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PACAP Protects Against Salsolinol-Induced Toxicity in Dopaminergic SH-SY5Y Cells: Implication for Parkinson's Disease

Dwayne Brown¹, Andrea Tamas², Dora Reglodi², and Yousef Tizabi¹

¹Department of Pharmacology, Howard University College of Medicine, Washington DC

²Department of Anatomy, PTE-MTA Lendulet PACAP Research Team, University of Pecs, Pecs, Hungary

Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an endogenous 38 amino acid containing neuropeptide with various cytoprotective functions including neuroprotection. Administration of PACAP has been shown to reduce damage induced by ischemia, trauma or exogenous toxic substances. Moreover, mice deficient in PACAP are more vulnerable to damaging insults. In this study we sought to determine whether PACAP may also be protective against salsolinol-induced toxicity in SH-SY5Y cells and if so, elucidate its mechanism(s) of action. Salsolinol (SALS) is an endogenous dopamine metabolite with selective toxicity to nigral dopaminergic neurons, which are directly implicated in Parkinson's disease (PD). SH-SY5Y cells, derived from human neuroblastoma cells express high levels of dopaminergic activity and are used extensively as a model to study these neurons. Exposure of SH-SY5Y cells to 400uM SALS for 24 h resulted in approximately 50% cell death that was mediated by apoptosis as determined by cell flow cytometry and increases in caspase 3 levels. Cellular toxicity was also associated with reductions in brain-derived neurotrophic factor (BDNF) and phosphorylated cyclic AMP response element-binding (p-CREB) protein. Pretreatment with PACAP dose-dependently attenuated SALS-induced toxicity and the associated apoptosis and the chemical changes. PACAP receptor antagonist PACAP 6-38 in turn, dose-dependently blocked the effects of PACAP. Neither PACAP nor PACAP antagonist had any effect of its own on cellular viability. These results suggest protective effects of PACAP in a cellular model of PD. Hence, PACAP or its agonists could be of therapeutic benefit in PD.

Keywords

PACAP; Salsolinol; SH-SY5Y cell line; Neuroprotection; Apoptosis; BDNF; p-CREB

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by movement disorders, resulting from damage or destruction of dopaminergic neurons in the substantia nigra. Later, cognitive and behavioral problems may also arise. Although available medications provide some symptomatic relief in almost all patients, none has been shown to significantly slow or stop the disease progression (Fernandez, 2012). Consequently, there is a dire need for more effective therapeutic interventions. The cause of

*Corresponding Author: Yousef Tizabi, Ph.D., Department of Pharmacology, Howard University College of Medicine, 520 W Street NW, Washington, DC 20059, Tel: 202-806-9719, Fax: 202-806-4453, ytizabi@howard.edu.

PD is unknown but some atypical cases seem to have a genetic origin. Although numerous genes responsible for familial PD have been identified, the etiology of sporadic PD, which accounts for the majority of PD cases, is still unknown (Healy et al. 2004; Morris, 2005). Recent advances in PD pathology suggest that the neuronal degeneration in this disease likely involves several cellular and molecular events, including oxidative stress, microglia-mediated inflammation, as well as proapoptotic mechanisms (von Bohlen et al. 2004). As such, there is a lot of effort being put into finding new therapies targeting these pathways.

Pituitary adenylate cyclase activating polypeptide (PACAP), originally isolated from the sheep hypothalamic extract, is a widespread neuropeptide with diverse actions. PACAP acts through the specific PAC1 receptor, and the VPAC1/2 receptors, which also bind vasoactive intestinal peptide (Vaudry, 2009). PACAP, and its closest structural related peptide VIP, have been shown to possess potent neuroprotective properties against ischemia, trauma or exogenous toxic substances such as 6-hydroxy-dopamine (6-OHDA), MPTP and rotenone both in-vivo and in neuronal cultures (Offen et al. 2000; Reglodi et al. 2004; Somogyvari-Vigh and Reglodi 2004; Wang et al. 2005, 2008; Botia et al. 2011; Rat et al. 2011; Reglodi et al. 2011; Nakamachi et al. 2012; Tamas et al. 2012; Tuncel et al. 2012; Tsuchikawa et al. 2012). Moreover, mice deficient in PACAP are more vulnerable to damaging insults (Reglodi et al. 2012; Szabadfi et al. 2012; Tamas et al. 2012a,b). Based on these findings, it has been suggested that PACAP may offer a novel therapeutic approach in the treatment of neurodegenerative diseases including Parkinson's disease (Dejda, 2005; Vaudry et al. 2009; Reglodi et al. 2011). PACAP and its major receptor PAC1, postulated to be the primary target of PACAP, are highly expressed in the substantia nigra (Masuo et al. 1992; Reglodi et al. 2011). It has been documented that at least some of the protective effects of PACAP are due to its anti-apoptotic effects, reflected in caspase 3 reduction, as well as up-regulation of brain derived neurotrophic factor (BDNF) and enhancement of its signal transduction mediated via phosphorylation of cyclic AMP response element-binding protein (CREB) (Vaudry et al. 2000; Frechilla et al. 2001; Yaka et al. 2003; Racz et al. 2006; Botia et al. 2011; Rat et al. 2011; Lazarovici et al. 2012). PACAP may also influence dopamine synthesis via activation of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (Reglodi et al. 2011).

In this study, we first sought to determine whether PACAP might have protective effects against salsolinol-induced toxicity in SH-SY5Y cells. Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, SALS) is an endogenous dopamine metabolite with selective toxicity to nigral dopaminergic neurons. Indeed, many Parkinson patients show high levels of SALS in their urine and cerebrospinal fluid, which has led to the suggestion that SALS might be involved in the etiology or loss of dopamine neurons in at least some of these patients (Storch et al. 2002; Maruyama et al. 2004). SH-SY5Y cells, derived from human neuroblastoma cells express high levels of dopaminergic activity and are used extensively as a cellular model to study mechanism(s) of toxicity and protection in nigral dopaminergic neurons (Storch et al. 2002; Maruyama et al. 2004; Naoi et al. 2004; Copeland et al. 2007; Das and Tizabi, 2009; Ramlochan Singh et al. 2011). Once the protective effects of PACAP were established, we were interested in determining the mechanism of such protection and whether the effects of PACAP could be attenuated or blocked by a PACAP antagonist.

METHODS

Drugs

Salsolinol, PACAP and its antagonist (PACAP6-38) as well as other analytical reagents were purchased from Sigma Chemical Company (Sigma Aldrich, St. Louis, MO).

Cell Culture

SH-SY5Y cells purchased from American Type Culture Collection (ATCC) in Manassas, VA and were cultivated in a 1:1 mixture of Dulbeccos Modified Eagle Medium and Ham's F12 supplemented with 10% fetal bovine serum, penicillin/streptomycin (1000 IU/ml) and gentamicin (50 ug/ml) at 37°C in an incubator. The cells were harvested approximately 5 days later when confluent and plated in a 96 well plate (1.6×10^4 cells/well). Cells were allowed to adhere to plate for 24 h.

Salsolinol Treatment

To determine the dose-response effects of salsolinol, following the adherence of the cells to the plate, the media was aspirated and fresh media containing different concentrations of salsolinol were added. After 24 h, cell viability was measured via MTT assay.

PACAP Treatment

To determine the protective effects of PACAP, 2 h prior to salsolinol (400 uM, a concentration that yielded approximately 50% toxicity), various concentrations of PACAP were added. Again, after 24 h cell viability was determined via MTT assay.

PACAP Antagonist Treatment

To determine the effects of PACAP antagonist on protective effects of PACAP, 2 h prior to PACAP, cells were treated with PACAP6-38, followed by salsolinol (400 uM) and determination of cell viability after 24 h via MTT assay.

MTT Assay for Cell Viability

Determination of cell viability was done by 3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The yellow MTT tetrazolium salt was dissolved in phosphate-buffered saline (PBS) with 10mM HEPES and 30ul of this reagent was added to each well for 3 h and was incubated at 37°C. The live cells caused a reduction of the yellow salt to insoluble purple formazan crystals. The wells were then aspirated and 50ul of dimethyl sulfoxide (DMSO) was added to the wells to solubilize the crystals. The plates were shaken for 1 h and read spectrophotometrically at 570 nm in a plate reader. The data was analyzed and is represented as percent cell viability.

Cell Flow Cytometry

Cell flow cytometry was used to detect apoptosis vs necrosis by measuring and sorting cells by fluorescent labeling of markers on cell surface. The cells were grown and treated as described above. In this case, however, following harvesting the cells were washed twice with cold PBS and then gently suspended in a solution that consisted of 100 ul Annexin V-Fluos labeling solution, 5 ul of fluorescein isothiocyanate labeled by Annexin V-FITC and 5 ul of propidium iodide (PI). Afterwards, the cells were incubated in the dark at room temperature for 15 minutes before adding 500 ul Annexin V-Fluos labeling solution to each well. Finally, the cells were subjected to flow cytometry using a cellometer machine (Nexcelom, Lawrence, MA) followed by analysis of apoptotic and necrotic fraction using FCS express software.

BDNF, P-CREB and Caspase-3 measurement

Western blot was used to determine the levels of BDNF, p-CREB and caspase-3 in SH-SY5Y cells. Briefly, cells were homogenized in lysis buffer (10 mM Tris-buffer, 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100 (v/v) with protease inhibitors (Sigma-Aldrich, St. Louis, MO). The protein concentration in each sample was determined using a BCA protein

Assay Kit (Pierce Biotechnology Inc., IL), and equal protein amount (as confirmed by β -actin) was loaded in each immunoblot. The proteins were separated using 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membranes were blocked with a blocking reagent (5% nonfat milk in TBS buffer) for ½ h and incubated at 4°C overnight with the primary antibody against BDNF, p-CREB and caspase-3 (1:1000). The membranes were washed with TBST (TBS buffer with 1% Tween-20) and blocked with the blocking reagent. Membranes were then incubated for 1 h at room temperature in Goat Anti-Rabbit-HRP conjugated secondary antibody (1:3000 in TBS, Bio-Rad Laboratories, CA). The membranes were then washed in the TBST washing solution and then visualized using enhanced chemiluminescent kits (Bio-Rad Laboratories, CA). The intensity of the protein bands on the gel was quantified using ChemiDoc XRS system (Bio-Rad Laboratories, CA).

Statistical Analysis

Statistical difference between treatment groups was determined by one-way ANOVA followed by post-hoc Tukey comparison test to determine which groups differed. Significant difference was considered a priori at $p < 0.05$. Data was analyzed using Graphpad Prism 3 (San Diego, CA), and is expressed as mean $\pm$ SEM.

RESULTS

Salsolinol (SALS) effect

As observed previously (Ramlochansingh et al. 2011), SALS caused a dose-dependent toxicity with near EC50 at 400 μ M concentration (Fig 1). Hence this concentration of SALS was used in subsequent studies.

PACAP effect

PACAP pre-treatment resulted in a dose-dependent protection against SALS-induced toxicity where the highest concentration (200 nM) completely blocked SALS effect (Fig 2). PACAP by itself had no effect on cell viability (data not shown).

PACAP antagonist effect

PACAP antagonist (PACAP6-38) dose-dependently blocked the effects of PACAP (200 nM). At 2 μ M, PACAP antagonist totally blocked the effect of PACAP (Fig 3). PACAP antagonist by itself had no effect on cell viability (data not shown).

Cell flow results

Cell flow cytometry indicates that major effects of SALS (400 μ M) were due to apoptotic mechanism. These effects were blocked by PACAP (200 nM), and PACAP antagonist (2 μ M) in turn, blocked the effects of PACAP (Fig 4).

BDNF and p-CREB results

Figures 5b and 5c depict that SALS resulted in approximately 25% reduction in BDNF and approximately 15% reduction in p-CREB levels. PACAP pretreatment (200 nM) completely blocked the effect of SALS. PACAP antagonist (2 μ M) in turn, blocked the effects of PACAP. PACAP or its antagonist did not have any effect of their own on levels of BDNF or p-CREB (data not shown).

Caspase 3 results

Figure 5d depicts that SALS resulted in approximately 40% increase in caspase-3 levels. PACAP pretreatment (200 nM) completely reversed the effect of SALS. PACAP antagonist

(2 μ M) in turn, blocked the effect of PACAP. PACAP or its antagonist did not have any effect of its own on caspase-3 levels (data not shown).

DISCUSSION

The results of the current study provide further proof of neuroprotective effects of PACAP and suggest that PACAP or its receptor agonist could be of therapeutic potential in retarding the progressive, neurodegenerative nature of PD. This contention is based on the finding that PACAP dose-dependently blocked the cytotoxicity induced by salsolinol and that PACAP effects in turn, were blocked by its antagonist, PACAP6-38. Although PACAP6-38 may also antagonize VPAC2 receptor it is likely that the protection seen in our studies are primarily mediated by PAC1 receptor as numerous studies have implicated this receptor in neuroprotective and cell survival promoting effects of PACAP (Somogyvari-Vigh and Reglodi, 2004; Vaudry et al. 2009). The results also confirm reported apoptotic effects of SALS via caspase-3 up-regulation (Bollimuntha et al. 2006; Copeland et al. 2007; Das and Tizabi, 2009; Jantas et al. 2008; Ramlochan Singh et al. 2011) and extend those findings to include involvement of neurotrophic pathway in SALS-induced neurotoxicity. Thus, SALS caused a reduction in BDNF and its signal transduction protein, p-CREB, both of which were attenuated by PACAP. Since stimulation of BDNF/CREB signaling can lead to down-regulation of caspase-3 levels and inhibition of neuronal apoptosis (Han et al. 2000; Kim and Zhao, 2005; Li and Liu, 2010), it may be suggested that the anti-apoptotic effects of PACAP may be a major mechanism of its neuroprotection against SALS-induced toxicity.

In addition to the aforementioned mechanisms, PACAP utility in PD may also be enhanced by its activation of TH. Interestingly, both BDNF and TH are regulated by the transduction signal CREB, phosphorylation of which can lead to enhanced synthesis of both proteins (Lewis-Tuffin et al. 2004; Purves et al. 2008). Hence, the dual effects of PACAP on prevention or delay of dopaminergic neuronal degeneration while simultaneously enhancing the DA synthesis capacity may offer a unique advantage in PD treatment. However, as with any peptide therapy, the questions of bioavailability and penetration of PACAP into the brain remain important concerns for the actual intervention in PD (Dogrukol-Ak et al. 2009). Nonetheless, possible development of ligands that may directly activate PAC1 receptor and/or enhance the signal transduction mechanisms involved in PACAP effectiveness may prove feasible and hence of significant therapeutic potential.

Our findings complement previous studies where PACAP has been shown to protect against various neuronal toxins in animal models of PD. Thus, PACAP can rescue dopaminergic neurons in rats subjected to unilateral injection of 6-hydroxydopamine, a commonly used model of PD (Reglodi et al. 2004, 2006). Additionally, PACAP is able to protect against methamphetamine as well as MPTP-induced toxicity in a mouse model of PD (Guillot et al. 2008; Wang et al. 2008). Altogether, the findings provide possible utility of this peptide in the treatment of PD.

It has been recently demonstrated that a number of established as well as novel drugs with utility in neurodegenerative conditions may share common mechanism of enhancing CREB-BDNF signaling (Li and Liu, 2010; Puerta et al. 2010; see also review by Bitner, 2012). In this regard, a combination of such drugs where the triggering mechanism for signal transduction may differ could prove of superior intervention than a mono-therapy (Priestley et al. 2012). Thus, combining PACAP which activates PAC1 receptor to inhibit caspase 3 (Dejda et al. 2005) with sildenafil, a phosphodiesterase 5 (PDE5) inhibitor which inhibits calpain, an intracellular Ca^{2+} -dependent Cys protease (Puerta et al. 2010; Sorimachi et al. 2012), both of which eventually lead to increase in signal transduction, may be a highly effective therapy in PD. PACAP utility in neurodegenerative disorders in general, and PD in

particular may also be aided by its suppression of inflammatory mediators that have been implicated in such diseases (Suk et al. 2004).

In summary, PACAP or its receptor agonists, by enhancing the neurotrophic pathway and inhibition of apoptosis may be of therapeutic benefit in PD.

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Figure 1.

Dose-response effect of salsolinol (SALS) on SH-SY5Y cells. MTT assay was conducted 24 h after SALS treatment. Values are mean $\pm$ SEM, n=5. * $P<0.05$ ** $P<0.01$, *** $P<0.001$ compared to control.

Figure 2.

Dose-response effect of PACAP (P) pre-treatment against salsolinol (SALS)-induced toxicity in SH-SY5Y cells. PACAP was applied 2 h prior to salsolinol treatment and the cell viability was determined 24 h after SALS. Values are mean $\pm$ SEM, $n=5$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control, # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared to SALS 400 μ M.

Figure 3.

Dose-response effect of PACAP antagonist (Antag) on protective effects of PACAP (P) against salsolinol (SALS)-induced toxicity in SH-SY5Y cells. PACAP antagonist was applied 2 h prior to PACAP, which was applied 2 h prior to SALS. Cell viability was determined 24 h after SALS. Values are mean $\pm$ SEM, $n=5$. ** $P<0.01$, *** $P<0.001$ compared to control, ## $P<0.01$, ### $P<0.001$ compared to SALS 400 uM, + $P<0.05$, +++ $P<0.001$ compared to P+SALS.

Fig 4a.

Fig. 4b

Figure 4.

Effects of PACAP (P, 200 nM), and PACAP antagonist (Antag, 2 μ M) on salsolinol (SALS)-induced apoptosis/necrosis as determined by cell flow cytometry (4a, cell flow diagram, 4b, % change in apoptosis/necrosis). Living cells (FITC⁻/PI⁻) are represented in lower left quadrant; early apoptotic cells (FITC⁺/PI⁻) are represented in lower right quadrant; late apoptotic cells (FITC⁺/PI⁺) are represented in upper right quadrant; and necrotic cells (FITC⁻/PI⁺) are represented in upper left quadrant. Antagonist was applied 2 h before PACAP, which was applied 2 h prior to SALS. Cell flow analysis was performed 24

h after treatment. Fig 4b depicts percent change in apoptosis/necrosis. Values are mean $\pm$ SEM, n=5. ***P<0.001 compared to control.

Fig. 5a

BDNF and p-CREB Measurement:

Fig. 5b

Fig. 5c

Fig. 5d

Figure 5.

Effects of PACAP (P, 200 nM), and PACAP antagonist (Antag, 2 μ M) on salsolinol (SALS)-induced changes in BDNF, p-CREB and Caspase 3 as determined by western blot (5a). Antag was applied 2 h before P, which was applied 2 h prior to SALS. Figures 5b, 5c and 5d represent BDNF, p-CREB and Caspase 3 relative optical density, respectively. Values are mean $\pm$ SEM, n=5. **P<0.01 compared to control, ##P<0.01 compared to SALS 400 μ M, ++P<0.01 compared to P+SALS.