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Anti-apoptotic actions of vasopressin in H32 neurons involve map kinase transactivation and bad phosphorylation

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ABSTRACT

Vasopressin (VP) secreted within the brain modulates neuronal function acting as a neurotransmitter. Based on the observation that VP prevented serum deprivation-induced cell death in the neuronal cell line, H32, which expresses endogenous V1 receptors, we tested the hypothesis that VP has anti-apoptotic properties. Flow cytometry experiments showed that 10 nM VP prevented serum deprivation-induced cell death and annexin V binding. Serum deprivation increased caspase-3 activity in a time and serum concentration dependent manner, and VP prevented these effects through interaction with receptors of V1 subtype. The signaling pathways mediating the anti-apoptotic effect of VP involve mitogen activated protein (MAP) kinase and extracellular signal-regulated kinases (ERK), Ca^{2+}/calmodulin dependent kinase (CaMK) and protein kinase C (PKC). Western blot analyses revealed time-dependent decreases of Bad phosphorylation and increases in cytosolic levels of cytochrome *c* following serum deprivation, effects which were prevented by 10 nM VP. These data demonstrate that activation of endogenous V1 VP receptors prevents serum deprivation-induced apoptosis, through phosphorylation-inactivation of the pro-apoptotic protein, Bad, and consequent decreases in cytosolic cytochrome *c* and caspase-3 activation. The data suggest that VP has anti-apoptotic activity in neurons and that VP may act as a neuroprotective agent in the brain.

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Introduction

Vasopressin (VP), produced mainly in magnocellular and parvocellular neurons of the hypothalamus, is an important neuropeptide involved in water conservation, blood pressure control and pituitary ACTH hormone secretion (Gilles et al., 1982; Rivier et al., 1984; Thibonnier, 1988). In addition, VP secreted within the central nervous system (CNS), modulates neuronal function acting as a neurotransmitter. The functions of VP are mediated through membrane VP receptors belonging to the G protein-coupled membrane receptor (GPCR) superfamily (Peter et al., 1995). There are two major VP receptor subtypes, V1, which is coupled to calcium phospholipid dependent pathways, and V2, which is coupled to cAMP-dependent pathways. While V2 receptors are responsible for the effects of VP on water homeostasis in the kidney, V1 receptors mediate the effects of VP in other tissues, including the brain (Winslow and Insel, 2004; Ostrowski et al., 1992). VP produced in the medial amygdala and the bed nucleus of the stria terminalis projects to the lateral septum and ventral hippocampal sites where VP acting through V1 VP receptors affects memory and behavior (Alescio-Lautier et al., 2000; Caffe et al., 1987; Szot et al., 1994). Previous studies showed that VP has trophic actions in a variety of cells and primary tissues including neurons (Zachary et al., 1987). Such trophic actions of VP have been implicated in the mechanism by which VP facilitates learning and memory in the hippocampus (Brinton et al., 1994). We have recently observed considerable amount of cell death in the neuronal cell line, H32, following overnight serum deprivation, but the effect was less evident when VP was present in the incubation medium (Chen et al., 2007). This observation suggested that VP protected H32 cells against serum deprivation-induced cell death and that VP may have protective properties.

The neuronal cell line H32 expresses functional V1 receptors (Volpi et al., 2006a), mostly V1a and a small proportion of V1b receptors. We have shown that in addition to stimulation of PKC and CaMK dependent pathways, activation of these receptors transactivate epidermal growth factor receptors (EGFR) resulting in activation of MAPK/ERK (Volpi et al., 2006a). The MAPK/ERK pathway is involved in neuronal development, memory formation, synaptic plasticity and neuronal survival (Davis, 2000; Sweatt, 2001). Stimulation of MAPK/ERK signaling pathway by growth factor receptors and GPCRs generally lead to a mitogenic and proliferative response (Seger and Krebs, 1995). In particular, activation of MAPK/ERK transduces a survival signal in a number of systems (Davis, 2000). Thus, it is possible that activation of the MAP kinase pathway by VP could mediate neuroprotective effects. Since H32 cells contain endogenous V1 VP receptors, this cell line provides a good model for studying possible functions of VP in neurons and the mechanisms by which VP exerts its effects.

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The objective of this study was to determine whether VP has anti-apoptotic effects on hypothalamic H32 neuronal cells, and to examine the signaling pathways and mechanisms involved in the effects of VP. We demonstrate that activation of endogenous V1 VP receptors by VP in H32 hypothalamic cells protects from serum deprivation-induced apoptosis, an effect which is mediated via phosphorylation-inactivation of the pro-apoptotic protein, Bad, and consequently decreases the release of cytochome *c* resulting in caspase-3 activation. The signaling pathways mediating this effect appear to involve the EGFR, MAPK/ERK, CaMK and PKC. This study suggests a novel action of VP in the brain as an anti-apoptotic and neuroprotective agent.

Materials and methods

Materials

Calphostin C, BIM, Gö 6983 and NK-93 were purchased from BIOMOL Research Lab. (Plymouth Meeting, PA); UO126, SL327, AG1478, SB203580 and H89 were from Calbiochem (San Diego, CA). Antibodies against phospho-Bad (Ser112), Bad, Phospho-p44/42 MAP Kinase (Thr202/Tyr204), p44/42 MAP Kinase, Phospho-RSK (Thr359/Ser363), RSK were purchased from Cell Signaling Technology™ (Beverly, MA); β-Tubulin antibody from Sigma (Saint Louis, MO). The non-peptide V1a VP receptor antagonist SR49059 and the V1b VP receptor antagonist SSR149415 were provided by Dr. Claudine Serradeil-Le Gal (Sanofi-Synthlab, Toulouse, France).

Cell culture and treatments

The hypothalamic cell line H32, provided by Dr Joachim Spiess, Goettingen, Germany, was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies, Inc.), 10% horse serum and 1% penicillin/streptomycin (Life Technologies, Inc.). After 24 h culture in 100 mm plates (1.5×10^6 cell per plate), at 37 °C, under 5% CO_2/95% air, the medium was changed to serum-free medium containing 0.1% BSA, with or without VP. To determine the signaling pathways and receptor subtypes mediating the effect of VP, cells were incubated in the presence and absence of inhibitors. After incubation for the time periods indicated in results and figure legends, cells were processed for caspase-3 activity or Western blot analysis.

Plasmids and transfection

Wild type Bad and Bad S112/136A mutant, cloned into pcDNA3 vector, were provided by Dr G. Kulik (Wake Forest University School of Medicine, Winston-Salem, NC). Ribosomal S6 kinase 90 kDa (RSK) wild type, RSK1 dominant negative mutant (RSK1 K112/464R) and RSK2 dominant negative mutant (RSK2 KR100) were obtained from Dr. M. E. Greenberg (Harvard Medical School, Boston, MA). Transient transfection was performed in Opti-MEM I Reduced Serum Medium (Invitrogen) using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's recommendations. Cells were used after 24 h transfection.

Flow cytometry assay

Cells were collected using trypsin-EDTA, centrifuged at 200 ×g for 5 min, washed twice with ice-cold PBS and resuspended in 0.3 ml of PBS containing 2% FBS. Forward Scatter (FSC) and Side Scatter (SSC) of cells were acquired by a FACSCalibur flow cytometer (Becton Dickinson, CA) and analyzed by FlowJo software (TreeStar, San Jose, CA). FSC indicates cell size, and SSC is related to cell granularity or internal complexity. Living cells were gated based on cell optic characteristics (FSC and SSC).

FACS detection of apoptotic cells

The degree of apoptosis following serum deprivation and VP treatment was examined by FACS, based on the ability of fluorescence-labeled annexin V to bind phosphatidyl serine, which is translocated to the outer membrane layer during early apoptosis, and the capacity of amino-actinomycin D to bind to the nuclei of late apoptotic cells. H32 cells (2.5×10^5) were incubated with 5 μl of annexin V-FITC (1 mg/ml) and 7-Amino-actinomycin D (7-AAD) (1 mg/ml) (BD Biosciences) for 15 min at room temperature, according to the manufacturer's instructions, and immediately analyzed by flow cytometry as described above. This method allows discrimination of early apoptotic cells (annexin V^+/7-AAD^-) and late apoptotic cells (annexin V^+/7-AAD^+) (Lecoeur et al., 1997). Early apoptotic cells (annexin V^+/7-AAD^-) and late apoptotic cells (annexin V^+/7-AAD^+) were counted for total apoptosis.

Caspase-3 activity measurement

Caspase-3 activity was measured using a Caspase-3/CPP32 fluorometric protease assay kit (BioSource International, Inc., Camarillo, CA) according to the manufacturer's protocol. Briefly, cells were washed with PBS, centrifuged for 5 min at 800 ×*g*, the supernatant removed and the pellet resuspended in ice-cold lysis buffer. After 20 min incubation at room temperature, samples were centrifuged at 16,000 ×*g* for 10 min at 4 °C, and protein concentrations in the supernatants determined using BCA™ protein Assay (PIERCE, Rockford, IL). Aliquots containing 100 μg of protein were incubated with substrate DEVD (Asp-Glue-Val-Asp)-AFC (7-amino-4-trifluoromethyl coumarin) for 90 min at 37 °C. Upon cleavage of the substrate by Caspase-3, free AFC, which emits a yellow-green fluorescence, was measured by using a FLUOStar OPTIMA microplate reader (BMG Labtechnologies Inc, Durham, NC), with a 405 nm excitation and 505 nm emission filter.

Cytosolic cytochrome c levels

The levels of cytosolic cytochrome *c* were measured using a Cytochrome *c* ELISA Kit (MBL, Watertown, MA). Briefly, H32 cells were cultured in 100 mm culture flasks, serum deprived for 0, 0.5, 1, 2, 4 and 6 h in the absence or in the presence of VP (10 nM). After treatment, the cells were harvested using trypsin-EDTA, spun down at 200 ×*g* for 5 min, washed twice with ice-cold PBS and resuspended in 500 μl ice-cold homogenization buffer (10 mM Tris/HCl (pH 7.5), 0.3 M sucrose, 25 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, and 10 μg/ml leupeptin). Cells were then homogenized on ice using a dounce homogenizer and centrifuged at 10,000 ×*g* for 60 min at 4 °C. Protein concentrations in the supernatants (cytosolic fractions) were determined using BCA™ protein Assay (Pierce, Rockford, IL). Cytosolic cytochrome *c* level was detected using peroxidase conjugated anti-cytochrome *c* polyclonal antibody, according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed essentially as described previously (Volpi et al., 2006b). Briefly, cells were lysed with T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with proteinase and phosphate inhibitor cocktail (Sigma). Protein concentrations were determined by BCA™ Protein Assay (Pierce) and 20 μg of protein were loaded and separated in a 4–20% SDS-PAGE (Invitrogen,). Proteins were transferred from the gel to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ), incubated with 5% nonfat dried milk in Tri-buffered saline (TBS plus 0.1% Tween-20 (TBST)) for 1 h and incubated with the antibodies at a 1:1000 dilution overnight. After washing in TBST, membranes were incubated for 2 h with peroxidase-linked anti-Rabbit IgG at a 1:10,000 dilution or anti-mouse IgG at a 1:5000. β-tubulin was used to correct for protein loading. Detection of immunoreactive band was performed by using ECL Plus TM reagents (Amersham Pharmacia Biotech) and exposure to BioMax MR film (Kodak, Rochester, NY). Densitometric quantification of the immunoblots was performed by using the public domain NIH Image program (ImageJ 1.36b developed at the US National Institutes of Health, and available on the Internet at: http://rsb.Info.nih.gov/nih-image).