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PACAP protects against TNFα-induced cell death in olfactory epithelium and olfactory placodal cell lines

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ABSTRACT

In mouse olfactory epithelium (OE), pituitary adenylate cyclase-activating peptide (PACAP) protects against axotomy-induced apoptosis. We used mouse OE to determine whether PACAP protects neurons *during* exposure to the inflammatory cytokine TNFα. Live slices of neonatal mouse OE were treated with 40 ng/ml TNFα ± 40 nM PACAP for 6 h and dying cells were live-labeled with 0.5% propidium iodide. TNFα significantly increased the percentage of dying cells while co-incubation with PACAP prevented cell death. PACAP also prevented TNFα-mediated cell death in the olfactory placodal (OP) cell lines, OP6 and OP27. Although OP cell lines express all three PACAP receptors (PAC1, VPAC1,VPAC2), PACAP's protection of these cells from TNFα was mimicked by the specific PAC1 receptor agonist maxadilan and abolished by the PAC1 antagonist PACAP6-38. Treatment of OP cell lines with blockers or activators of the PLC and AC/MAPKK pathways revealed that PACAP-mediated protection from TNFα involved both pathways. PACAP may therefore function through PAC1 receptors to protect neurons from cell death during inflammatory cytokine release *in vivo* as would occur upon viral infection or allergic rhinitis-associated injury.

Introduction

During CNS injury, microglia become activated and release cytokines such as TNFα and IL1β (Clausen et al., 2008). Cytokines then cause a series of inflammatory responses in the tissue that are often detrimental, and can result in exacerbation of the original injury. Several studies have documented the ability of pituitary adenylate cyclase-activating polypeptide (PACAP) to reduce expression and release of TNFα and other cytokines following lipopolysaccharide stimulation of microglial cell lines (Kim et al., 2000) or purified macrophage cultures (Delgado et al., 2003b). PACAP belongs to the vasoactive intestinal peptide (VIP)-secretin–glucagon superfamily of peptides (Kieffer and Habener, 1999) and acts through G-protein coupled PAC1, VPAC1 and VPAC2 receptors to increase cAMP and reduce TNFα expression in microglia (Kim et al., 2000). We used the murine olfactory epithelium (OE) as a model to analyze the effect of PACAP administration during cytokine release. Unlike in the CNS, the neurons of the OE are directly exposed to the external environment and are continually damaged by airborne toxins and xenobiotics. In response to the continual damage, the OE is able to regenerate and does so throughout adulthood (Graziadei and Graziadei, 1979). Both TNFα and TNFα receptors (TNFR1 and TNFR2) are expressed in the OE (Farbman et al., 1999) and TNFα can induce apoptosis in explants

Abbreviations: AA, amino acid; AC, adenylate cyclase; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; dNTP, deoxynucleotide triphosphate; dT, deoxythymidine; EC1, first extracellular domain of PAC1R sequence; FBS, fetal bovine serum; FSK, forskolin; GPCR, G-protein-coupled receptor; IC3, third intracellular domain of PAC1R sequence; INP, immediate neuronal precursor; IRN, immature receptor neuron; K, potassium; MAPKK, mitochondrial activated protein kinase kinase; N, normal variant of EC1 domain of PAC1R; *n*, number of animals used, or cell-based experiments done; NST, neuron specific tubulin; OE, olfactory epithelium; OMP, olfactory marker protein; OP, olfactory placodal; OSN, olfactory sensory neuron; P1, post-natal day 1; PACAP, pituitary adenylate cyclase-activating peptide; PAC1R, PACAP-specific receptor; PCR, polymerase chain reaction; PI, propidium iodide; PLC, phospholipase C; PMA, phorbol 12-myristate13-acetate; PKC, protein kinase C; R, regular variant of IC3 domain of PAC1R; ROI, regions of interest; RT-PCR, reverse transcriptase polymerase chain reaction; S, short variant EC1 domain of PAC1R; TNFα, tumor necrosis factor α; TNFR1, TNFR2, TNFα receptors; VIP, vasointestinal peptide; VPAC1,VPAC2, receptors for VIP; VS, very short variant of EC1 domain of PAC1R.

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of the embryonic OE (Suzuki and Farbman, 2000). TNFR2 activates pro-survival signaling pathways, while TNFR1 contains a cytoplasmic death domain which allows recruitment of several death domain and other proteins which can then activate caspases and induce apoptosis (McCoy and Tansey, 2008).

PACAP is expressed in the OE (Hegg et al., 2003a; Hansel et al., 2001) and protects against axotomy-induced apoptosis of neonatal rat olfactory sensory neurons (OSNs) in primary cultures (Hansel et al., 2001). In addition, PACAP protects against axotomy-induced apoptosis of adult mouse OSNs via a phospholipase C (PLC) and calcium-dependent reduction in A-type potassium channels (Han and Lucero, 2005). While axotomy is a well-characterized model for study of olfactory neurodegeneration, it differs considerably from apoptosis induced by direct damage to the OE. Studies using cytokine-induced apoptosis more closely mimic an inflammatory response to direct damage to the OE and are likely to yield more physiologically-relevant information.

In this study, we therefore used a live slice model of the OE (Hegg et al., 2003a) to demonstrate that PACAP can protect against cytokine-induced cell death. As this model does not distinguish between effects of PACAP on neurons or immune cells, the majority of our study involved using two olfactory placodal (OP) cell lines to examine the effects of PACAP on neuronal cells in the absence of immune cells. OP6 and OP27 are olfactory neuronal precursor cell lines clonally derived from the olfactory placode of embryonic mice (Illing et al., 2002). These cell lines are well-characterized and shown to be a good model to study both immature and mature olfactory neuron function. OP27 cells contain the expression profile of immediate neuronal precursors (INPs) while OP6 cells express markers of immature receptor neurons (IRNs) (Illing et al., 2002). When placed in differentiating conditions, both OP cell lines develop a bipolar neuronal morphology, express odorant receptors, upregulate G_{olf} and OMP, and down regulate NST in a manner similar to olfactory neurons *in vivo* (Illing et al., 2002; Regad et al., 2007; Pathak et al., 2009).

We examined the role of PACAP receptors and identified PAC1R subtypes expressed by the OP cell lines. The PACAP receptors PAC1, VPAC1 and VPAC2 have different agonist and antagonist profiles. Maxadilan is a potent vasodilatory peptide isolated from sandfly saliva (Lerner et al., 1991) which functions by activating the PAC1 receptor (PAC1R) (Lerner et al., 2007). Unlike PACAP itself, maxadilan binds specifically to PAC1R, and does not activate either of the VPAC receptors (Moro and Lerner, 1997). PACAP6-38 on the other hand is a truncated version of PACAP which functions as a PACAP receptor antagonist at both PAC1 and VPAC2 receptors, but does not block VPAC1 (Moro et al., 1999). Maxadilan and PACAP6-38 were therefore used to probe receptor specificity in this study. In addition, the PAC1 receptor occurs as several subtypes resulting from alternative splicing of mRNA encoding the first extracellular domain (EC1) (Pantaloni et al., 1996), and the third intracellular cytoplasmic loop (IC3) (Spengler et al., 1993). The most common EC1 variants are called normal (N, with no deletion) and short (S, with a 21 AA deletion), and these primarily regulate agonist binding (Pantaloni et al., 1996). IC3 splice variants occur as the regular form (R) without insertions, or with the inserted cassettes of Hip, Hop1, Hop2, or HipHop1/2 (Spengler et al., 1993). The IC3 splice variants govern coupling to either or both of the adenyl cyclase (AC) or phospholipase C (PLC) transduction pathways (Spengler et al., 1993; Ushiyama et al., 2007). We determined the PAC1R splice variants expressed by the OP cells to aid in identifying the second messenger systems activated by PACAP in this model.

In the present work, we use live-labeling with propidium iodide (PI) to show that TNFα induces cell death in neuronal precursors, and that TNFα-mediated cell death is reduced by PACAP via PAC1 receptor binding and activation of either the PLC or the AC/MAPKK second messenger systems. We also show that TNFα induced caspase activity is blocked by PACAP treatment in OP cells. These studies suggest that PACAP can be neuroprotective even if it does not precede TNFα release.

Results

PACAP protects against TNFα-induced cell death in live slices of the OE

We treated live slices of OE from P1 mice with 40 ng/ml TNFα, TNFα+40 nM PACAP or vehicle for 6 h, and then live-labeled for 20 min with 0.05% propidium iodide (PI), a dye that only enters dead or dying cells. Slices treated with TNFα (Fig. 1B, E) exhibited a significantly higher percentage of PI labeling (38 ± 6%, Fig. 1G) than vehicle controls (10 ± 3%, $p = 0.04$, Fig. 1A, D). Co-incubating slices in both TNFα and PACAP (Fig. 1C, F) significantly reduced the percentage of PI labeling compared to TNFα alone ($p = 0.003$) to a value that was not different from control (7 ± 1%, $p = 0.5$, Fig. 1G). While TNFα treatment affected cells in all layers of the OE (Fig. 1E), the majority of PI labeling was in the basal to lower middle layers of the OE (Fig. 1D–F), which contain mainly basal progenitor cells, immediate neural precursors and immature neurons. These immature OE cells may thus be more vulnerable to the cumulative damaging effects of slicing and cytokine treatment than the mature neurons and sustentacular cells in the apical OE. PACAP reduced TNFα-induced PI labeling throughout the OE, demonstrating that PACAP can protect olfactory epithelial cells against cell death induced by TNFα, a cytokine endogenous to the OE (Farbman et al., 1999).

OE slice experiments however were relatively qualitative and did not differentiate between necrotic cell death and apoptosis nor distinguish between the different cell types of the olfactory mucosa. To focus our studies on neuronal cells of the OE, we assayed olfactory placodal cell lines of neuronal lineage.

OP6 and OP27 cells express TNFα, PACAP and their receptors

OP6 and OP27 cells are clonal olfactory neuronal precursor cell lines derived from E10 mouse olfactory placode (Illing et al., 2002). Prior to using the OP cell lines in the apoptosis assay, we first determined whether they express receptors for the cytokine TNFα as well as PACAP and its receptors. RT-PCR showed that both OP6 and OP27 cells express both TNFR1 and TNFR2 (Fig. 2). RT-PCR also showed that OP6 and OP27 cells express PACAP and its receptors PAC1, VPAC1 and VPAC2 (Fig. 2). These data therefore indicate that the OP cell lines could function as an appropriate model system to examine a potential role for PACAP in protection against TNFα-induced neuronal cell death.

PACAP protects olfactory placodal cell lines against TNFα-induced cell death

To test the effects of PACAP on survival of cells of neuronal lineage, we cultured OP6 and OP27 cells for 5 h in the presence or absence of 40 ng/ml TNFα ± 40 nM PACAP. The percentage of PI-labeled to total cells was calculated for cultures treated with TNFα, TNFα + PACAP or vehicle. Under control conditions, PI-labeled cells comprised 4 ± 0.7% of total in OP6 cells (Fig. 3A, G) and 2.3 ± 0.4% of total in OP27 cells (Fig. 3B, H). Treatment with TNFα significantly increased the level of cell death to 5.7 ± 0.8% in OP6 cells (Fig. 3C, G, $n = 15$, $p = 0.003$) and 3.5 ± 0.6% in OP27 cells (Fig. 3D, H, $n = 15$, $p = 0.004$). TNFα therefore increased cell death 42% above control in OP6 cells and 52% above control in OP27 cells.

Co-incubating cells with TNFα and PACAP reduced the percentage of PI-labeled cells down to or below that seen in control: to 2.9 ± 0.5% in OP6 cells (Fig. 3E, G) and 2.3 ± 0.4% in OP27 cells (Fig. 3F, H). TNFα + PACAP treatment significantly lowered cell death from that seen with TNFα alone ($n = 15$, $p < 0.001$ (OP6); $p = 0.002$ (OP27)).