effects of PACAP1-38 were inhibited by the PACAP antagonist PACAP6-38. In summary, our results show that PACAP1-38 activates the PKA-Bad-14-3-3 pathway which is inhibited by MSG treatment. Our results also provide new insights into the signaling mechanisms possibly involved in the PACAP-mediated anti-apoptotic effects.

Keywords: Protein kinase A; Phospho-Bad; 14-3-3; Bcl-Xl

INTRODUCTION

The neuropeptide PACAP (pituitary adenylate cyclase activating polypeptide) was originally isolated from ovine hypothalamic extracts based on its stimulatory effect on adenylate cyclase activity in pituitary cells (Vaudry *et al.*, 2000). PACAP exists in two forms, with 27 and 38 amino acid

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residues. PACAP acts through G protein-coupled receptors: the specific PAC1 receptor as well as the VPAC1 and VPAC2 receptors, which also bind vasoactive intestinal peptide (VIP) with similar affinity (Vaudry *et al.*, 2000). Numerous actions of PACAP in the nervous system and in peripheral organs have been described and reviewed (Vaudry *et al.*, 2000; Somogyvari-Vigh and Reglodi, 2004; Shioda *et al.*, 2006).

In the retina, PACAP and its receptors are present in most layers: in the amacrine and horizontal cells, in the inner plexiform layer, in the ganglionic cell layer, in the nerve fiber layer and also in Muller glial cells (D`Agata and Cavallaro, 1998; Izumi *et al.*, 2000; Seki *et al.*, 2000a,b; Silveira *et al.*, 2002; Kubrusly *et al.*, 2005; Nakatani *et al.*, 2006). PACAP is a potent stimulator of adenylate cyclase in the retina (Onali and Olianas, 1994) and is involved in the retinal regulation of circadian rhythmic functions (Jozsa *et al.*, 2001; Hannibal and Fahrenkrug, 2004; Vereczki *et al.*, 2006).

PACAP is a member of the growing family of neurotrophic factors (Waschek, 2002; Falluel-Morel *et al.*, 2007). Besides the numerous reports on its trophic effects on neuronal cultures, these actions have also been reported in the retina (Borba *et al.*, 2005). PACAP is neuroprotective *in vitro* and *in vivo*, in various models of nervous injuries (Somogyvari-Vigh and Reglodi, 2004). Its retinoprotective effects have been shown against anisomycin-induced cell death, following optic nerve transection and in ischemic retinal lesion (Silveira *et al.*, 2002; Seki *et al.*, 2003; Atlasz *et al.*, 2007).

Elevations of extracellular glutamate concentrations lead to retinal degeneration, and several diseases of the retina are likely to involve glutamate receptor-mediated toxicity (Sucher *et al.*, 1997). *In vitro*, PACAP attenuates glutamate-induced neurotoxicity in retinal culture (Shoge *et al.*, 1999). We have recently shown that this protective effect is present also *in vivo*: intravitreal and even systemic injection of PACAP significantly reduced the monosodium glutamate (MSG)-induced retinal degeneration (Tamas *et al.*, 2004; Reglodi *et al.*, 2005; Babai *et al.*, 2005; 2006; Kiss *et al.*, 2006). MSG treatment of neonatal rats leads to a severe degeneration of inner retinal layers, which can be counteracted by PACAP. The retinoprotective effect of PACAP has also been shown against kainic acid, another glutamate receptor agonist (Seki *et al.*, 2006).

Retinal cell death induced by over-stimulation of glutamate receptors is related to apoptosis (Lam *et al.*, 1999; Chen *et al.*, 2001; Baptiste *et al.*, 2004). PACAP is a very potent anti-apoptotic agent, and according to most studies, these effects are mediated through the cAMP/protein kinase A (PKA)/mitogen activated protein kinase (MAPK) pathways (Vaudry *et al.*, 2000; Somogyvari-Vigh and Reglodi, 2004; Falluel-Morel *et al.*, 2007). Currently available data indicate that PACAP is able to interfere with apoptotic pathways at various levels: it stimulates proteins with anti-apoptotic effects such as extracellular signal-regulated kinase (ERK1/2), cAMP-responsive element binding protein (CREB), phospho-Bad, Bcl-2 and inhibits pro-apoptotic molecules such as JNK1/2, p38 MAPK, apoptosis signal-regulating kinase-1 and caspase-3 (Vaudry *et al.*, 2003; Somogyvari-Vigh and Reglodi, 2004; Gasz *et al.*, 2006a,b; Ravni *et al.*, 2006; Shioda *et al.*, 2006; Falluel-Morel *et al.*, 2007).

In our previous studies, we have demonstrated that PACAP induces anti-apoptotic mechanisms and inhibits pro-apoptotic signaling pathways in the MSG-treated retinas *in vivo*: it increases levels of ERK1/2 and CREB, and decreases activation of caspase-3, JNK1/2, and the cytosolic translocation of apoptosis inducing factor and cytochrome *c* (Racz *et al.*, 2006a,b,c). Although some apoptotic pathways are known to be specific to certain cell lines, many of them are organized in a ubiquitous manner in different tissues. We have recently shown the anti-apoptotic effects of PACAP in oxidative stress-induced apoptosis of cardiomyocytes and endothelial cells, where similar influence on the anti-apoptotic signal transduction pathways has been found (Gasz *et al.*, 2006a,b; Racz *et al.*, 2007a).

Most recently we have shown that PACAP activates the PKA-Bad-14-3-3 signal transduction pathway, which may be involved in its protective effects against cardiomyocyte apoptosis (Racz *et al.*, 2007b). Bad belongs to the pro-apoptotic members of the Bcl-2 family, which forms a complex with the anti-apoptotic member of the family, Bcl-xL. The bound Bcl-xL is not able to exert its anti-apoptotic effects. When Bad is phosphorylated at Ser112, Ser136 or Ser155, it forms a complex with the 14-3-3 protein in the cytosol thereby pre-

venting binding to Bcl-2/Bcl-xL and so promoting cell survival (Wang *et al.*, 1999). Phosphorylation of Bad can be triggered by survival factors, such as PKA. Given the common anti-apoptotic mechanism exerted by PACAP in different tissues, the aim of the present study was to investigate the changes caused by MSG treatment in the PKA-Bad-14-3-3 signal transduction pathway and whether it can be influenced by PACAP administration.

MATERIALS AND METHODS

Newborn Wistar rat pups were used for the experiments. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (No: BA02/2000-20/2006). Pups were injected subcutaneously with 4 mg/g bodyweight MSG on postnatal days 1 and 5 (Babai *et al.*, 2005; 2006; Racz *et al.*, 2006b). 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 control. In order to investigate the effects of PACAP1-38 and the PACAP 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; Racz *et al.*, 2006b), and the peptides were synthesized as previously described (Gasz *et al.*, 2006).

Animals were randomly distributed and were sacrificed 12 or 24 hours after treatments from each group. Retinas were removed and processed for Western blot analysis. Samples were homogenized in ice-cold isotonic Tris buffer (50 mM, pH: 8.0) containing 0.5 mM sodium-metavanadate, 1 mM EDTA and Protease Inhibitor Coctail (1:1000, Sigma-Aldrich Co., Hungary). Antigens were determined from the tissue homogenates following sonication. Proteins were precipitated by TCA, washed 3 times with -20ºC 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 4ºC with antibodies recognizing the following antigens: phospho-specific PKA, phospho-specific Bad(Ser 112), Bcl-xL and 14-3-3 (Cell Signaling Technology, Hungary). 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. Even loading of samples was confirmed by anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon International, Inc.) immunoblotting (not shown). Pixel volumes of the bands of interest were normalized to that of the appropriate loading control (Bcl-xL, 14-3-3) or the respective total protein (phospho-PKA, phospho-Bad Ser112). Each experiment was repeated a minimum of three times. All data were expressed as the mean ± S.D. Statistical analysis was performed by analysis of variance and Student's t test.

RESULTS

PKA activity was significantly increased by PACAP1-38 treatment alone 12 hours after the treatment while PACAP antagonist alone had no effect (FIG. 1A). The level of phospho-PKA was significantly decreased after 12 and 24 hours after the first treatment with MSG (FIG. 1B). This inhibiting effect of MSG on PKA phosphorylation was prevented by PACAP1-38 treatment which could be counteracted by the PACAP antagonist PACAP6-38 (FIG. 1B). PKA phosphorylation was not significantly altered after the second MSG treatment (FIG. 1C).

Bad phosphorylation at Ser112 was slightly increased by PACAP1-38 treatment alone and decreased by PACAP6-38 treatment alone at 12 and 24 hours (FIG. 2A). However, compared to control groups, significant results were obtained only with PACAP6-38 administration at 24 hours (FIG. 2A). MSG induced a significant decrease in Bad phosphorylation 12 and 24 hours after the first

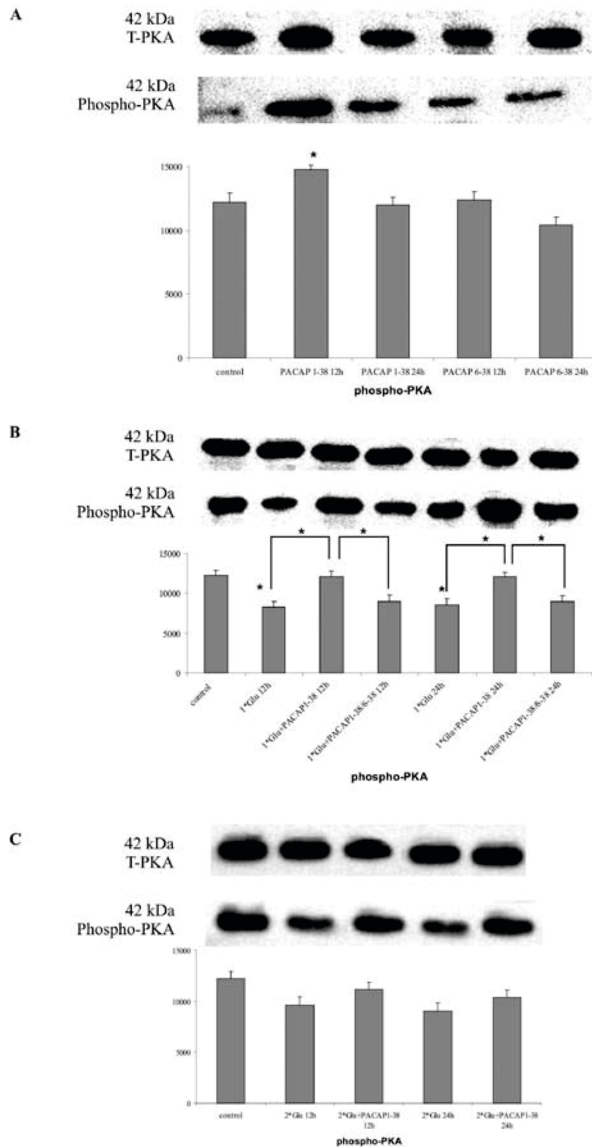

FIGURE 1 (**A**) Effects of intravitreal PACAP1-38 and PACAP6-38 on phosphorylation and thus activation of PKA in the retina. (**B,C**) Effects of PACAP on phosphorylation of PKA in retinas treated with MSG on postnatal day 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 hours after treatment. Besides representative immunoblots of total (phosphorylated + non-phosphorylated) PKA (upper panel) and phosphorylated PKA (lower panel), bar diagrams representing pixel volumes of the phosphorylated PKA bands (normalized to the appropriate total PKA value) from at least 3 independent experiments are presented. *P <0.05 compared to control levels unless otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.

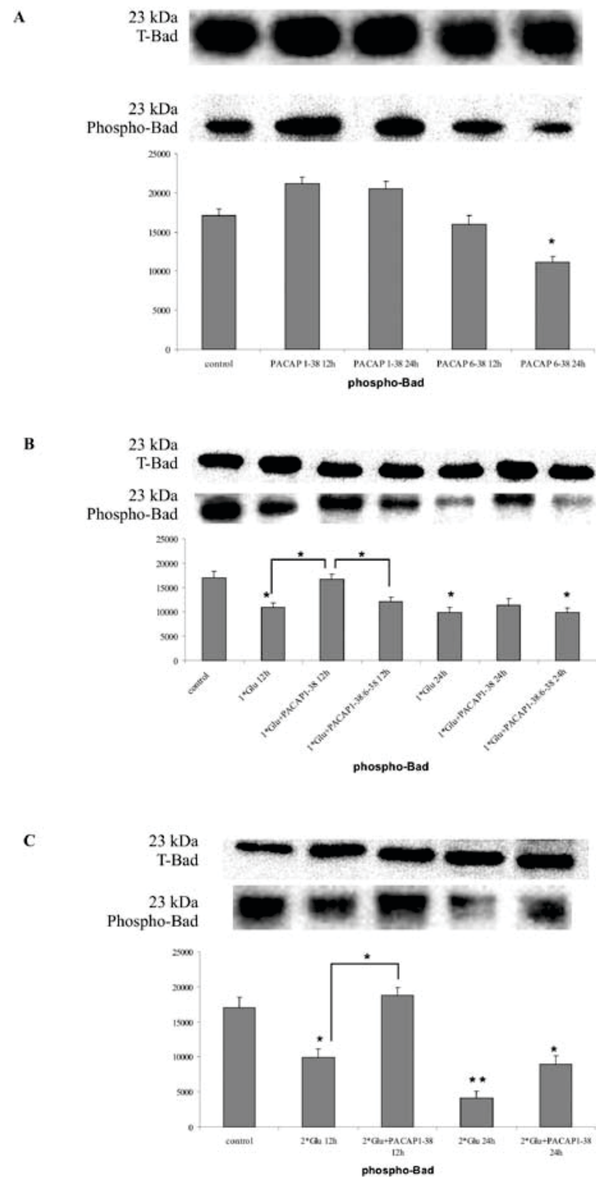

FIGURE 2 (**A**) Effects of intravitreal PACAP1-38 and PACAP6-38 on phosphorylation of Bad at Ser112 in the retina. (**B,C**) Effects of PACAP on phosphorylation of Bad at Ser112 in retinas treated with MSG on postnatal day 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 hours after treatment. Besides 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 compared to control levels unless otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.

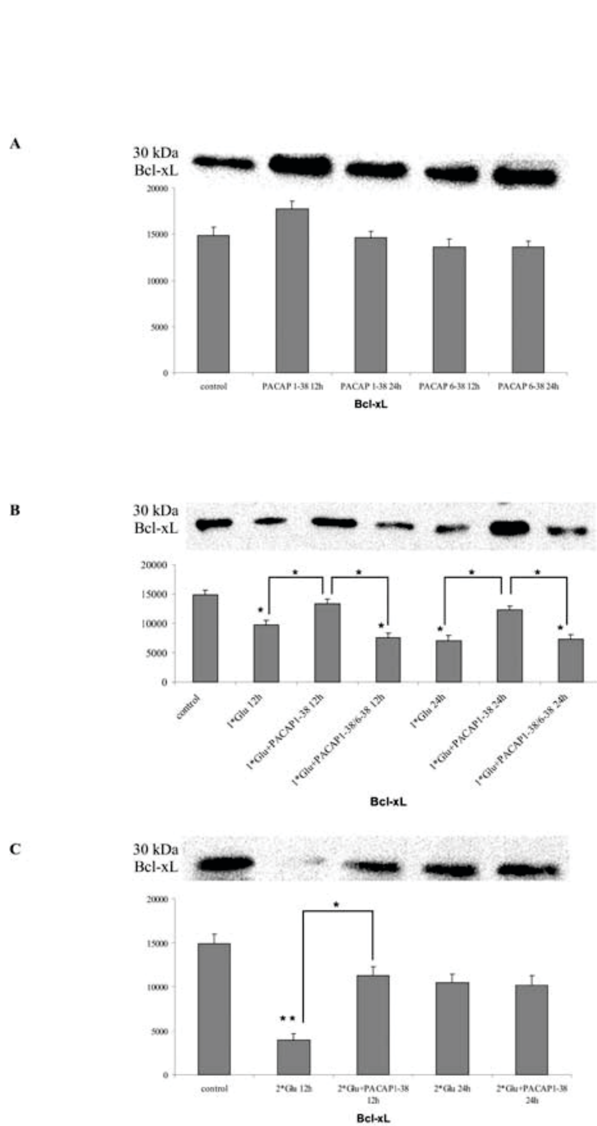

FIGURE 3 (**A**) Effects of intravitreal PACAP1-38 and PACAP6-38 on levels of Bcl-xL in the retina. (**B,C**) Effects of PACAP on Bcl-xL translocation in retinas treated with MSG on postnatal day 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 hours after treatment. Besides representative immunoblots, bar diagrams representing pixel volumes of the Bcl-xL 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 compared to control levels unless otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.

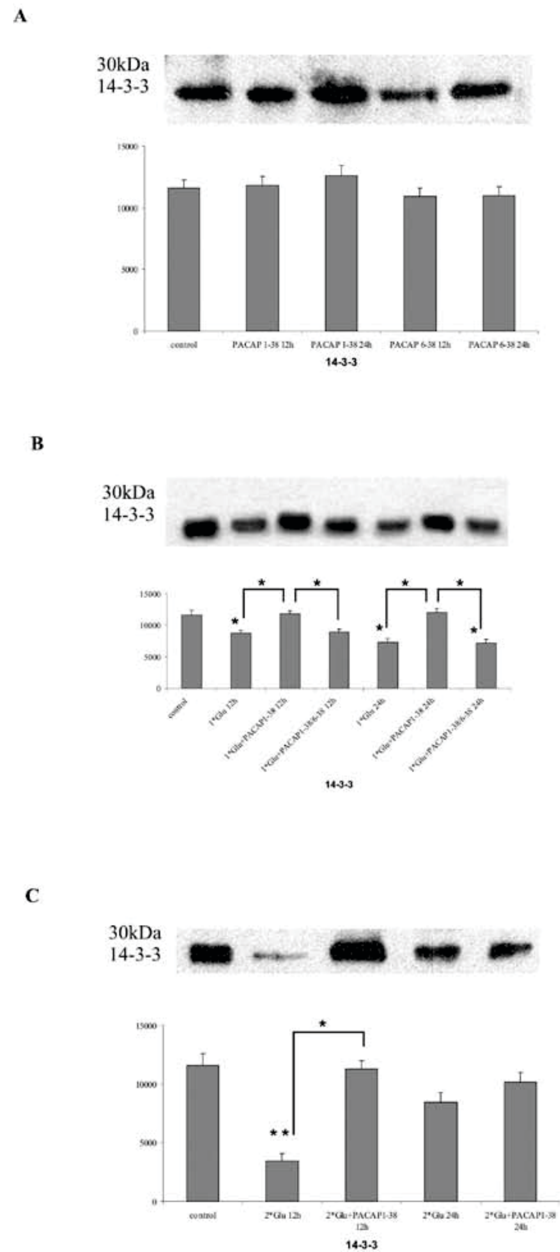

FIGURE 4 (**A**) Effects of intravitreal PACAP1-38 and PACAP6-38 on levels of 14-3-3 in the retina. (**B,C**) Effects of PACAP on 14-3-3 translocation in retinas treated with MSG on postnatal day 1 (1*Glu) and on days 1 and 5 (2*Glu), 12 and 24 hours after treatment. Besides representative immunoblots, bar diagrams representing pixel volumes of the 14-3-3 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 compared to control levels unless otherwise indicated. Vertical axes represent pixel volumes of the scanned bands on the Western blots in arbitrary units.

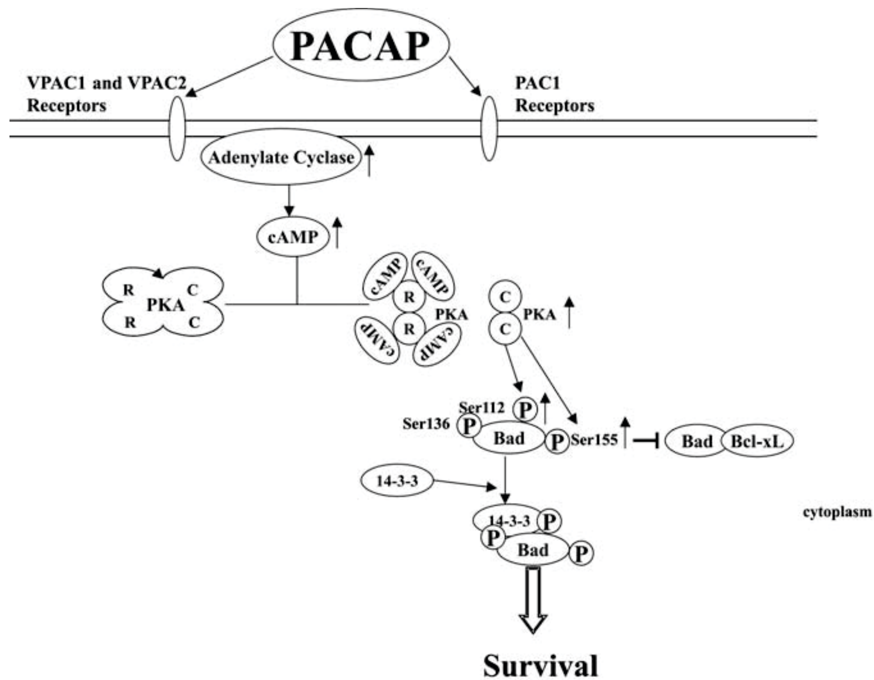

FIGURE 5 Schematic drawing about the PKA-Bad-14-3-3 signaling pathway induced by PACAP. The arrows indicate increased or decreased levels of investigated proteins. PACAP effectively stimulates cAMP in the retina, where the second messenger cyclic AMP (cAMP) activates cAMP-dependent protein kinase A (PKA) in mammalian cells. Inactive PKA is a heterotetramer composed of regulatory subunit (R) dimer and a catalytic subunit (C) dimer, and in this inactive state the pseudosubstrate sequence on the R subunits blocks the active sites on the C subunits. Upon binding of cAMP to the R subunits, the autoinhibitory contact is eased, and active monomeric C subunits are released. Substrates that contain this consensus sequence and have been shown to be phosphorylated by PKA include Bad (Ser155 and Ser 112). Bad is a pro-apoptotic member of the Bcl-2 family, which impairs the function of the protective Bcl-xL. Phosphorylation at Ser112 and Ser136 results in the binding of Bad to 14-3-3 proteins and inhibition of Bad binding to Bcl-2 and Bcl-xL.

treatment, which was abolished by PACAP administration after the first 12 hours, while it was not significantly altered at 24 hours (FIG. 2B). These effects could be blocked by adding PACAP6-38 (FIG. 2B). Similar decreases in Bad phosphorylation were observed after the second MSG treatment, which was counteracted by PACAP1-38 both 12 and 24 hours after treatments (FIG. 2C).

Bcl-xL was slightly elevated 12 hours after PACAP1-38 treatment alone, although it was not significant compared to control values (FIG. 3A). Reduction in level of Bcl-xL was induced by the first MSG treatment (FIG. 3B). PACAP1-38 was able to up-regulate the level of Bcl-xL in the retina after the MSG treatment which could be blocked by PACAP antagonist (FIG. 3B). MSG also induced a significant decrease in Bcl-xL after the second treatment at 12 hours, which was moderated by PACAP1-38 (FIG. 3C). No changes were observed 24 hours after the second MSG treatment (FIG. 3C).

The level of 14-3-3 protein was not altered by PACAP1-38 or PACAP antagonist treatments alone (FIG. 4A). However, it was significantly decreased 12 and 24 hours after the first, and 12 hours after the second MSG treatment (FIGs. 4B,4C). At all time-points, where MSG-induced decreases of 14-3-3 protein were observed, PACAP could block these effects (FIGs. 4B,4C). The 14-3-3 protein-inducing effects of PACAP could be abolished with PACAP6-38 administration (FIG. 4B).

DISCUSSION

The present study demonstrated that MSG treatment inhibited the PKA-Bad-14-3-3 signal transduction pathway, a survival-promoting pathway, in neonatal retinas *in vivo*. PACAP1-38 counteracted

in vivo against the MSG-induced changes by inducing phosphorylation of PKA and Bad at Ser 112, thereby increasing the levels of the protective Bcl-xL and 14-3-3 proteins (FIG. 5). Treatment with the PACAP antagonist, PACAP6-38, could block the protective effects of PACAP1-38 indicating that the effects of PACAP were specific.

Neurotoxins, in a stricter sense, represent exogenous compounds that produce specific neuronal damage when given in relatively small amounts (Kostrzewa, 1999; Segura-Aguilar and Kostrzewa, 2004; 2006). In a broader sense, endogenous substances can produce toxic effects when present at higher concentrations, like the overexcitation of glutamate receptors, which leads to excitotoxic injury in the nervous system (Smythies, 1999). The protective effects of PACAP against glutamate-induced neuronal cell death have been described in cortical neurons (Morio *et al.*, 1996) and in PC12 cells (Said *et al.*, 1998). Glutamate toxicity is also implicated in various ocular diseases (Sucher *et al.*, 1997; Vidal-Sanz *et al.*, 2000; Munemasa *et al.*, 2005). The protective effects of PACAP in the retina have first been shown in cultured retinal neurons (Shoge *et al.*, 1999). In the past few years, we have provided evidence that the protective effects of PACAP against glutamate-induced retinotoxicity are also present *in vivo*, in rats treated neonatally with MSG, where the almost entire degeneration of the inner retinal layers can be prevented by PACAP treatment (Tamas *et al.*, 2004; Babai *et al.*, 2005; 2006; Reglodi *et al.*, 2005; Kiss *et al.*, 2006).

Recent studies have indicated that excitotoxicity leads to apoptotic cell death in the retina (Lam *et al.*, 1999; Chen *et al.*, 2001; Baptiste *et al.*, 2004). Data accumulated since the discovery of PACAP have established that PACAP is a very potent anti-apoptotic agent in various neuronal and non-neuronal cells (Vaudry *et al.*, 2000; Somogyvari-Vigh and Reglodi, 2004). Evidence shows that the anti-apoptotic effects of PACAP play an important role during neurogenesis and differentiation of the nervous system and in neuronal repair following injuries (Waschek, 2002; Castel *et al.*, 2006; Shioda *et al.*, 2006; Falluel-Morel *et al.*, 2007). Exogenously given PACAP can exert neuroprotective effects which involve, among others, anti-apoptotic actions (Somogyvari-Vigh and Reglodi, 2004; Shioda *et al.*, 2006).

Various, partially interacting, signaling mechanisms underlying the anti-apoptotic effects of PACAP have been shown which lead to inhibition of the executor caspases (Vaudry *et al.*, 2003; Somogyvari-Vigh and Reglodi, 2004; Ravni *et al.*, 2006). The PKA/MAPK pathway seems to be an important contributor to these protective effects: the involvement of various members of the MAPK family has been demonstrated (Vaudry *et al.*, 2000; Somogyvari-Vigh and Reglodi, 2004; Shioda *et al.*, 2006). PACAP stimulates the production of the generally anti-apoptotic ERK1/2, and inhibits the production of the pro-apoptotic JNK1/2 and p38MAPK (Shioda *et al.*, 1998; Dohi *et al.*, 2002; Vaudry *et al.*, 2003; Aubert *et al.*, 2006; Falluel-Morel *et al.*, 2007). PACAP also influences the mitochondrial apoptotic pathway: it inhibits the mitochondrial release of cytochrome *c* and apoptosis inducing factor, both of which are pro-apoptotic signals (Falluel-Morel *et al.*, 2004). In previous studies, we have shown that MSG treatment induces activation of caspase-3 and JNK, promotes the cytosolic translocation of cytochrome *c* and apoptosis inducing factor from mitochondria, and inhibits ERK1/2 and CREB, while PACAP treatment counteracts these effects of MSG treatment (Racz *et al.*, 2006a,b,c).

The focus of our present study was the survival-promoting PKA-Bad-14-3-3 pathway which, according to our results, is also activated by *in vivo* PACAP treatment. The Bcl-2 family consists of both pro- and anti-apoptotic proteins that play a key role in cell death-survival also in the retina (Chen *et al.*, 2003; Doonan and Cotter, 2004; Charles *et al.*, 2005; Wang *et al.*, 2005). Previous studies have shown that PACAP stimulates the expression of the protective Bcl-2 protein (Falluel-Morel *et al.*, 2004; Aubert *et al.*, 2006; Gasz *et al.*, 2006a). In the present study we investigated levels of other members of the Bcl-2 family: Bad and Bcl-xL. The dephosphorylated form of Bad promotes cell death by binding to Bcl-2 or Bcl-xL, thus impairing the function of these anti-apoptotic proteins (Wang *et al.*, 1999). Survival factors, such as PKA, induce the phosphorylation of Bad, leading to a conformational change in Bad which causes dissociation of the Bad-Bcl-xL complex in the cytosol, promoting cell survival (Harada *et al.*, 1999; Datta *et al.*, 2000). The phosphorylation of Bad is also part of an intrin-

sic cell survival program in the retina (Kim and Park, 2005). Our present observations, that PACAP increased the phosphorylation of PKA, are in accordance with numerous earlier studies showing the same effect (Vaudry *et al.*, 2000; Somogyvari-Vigh and Reglodi, 2004). The PACAP-induced increased phosphorylation of PKA in the present study could be responsible for the increased phosphorylation of Bad at Ser112. 14-3-3 proteins play an essential role in many biological processes, including the inhibition of apoptosis. Also, the finding that PACAP6-38 treatment alone decreased Bad phosphorylation indicates the involvement of endogenous PACAP in this process. 14-3-3 binds to phosphorylated Bad, which prevents the formation of the Bad/Bcl-xL complex (Datta *et al.*, 2000). Our results show that PACAP increased the level of 14-3-3 and thus also the protective Bcl-xL proteins.

In summary, we demonstrated that MSG treatment inhibited the survival-promoting PKA-Bad-14-3-3 signal transduction pathway. Neurotrophic factors represent an important endogenous way to exert neuro- and retinoprotection (Barkana and Belkin, 2004; Segura-Aguilar and Kostrzewa, 2006). Our present study shows that PACAP, a neurotrophic factor, stimulates the PKA-Bad(Ser112)-14-3-3 signaling pathway. These results provide new insights into the signaling mechanism mediated by PACAP and give further evidence for the *in vivo* survival-promoting effects of PACAP in MSG-induced retinal injury.

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