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ScienceDirect
Neuropeptides 43 (2009) 53–61
Neuropeptides
www.elsevier.com/locate/npep

Calmodulin interacts with PAC1 and VPAC2 receptors and regulates PACAP-induced *FOS* expression in human neuroblastoma cells ☆

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Received 7 January 2009; accepted 5 February 2009
Available online 6 March 2009

Abstract

The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) mediates its physiological functions through activation of PAC1, VPAC1 and VPAC2 receptors, and the ubiquitous Ca^{2+}-sensor calmodulin has been implicated in PACAP-induced signaling. The immediate early response gene *FOS* is a well-known marker of neuronal activation, so we used a human neuroblastoma cell line NB-1 to explore the role of calmodulin in PACAP-induced *FOS* gene expression. We observed both short-term and prolonged altered PACAP-mediated activation of the *FOS* gene in the presence of the calmodulin-antagonist W-7. NB-1 cells were shown to express PAC1 and VPAC2 receptors, and immunoprecipitation of both receptors displayed a co-association with calmodulin in the absence of Ca^{2+}. Our findings indicate a novel mechanism of calmodulin in regulating PACAP signaling by possible interaction with the inactive state of PAC1 and VPAC2 receptors.

Keywords: Neuropeptides; Chinese hamster ovary cells; FOS; co-immunoprecipitation; W-7; W-5; G-protein-coupled receptors; Gene expression; Protein interaction; Signaling

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide expressed in the central and peripheral nervous system. PACAP mediates its functions as a neurotransmitter, neuromodulator, and neurotrophic factor through PACAP receptors expressed in both neuronal and non-neuronal cell types (Vaudry et al., 2000). PACAP activates three receptors: the PAC1, VPAC1 and VPAC2 receptors, which belong to a subfamily of the class B G-protein-coupled receptors (Harmar et al., 1998). Activation of PACAP receptors is coupled to the synthesis of cAMP and also associated with an increase in intracellular Ca^{2+} levels (Harmar et al., 1998; Vaudry et al., 2000; Ravni et al., 2006a), and changes in transcriptional activity, e.g. enhanced expression of the immediate early response gene *Fos,* are often observed (Vaudry et al., 1998; Grumolato et al., 2003; Hayez et al., 2004; Ishido and Masuo, 2004; Ravni et al., 2006b; Braas et al., 2007). The ubiquitous Ca^{2+}-sensor calmodulin (CaM) is a PACAP-activated protein (Murthy and Makhlouf, 1994; Akiyama et al., 2001; Lebon et al., 2006), and PACAP-induced *Fos* expression has been shown to be sensitive towards inhibition of CaM (Schäfer et al., 1996).

☆ This work was supported by The Danish Biotechnology Center for Cellular Communication and the Copenhagen Hospital Corporation Research Council.
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doi:10.1016/j.npep.2009.02.001

The brain's biological clock is located in the hypothalamic suprachiasmatic nucleus (SCN) and contains vasoactive intestinal peptide (VIP)-expressing neurons innervated by PACAP-expressing retinal ganglion cells by which environmental light signals to the brain's clock (Sanggaard et al., 2003). Light stimuli have been found to induce *Fos* expression in the SCN neurons (Kornhauser et al., 1990), and mice carrying a null mutant in the *Fos* gene exhibit diminished behavioural responses to light (Honrado et al., 1996). Others and we have demonstrated that mice deficient of either PACAP or the PAC1 receptor exhibit attenuated light-induced *Fos* expression in the SCN (Hannibal et al., 2001; Kawaguchi et al., 2003). Furthermore, we have identified a change in CaM expression as a key feature of light-induced PAC1 receptor activation in the SCN (Fahrenkrug et al., 2005).

We have previously used human NB-1 neuroblastoma cells to study PACAP-induced *VIP* expression (Georg and Fahrenkrug, 2000), so this cell model was chosen to elucidate the role of CaM in the PACAP-induced *FOS* expression in neuronal cells. We here report that inhibiting CaM activity prior to addition of PACAP to NB-1 cells resulted in both an initial and long-term altered *FOS* gene regulation. Immunoprecipitating PAC1 and VPAC2 receptors revealed that Ca^{2+} modulates a co-association with CaM thus indicating a direct regulation of CaM on PACAP receptor activities.

2. Materials and methods

2.1. Cell culture

All reagents and media used for cell culture were obtained from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Human NB-1 neuroblastoma cells were grown in RPMI1640 media supplemented with 10% foetal calf serum and 50 μg/mL gentamycin. Stably transfected Chinese hamster ovary (CHO) cells were cultured as NB-1 cells but with inclusion of 0.8 mg/mL G418. Human T98G glioblastoma cells were grown in MEM-EAGLE media supplemented with 10% FCS, 1 mM sodium pyruvate, non-essential amino acids and with 50 μg/mL gentamycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air and tested negative for mycoplasma infection by the MycoSensor™ QPCR Assay Kit (Stratagene, La Jolla, CA, USA).

2.2. Reagents

PACAP38 was from Bachem (Bubendorf, Switzerland) and was dissolved in sterile water to 1 mg/mL. The calmodulin antagonists W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl) and W-5 (N-(6-Aminohexyl)-1-naphthalenesulfonamide hydrochloride) were dissolved in dimethylsulfoxide to 50 mM and were obtained from Calbiochem (Merck Chemicals Ltd., Nottingham, UK) and Tocris Biosciences (Bristol, UK), respectively.

2.3. Generation of PAC1 expressing CHO cells

A full-length rat PAC1 cDNA clone was obtained from Dr. S.A. Wank Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD, USA. A nucleotide substitution leading to a Cys to Arg mutation in amino acid 24 of this cDNA was corrected and the full-length cDNA was inserted in sense orientation in pcDNA3 (nt: 30-1546 of L16680). The resulting plasmid (BG30-1) was used to transfect CHO 591 cells. A stably transfected cell clone (81-13) was obtained by selection with 0.8 mg/ml G418. Stable empty vector-expressing CHO cells were obtained by transfection with empty pcDNA3 and by selection with G418.

2.4. PACAP-induced FOS expression and extraction of RNA

NB-1 cells were grown to near-confluence, serum starved for 2–3 days and the medium was renewed the day before experiments. On the day of experiments, cells were incubated with either 10 nM PACAP or vehicle for periods of 30 min to 6 h at 37 °C. Calmodulin antagonists W-7 (25 μM) or W-5 (25 μM) were added to cells 30 min prior to incubation with PACAP. Cells were harvested by removal of incubation media, detachment with trypsin-EDTA and by suspension in ice-cold Ca^{2+}-containing PBS buffer. Cells were collected by centrifugation at 1200 *g* for 5 min and were lysed by adding LysisBinding Solution (supplied with the RNAqueous Kit). Extracts were kept at −20 °C until isolation of total RNA with the RNAqueous Kit according to the manufacturers' instructions (Ambion, Applied Biosystems, Foster City, CA, USA).

2.5. Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

cDNA was synthesized from an input of 250 ng total RNA in a 50 μL assay volume using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A TaqMan® Gene Expression assay (ID no.: Hs00170630_m1, Applied Biosystems) was applied to analyze *FOS* expression by real-time PCR. Expression levels of the *FOS* gene (RefSeq: NM_005252.2) were normalised to the expression of the ß2-microglobulin (*B2M*) gene (RefSeq: NM_004048). *B2M* primers and probe were synthesized by TAG Copenhagen A/S