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IMMUNOHISTOCHEMICAL LOCALIZATION OF THE CALCITONIN GENE-RELATED PEPTIDE BINDING SITE IN THE PRIMATE TRIGEMINOVASCULAR SYSTEM USING FUNCTIONAL ANTAGONIST ANTIBODIES☆

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Abstract—Calcitonin gene-related peptide (CGRP) is a potent vasodilator and a neuromodulator implicated in the pathophysiology of migraine. It binds to the extracellular domains of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein (RAMP) 1 that together form the CGRP receptor. Antagonist antibodies against CGRP and its binding site at the receptor are clinically effective in preventing migraine attacks. The blood–brain barrier penetration of these antagonist antibodies is limited, suggesting that a potential peripheral site of action is sufficient to prevent migraine attacks. To further understand the sites of CGRP-mediated signaling in migraine, we used immunohistochemical staining with recently developed antagonist antibodies specifically recognizing a fusion protein of the extracellular domains of RAMP1 and CLR that comprise the CGRP binding pocket at the CGRP receptor in monkey and man. We confirmed binding of the antagonist antibodies to human vascular smooth muscle cells (VSMCs) of dural meningeal arteries and neurons in the trigeminal ganglion, both of which are likely sites of action for therapeutic antibodies in migraine patients. We further used one of these antibodies for detailed mapping on cynomolgus monkey tissue and found antagonist antibody binding sites at multiple levels in the trigeminovascular system: in the dura mater VSMCs, in neurons and satellite glial cells in the trigeminal ganglion, and in neurons in the spinal trigeminal nucleus caudalis. These data reinforce and clarify our understanding of CGRP receptor localization in a pattern consistent with a role for CGRP receptors in trigeminal sensitization and migraine pathology. © 2016 Published by Elsevier Ltd on behalf of IBRO.

Key words: migraine, cynomolgus monkey, human, antibody, immunohistochemistry, trigeminal system.

INTRODUCTION

Migraine is a painful, disabling neurological disease affecting up to 16% of the adult population in Western countries (Lipton et al., 2007). While the primary cause of migraine is still unknown, scientific research over the past three decades has established the neuropeptide calcitonin gene-related peptide (CGRP) as a key player in the pathophysiology of migraine (Edvinsson, 2015b; Karsan and Goadsby, 2015). Clinical studies have shown that CGRP levels are elevated in the plasma and saliva during spontaneous migraine attacks (Goadsby et al., 1990; Goadsby and Edvinsson, 1993; Gallai et al., 1995; Juhasz et al., 2003) and restored to baseline after administration of triptan drugs coincident with pain relief (Goadsby and Edvinsson, 1993; Juhasz et al., 2003; Cady et al., 2009). Moreover, intravenous injection of CGRP induced a delayed migraine-like headache in migraineurs (Lassen et al., 2002). These findings led to the development and clinical proof of efficacy of small-molecule antagonists of the CGRP receptor (gepants) (Olesen et al., 2004; Ho et al., 2008, 2010; Connor et al., 2009; Diener et al., 2011; Hewitt et al., 2011) and, more recently, monoclonal antibodies against CGRP and its receptor (Dodick et al., 2014a,b; Bigal et al., 2015a,b,c; Sun-Edelstein and Rapoport, 2016; Sun et al., 2016). Interestingly, even though migraine attacks are considered to originate in the brain (Akerman et al., 2011; Maniyar et al., 2014), monoclonal antibodies are unlikely to penetrate the blood–brain barrier (BBB) in clinically relevant concentrations (Dolgin,

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Abbreviations: BBB, blood–brain barrier; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; CLR, calcitonin receptor-like receptor; CNS, central nervous system; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; HEK, human embryonic kidney; HRP, horseradish peroxidase; IgG, immunoglobulin G; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; MCF-7, Michigan Cancer Foundation-7; MCT, mast cell tryptase; MEM, minimum essential medium; NEAA, non-essential amino acids; NF200, neurofilament 200; NGS, normal goat serum; PBS, phosphate-buffered saline; PSD-95, post-synaptic density protein 95; RAMP, receptor activity-modifying protein; SMA, smooth muscle actin; VSMC, vascular smooth muscle cell; VWF, von Willebrand factor.

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2013; Eftekhari et al., 2015; Lundblad et al., 2015). Further, significant brain exposure of the small-molecule antagonist telcagepant was not required for its anti-migraine efficacy (Hostetler et al., 2013). Therefore, the clinical efficacy of the novel CGRP and CGRP receptor antagonists has re-ignited the debate about the site of action of CGRP and its receptor in migraine (Edvinsson, 2015a).

CGRP and its receptor are widely expressed in the central nervous system (CNS) and throughout the trigeminovascular system (Eftekhari and Edvinsson, 2010; Raddant and Russo, 2011; Eftekhari et al., 2015, 2016). CGRP receptors are heteromeric complexes consisting of the G-protein-coupled receptor calcitonin receptor-like receptor (CLR) and the transmembrane protein receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998; Archbold et al., 2011). A specific CGRP antagonist ligand, ^{3}H-MK3207, has been used for autoradiographic localization of the CGRP receptor complex, but does not provide cellular resolution (Eftekhari et al., 2013a, 2016). Co-localization experiments with antibodies against the individual receptor components CLR and RAMP1 have been performed to map the CGRP receptor at a cellular level in rats, rhesus monkeys, and human tissues (Oliver et al., 2002; Marvizon et al., 2007; Lennerz et al., 2008; Eftekhari et al., 2010; Eftekhari and Edvinsson, 2011; Eftekhari et al., 2013a,b, 2015, 2016). Species differences and other discrepancies exist between these studies. Therefore, further investigation of the binding sites of CGRP and CGRP receptor antagonists is necessary.

In this study, we explored the potential of using functional CGRP receptor antagonist antibodies to map the localization of CGRP binding sites in tissues by immunohistochemistry. We used two monoclonal human immunoglobulin G1 (IgG1) antibodies, AA32 and AA58, raised against a fusion protein of the extracellular domains of CLR and RAMP1, which comprise the CGRP binding pocket of the CGRP receptor (ter Haar et al., 2010). Both antibodies bind to CGRP receptors expressed by cells, inhibit CGRP-induced cyclic adenosine monophosphate (cAMP) production *in vitro*, detect a single band in Western blot, and specifically bind to cells overexpressing CGRP receptors after fixation for immunohistochemistry. We confirm antagonist antibody binding to human dura and trigeminal ganglion. To further map the binding sites of antagonist antibodies in the trigeminovascular system, we used one of these antibodies, AA32, on formalin-fixed, paraffin-embedded cynomolgus monkey tissues to improve resolution and background staining due to non-specific binding to human tissue epitopes.

EXPERIMENTAL PROCEDURES

Cells, tissues, and antibodies

Cell lines. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) unless stated otherwise. Human neuroblastoma (SK-N-MC) cells that endogenously express CGRP receptors were grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1% glutamine/penicillin/streptomycin, MEM non-essential amino acids (NEAA), and MEM sodium pyruvate. Michigan Cancer Foundation-7 (MCF-7) epithelial cells that endogenously express amylin receptors were cultured in Eagle's MEM plus the same additives as for the SK-N-MC cells. Stable human CGRP receptor and human adrenomedullin-1 receptor expressing cells were generated by co-transfection of CLR and RAMP1 into human embryonic kidney (HEK) 293 cells and CLR and RAMP2 cDNAs into HEK/EBNA cells (Amgen Inc., Thousand Oaks, CA, USA), respectively. HEK cells were cultured in Dulbecco's modified Eagle's medium, 5% FBS, 1% glutamine/penicillin/streptomycin, MEM NEAA, MEM sodium pyruvate, 200 μg/mL Zeocin™, 250 μg/mL Geneticin™ (G418), and 200 μg/mL hygromycin. Chinese hamster ovary (CHO) cell lines (PerkinElmer, Waltham, MA, USA) stably expressing human adrenomedullin-2 receptors were grown in Ham's F12, 10% FBS, 1% glutamine/penicillin/streptomycin, 10 μg/mL Blasticidin, and 400 μg/mL G418. For immunohistochemistry experiments, 3×10^{6} cells were injected into collaplugs™ (Zimmer Dental Inc., Warsaw, IN, USA) and fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), pH = 7.4, for 1 h, cryoprotected with 30% sucrose overnight at 4 °C, and sectioned at 12 μm using a cryostat.

Frozen human tissue. Human specimens were obtained from the University Hospital in Lund. Human meningeal vessels were obtained in conjunction with neurosurgery operations for tumor removal or epilepsy (n = 4: two male and two female patients ages between 48 and 72 years; diameter 0.6–1 mm), and human subcutaneous vessels were obtained in conjunction with surgical removal of excess of skin (n = 4). The specimens were put in buffer solution and immediately transported to the laboratory for fixation in 4% formalin. Human trigeminal ganglia were removed at autopsy from three patients (who died of a non-cerebral cause) within 24 h of death. The vessels with adjacent tissue and the trigeminal ganglia were fixed in 4% paraformaldehyde in PBS (Sigma–Aldrich, St. Louis, MO, USA) for 4 h, cryoprotected by sucrose series, and sectioned at 10 μm using a cryostat as described previously (Edvinsson et al., 2010; Eftekhari et al., 2010). The tissues were removed in accordance with the University of Lund Ethics permission (LU99). In addition, the resected vessels were used with written consent from the living patients as described previously (Edvinsson et al., 2010).

Paraffin cynomolgus monkey tissue. The supratentorial dura mater, medulla, and trigeminal ganglia, including processes and sphenopalatine ganglia, from three adult male cynomolgus monkeys (*Macaca fascicularis*) were procured from Covance (Madison, WI, USA). Tissue samples were preserved in 10% neutral buffered formalin and embedded in paraffin.

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