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STIMULATORY EFFECT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE 6-38, M65 AND VASOACTIVE INTESTINAL POLYPEPTIDE 6-28 ON TRIGEMINAL SENSORY NEURONS

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Abstract—Pituitary adenylate cyclase-activating polypeptide (PACAP) acts on G protein-coupled receptors: the specific PAC1 and VPAC1/VPAC2 receptors. PACAP6-38 was described as a potent PAC1/VPAC2 antagonist in several models, but recent studies reported its agonistic behaviors proposing novel receptorial mechanisms. Since PACAP in migraine is an important research tool, we investigated the effect of PACAP and its peptide fragments on trigeminal primary sensory neurons. Effect of the peptides was studied with ratiometric Ca-imaging technique using the fluorescent indicator fura-2 AM on primary cultures of rat and mouse trigeminal ganglia (TRGs) neurons. Specificity testing was performed on PAC1, VPAC1 and VPAC2 receptor-expressing cell lines with both fluorescent and radioactive Ca-uptake methods. Slowly increasing intracellular free calcium concentration $[Ca^{2+}]_i$ was detected after PACAP1-38, PACAP1-27, vasoactive intestinal polypeptide (VIP) and the selective PAC1 receptor agonist maxadilan administration on TRG neurons, but interestingly, PACAP6-38, VIP6-28 and the PAC1 receptor antagonist M65 also caused similar activation. The VPAC2 receptor agonist BAY 55-9837 induced similar activation, while the VPAC1 receptor agonist $Ala^{11,22,28}$VIP had no significant effect on $[Ca^{2+}]_i$. It was proven that the Ca^{2+}-influx originated from intracellular stores using radioactive calcium-45 uptake experiment and Ca-free solution. On the specific receptor-expressing cell lines the antagonists inhibited the stimulating actions of the respective agonists, but had no effects by themselves. PACAP6-38, M65 and VIP6-28, which were described as antagonists in numerous studies in several model systems, act as agonists on TRG primary sensory neurons. Currently unknown receptors or splice variants linked to distinct signal transduction pathways might explain these differences.
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Key words: G protein-coupled receptor, PACAP6-38, PAC1, VPAC1, VPAC2, trigeminal ganglia.

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the vasoactive intestinal polypeptide (VIP) secretin family. The peptide was isolated from ovine hypothalamus extracts based on its ability to stimulate cyclic adenosine 3′,5′-monophosphate (cAMP) in rat anterior pituitary cells (Miyata et al., 1989; Arimura, 1998). PACAP has the same amino acid sequence in humans and other mammals (Sherwood et al., 2000). Two major forms are known, a 38-amino acid form (PACAP1-38) and a 27-amino acid form (PACAP1-27) which is a C-terminally truncated variant (Miyata et al., 1989; Vaudry et al., 2009). The effects of PACAP in the central and peripheral nervous systems have been widely described (Arimura, 1998; Sherwood et al., 2000; Pisegna and Oh, 2007). PACAP receptors are class B G protein-coupled receptors (GPCRs), the specific PAC1 receptor binds VIP with much less affinity. In contrast, VPAC1 and VPAC2 receptors show similar affinity for VIP and PACAP (Lutz et al., 1999; Laburthe and Couvineau, 2002; Laburthe et al., 2007; Muller et al., 2007). Several isoforms of the PAC1 receptor exist due to alternative splicing within two regions of the PAC1

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Abbreviations: AC, adenylate cyclase; $[Ca^{2+}]_i$, intracellular free calcium concentration; cAMP, cyclic adenosine 3′,5′-monophosphate; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary cell line; CPM, count per minute; DAG, diacylglycerol; D-MEM, Dulbecco's-Modified Eagle Medium; DMSO, dimethyl sulfoxide; ECS, extracellular solution; GDP, guanosine 5'-diphosphate; GPCRs, G protein-coupled receptors; GTP, guanosine 5'-triphosphate; GTPγS, guanosine-5'-O-[γ-thio] triphosphate; IP3, inositol 1,4,5-trisphosphate; NGF, nerve growth factor; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered solution; PLC, phospholipase C; PLD, phospholipase D; S.E.M., standard error of means; TNC, trigeminal nucleus caudalis; TRG, trigeminal ganglion; Tris-EGTA, tris-ethylene glycol-tetraacetate; Tris-HCl, tris-(hydroxymethyl)-aminomethane hydrochloride; TRPV1, Transient Receptor Potential Vanilloid 1; VIP, vasoactive intestinal polypeptide.

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gene: the N-terminus and the third intracellular loop. The deletions at the N-terminal extracellular domain affect ligand binding and second messenger stimulation after ligand binding (Pantaloni et al., 1996; Dautzenberg et al., 1999; Holighaus et al., 2011). Binding of PACAP and its analogs to PACAP receptors stimulates adenylate cyclase (AC) and elevates the intracellular cAMP level through Gs. In addition, other intracellular messengers and pathways are also involved, the binding can regulate inositol 1,4,5-trisphosphate (IP3) production by coupling to phospholipase C (PLC) through Gq (Holighaus et al., 2011), intracellular Ca^{2+} concentration, phospholipase D (PLD) or diacylglycerol (DAG) in various cell types (Vaudry et al., 2009). Class B GPCRs may interact with a few accessory proteins which play a role in cell signaling (Couvineau and Laburthe, 2012a,b).

Several agonists and antagonists of the PAC1 and VPAC receptors have been described. PACAP6-38 is a peptide fragment in which the five first residues are deleted. Based on numerous studies, PACAP6-38 is considered to be a PAC1/VPAC2 antagonist in several cell lines, including human neuroblastoma NB-OK-1 cells (Robberecht et al., 1992; Laburthe et al., 2007). Its antagonistic effects have been described also on survival of cortical neurons (Shintani et al., 2005) and differentiation of sensory neurons (Nielsen et al., 2004). Surprisingly, we have found that in certain cells/ tissues, PACAP6-38 behaves as an agonist: instead of inhibiting PACAP1-38-evoked responses, it in fact induces similar effects. Our group described that similar to PACAP1-38, PACAP6-38 also decreased the electrical-field stimulation-induced release of the sensory neuropeptide calcitonin gene-related peptide (CGRP) from sensory nerve endings of isolated rat trachea (Nemeth et al., 2006; Reglodi et al., 2008). Furthermore, we reported the agonistic behavior of PACAP6-38 on a human cytotrophoblast cell line (JAR cells) (Boronkai et al., 2007).

Maxadilan is a potent 61 amino acid-containing vasodilator peptide that was isolated from the salivary glands of the sand fly (Lerner et al., 1991). Maxadilan specifically activates the PAC1 receptor, while its fragment, the deleted peptide (#25-41) of maxadilan (M65) has been considered to be a specific PAC1 receptor antagonist. M65 selectively displaced the binding of [^{125}I]PACAP27 in PVRI-Chinese hamster ovary cell line (CHO) (Uchida et al., 1998). The pharmacodynamic characteristics of VIP6-28 is partial agonism on the VPAC1/ VPAC2 receptors; therefore, it has antagonistic actions in several models (Schuelert and McDougall, 2006). Ala^{11,22,28} VIP is known as a selective VPAC1 agonist with three substitutions at Thr^{11}, Tyr^{22} and Asn^{28}. It elicits the maximal response on the VPAC1 receptor using 10^{-8} M concentration but it is inactive on the VPAC2 receptor (Nicole et al., 2000). BAY 55-9837 consisting of 31 amino acid residues is a VPAC2-selective agonist that displays high selectivity for VPAC2 in receptor binding and functional assays. BAY 55-9837 was described as a potential therapeutic agent for the treatment of type 2 diabetes (Tsutsumi et al., 2002).

The role of PACAP in migraine and other trigeminal sensory functions has been extensively investigated recently. The expression of PACAP in several "migraine generator" brain regions of humans and animals related to the trigeminal system (Tajti et al., 2001; Christiansen et al., 2003) as well as the localization of PACAP and PAC1 receptors in the smooth muscle of human and rat intracranial vasculature (Boni et al., 2009; Chan et al., 2011) are well documented. PACAP and PACAP receptors have been described on primary sensory neurons of the trigeminal ganglia (TRGs) (Tajti et al., 1999; Zhou et al., 2002; Kuris et al., 2007; Nakajima et al., 2013) and peripheral terminals of capsaicin-sensitive sensory nerves and vascular smooth muscle cells (Mulder et al., 1994; Fahrenkrug and Hannibal, 1998; Zhou et al., 2002; Vaudry et al., 2009). Schytz and co-workers (2009) reported that PACAP infusion triggered migraine-like headache without aura in migraineurs. According to this human observation, PACAP has been proposed as a mediator of trigeminovascular activation which plays a significant role in migraine (Schytz et al., 2010). In another human study, plasma PACAP levels were elevated during the ictal phase of migraine (Tuka et al., 2013). Our research group provided the first complex experimental data with behavioral, functional and morphological techniques using gene-deleted mice that PACAP plays a pivotal role in migraine-related pathophysiological processes at the levels of the meningeal vessels, as well as the TRGs and nucleus caudalis (Markovics et al., 2012). Clarification of the precise mechanism of trigeminovascular activation and identification of the targets might open promising future perspectives in novel anti-migraine therapy. This is the reason why we chose the TRG neurons as experimental tools to analyze the effects of PACAP and PACAP fragments on primary sensory neurons. The culture of TRG neurons might be a translational model system in anti-migraine drug development. In the present study we aimed at analyzing the actions of this peptide and its analogs considered as agonists and antagonists on sensory neural responses *in vitro*. In order to investigate the selectivity of these peptides, experiments were also performed on CHO cells stably expressing the human PAC1, VPAC1 and VPAC2 receptors (Nicole et al., 2000; Bourgault et al., 2008).

EXPERIMENTAL PROCEDURES

Primary cultures of TRG neurons and CHO cells stably expressing the human PAC1, VPAC1 and VPAC2 receptors

Cultures were made from 1–4-day-old Wistar rat pups and CD1 mice pups as described elsewhere (Szőke et al., 2000). TRGs were dissected in ice-cold phosphate-buffered solution (PBS), incubated for 35 min at 37 °C in PBS containing collagenase (Type XI, 1 mg/ml) and then in PBS with deoxyribonuclease I (1000 units/ml) for 8 min. The ganglia were then rinsed with Ca^{2+} and Mg^{2+}-free PBS and dissociated by trituration. TRG cells were plated on poly-D-lysin-coated glass coverslips and grown in a nutrient-supplemented medium. The cell culture medium contained 180 ml Dulbecco's-Modified Eagle Medium (D-MEM), 20 ml horse serum,

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