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EVIDENCE FOR ANTI-INFLAMMATORY AND PUTATIVE ANALGESIC EFFECTS OF A MONOCLONAL ANTIBODY TO CALCITONIN GENE-RELATED PEPTIDE

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Abstract—*Background:* Calcitonin gene-related peptide (CGRP) is a powerful pro-inflammatory mediator thought to play a significant role in the development of inflammation and pain. We investigated the role of CGRP in trigeminal inflammatory pain by determining the ability of a monoclonal antibody to CGRP to modify central Fos expression in response to stimulation of the inflamed ferret tooth pulp. We also assessed the effect of the antibody on pulpal inflammation.

***Methods:* Ten adult ferrets were prepared under anaesthesia to allow stimulation of the upper and lower left canine pulps, recording from the digastric muscle and intravenous injections at subsequent experiments. In all animals, pulpal inflammation was induced by introducing human caries into a deep buccal cavity. Four days later animals were treated intravenously with either CGRP antibody ($n = 5$) or vehicle ($n = 5$). After a further 2 days animals were re-anaesthetised and the tooth pulps stimulated at 10 times jaw-opening reflex threshold. Brainstems and tooth pulps were processed immunohistochemically for Fos and the common leucocyte marker CD45, respectively.**

***Results:* Fos was expressed in ipsilateral trigeminal subnuclei caudalis (Vc) and oralis (Vo). Significantly fewer Fos-positive nuclei were present within Vc of CGRP antibody-treated animals ($p = 0.003$ vs vehicle-treated). Mean percentage area of staining for CD45 was significantly less in antibody-treated animals ($p = 0.04$ vs vehicle-treated).**

***Conclusions:* This is the first direct evidence that sequestration of CGRP has anti-inflammatory and putative analgesic effects. Previous studies using this Fos model have demonstrated that it is able to predict clinical analgesic efficacy. Thus these data indicate that this antibody may have analgesic effects in dental pain and other types of inflammatory-mediated transmission, and suggest that this is in part due to peripheral anti-inflammatory effects.**

Key words: trigeminal, calcitonin gene-related peptide, Fos, antibody therapy, inflammatory pain, immunoneutralisation.

INTRODUCTION

Inflammation of the tooth pulp in man produces a variety of symptoms, including spontaneous pain and hypersensitivity to thermal stimuli. These symptoms are thought to result from a combination of peripheral and central changes, which lead to altered neuronal excitability (Sessle, 2000). Neuronal excitability is influenced by a wide variety of factors including the presence of neuropeptides such as calcitonin gene-related peptide (CGRP). CGRP is widely distributed within neurons of the central and peripheral nervous systems (van Rossum et al., 1997), and is known to have a variety of pro-inflammatory effects via its influence on immune cells (Tang et al., 1998; Cuesta et al., 2002; Yaraee et al., 2003) and the vasculature (Lennerz et al., 2008). CGRP is also thought to play a significant role in the development of inflammatory pain. It is released from peripheral and central terminals of nerve fibres in response to nociceptor activation (Lundberg et al., 1992; Averbeck and Reeh, 2001; Bernardini et al., 2004) and is known to modulate the excitability of dorsal horn neurons (Leem et al., 2001). CGRP has also been implicated in the development of adjuvant-induced inflammatory pain and central pain states (Bennett et al., 2000; Leem et al., 2001; Ohtori et al., 2001).

Within the trigeminal system, CGRP has a well-established role in the pathophysiology of migraine. Recent studies suggest that CGRP has four sites of action, including blood vessels, mast cells, second-order sensory neurons and in the trigeminal ganglion (Villalon and Olesen, 2009). Intracranial blood vessels are innervated by trigeminal neurons enriched with CGRP

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Abbreviations: AOI, area of interest; CGRP, calcitonin gene-related peptide; ELISA, enzyme-linked immunosorbent assay; EMG, electromyogram; IR, immunoreactive; JOR, jaw opening reflex; MSN, main sensory nucleus [of the trigeminal nucleus]; NDS, normal donkey serum; NGS, normal goat serum; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Triton-X; PGP, protein gene product; SP, substance P; Vc, trigeminal subnucleus caudalis; Vi, trigeminal subnucleus interpolaris; VIP, vasoactive intestinal polypeptide; Vo, trigeminal subnucleus oralis.

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(Uddman et al., 1986) and patients suffering from migraine are seen to have increased CGRP plasma levels during a severe attack (Goadsby et al., 1988; Edvinsson and Goadsby, 1995; Ashina et al., 2000).

There is also evidence from this and other laboratories, that CGRP plays a role in trigeminal neuropathic and inflammatory pain (Loescher et al., 2001; Rodd and Boissonade, 2002; Bird et al., 2003). Within the tooth pulp CGRP is known to cause vasodilation and subsequent increased blood flow following electrical stimulation (Heyeraas et al., 1994). Studies of human and animal tissues have demonstrated that inflammation of the tooth pulp induces a significant increase in CGRP expression (Kimberly and Byers, 1988; Rodd and Boissonade, 2002). These data suggest a role for CGRP in pain from the tooth pulp.

Studies of inflammation in the tooth pulp have shown that there is an increase in leucocyte infiltration, which leads to an increase in leucocyte density (Kimberly and Byers, 1988; Rodd and Boissonade, 2006). Inflammation of the tooth pulp is also associated with an increase in neural density (Plackova, 1966; Torneck, 1977; Byers, 1992; Hong et al., 1993; Rodd and Boissonade, 2001). More recent studies have identified that CGRP and other neuropeptides including substance P (SP), vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY) are significantly increased during inflammation of the tooth pulp (Rodd and Boissonade, 2002). Thus there is significant evidence supporting the hypothesis that CGRP plays a significant role in the development of pulpal inflammation.

In this laboratory we have developed a model to investigate neuronal activation in the trigeminal nucleus following inflammation and stimulation of the tooth pulp, by quantifying expression of the immediate-early gene product Fos (Worsley et al., 2007). In the present study we have used this model to investigate the role of CGRP in inflammatory pain from the tooth pulp, by determining the ability of a monoclonal antibody to CGRP to modify Fos expression. We have also investigated the ability of the CGRP antibody to modify pulpal inflammation, and assessed its impact on neural density and CGRP expression in the inflamed tooth pulp.

EXPERIMENTAL PROCEDURES

Experiments were carried out in 10 adult female ferrets (5–8 months old, 554–1141 g; Highgate Farm, UK), under UK Home Office Licence regulation and approval. The ferrets were prepared under anaesthesia (ketamine, Fort Dodge, Southampton, UK; 25 mg/kg; xylazine, Bayer, Newbury, UK; 2 mg/kg; intramuscularly) in order to allow tooth pulp inflammation and stimulation of the upper and lower left canine teeth, and an electromyogram (EMG) to be recorded from the digastric muscle, during a subsequent experiment. Briefly, a connector block composed of a mini 9-way socket and an intravenous injection port was attached to the skull. Leads from this block were passed subcutaneously to insulated Ag–AgCl fillings in the left canine teeth for stimulation, and to the left digastric muscle for recording its EMG. A cannula from the injection port was inserted into the left jugular vein. Following cannula insertion anaesthesia was maintained intravenously, using alfaxalone (Vétoquinol; Buckingham, UK) at 6 mg/kg/h. These procedures have been described in greater detail previously (Oakden and Boissonade, 1998; Worsley et al., 2007). The animals also had pulpal inflammation induced by the introduction of human caries into deep buccal cavities in the upper and lower left canine teeth as described previously (Worsley et al., 2007).

The animals were allowed to recover for 6 days following the preparatory surgery. Previous studies in this laboratory (Worsley et al., 2007) have demonstrated inflammation-induced changes in the tooth pulp and in Fos expression at this time point following induction of pulpal inflammation. On day 4 the animals received an intravenous injection via the venous injection port, of either a monoclonal antibody to CGRP ($n = 5$; Clone 4901, Sigma Aldrich, USA; 2 mg/ml administered at 1 ml/kg) or the phosphate-buffered saline (PBS) vehicle ($n = 5$). The antibody had previously been dialysed in PBS to remove 15 mM sodium azide (Slide-a-lyzer Dialysis Cassettes; Pierce, Rockford, IL, USA). The dose of antibody and the time course of administration were based on previous functional studies using this antibody (Benschop et al., 2005; Zeller et al., 2008); further details are provided in the Discussion.

On day 6 the animals were anaesthetised with alfaxalone (4 mg/kg) via the indwelling cannula. Light anaesthesia was then maintained by continuous infusion of alfaxalone (10.5–16 mg/kg/h), adjusted to allow a withdrawal reflex to be seen following a paw squeeze. Body temperature was maintained at 38 ± 0.5 °C. The tooth pulps were then electrically stimulated via the digastric EMG electrodes (a train of three 0.5-ms duration stimuli at 200 Hz) and the amplitude of the stimulus required to produce the jaw opening reflex (JOR) was determined for each tooth. The tooth pulps were then stimulated at ten times the JOR threshold, once per second for 90 min. Following stimulation, anaesthesia was maintained for a further 30 min, during which time blood samples were taken for analysis of the antibody concentrations (see below). The animals were then deeply anaesthetised with sodium pentobarbitone (Sagatal; Rhone Merieux, Harlow, UK; 42 mg/kg, intraperitoneally) and perfused with 1000 ml of PBS, followed by 1000 ml of 4% paraformaldehyde fixative.

Following perfusion the brainstem and tooth pulps were removed, post-fixed in paraformaldehyde for 4 h at 4 °C and immersed in a 30% sucrose solution overnight for cryoprotection. The pulps taken from both the upper and lower left canine teeth were used for the subsequent immunohistochemistry giving 10 pulps from each experimental group.

Collection and processing of blood samples

Approximately 1 ml of blood was collected from a superficial vein in the hind limb, into ethylenediaminetetraacetic acid (EDTA)-coated tubes, centrifuged at 4 °C for 5 min at 5000 rpm, and the plasma stored at −80 °C. Analysis to quantify antibody levels was undertaken using an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated overnight (4 °C) with anti-mouse IgG1 (BD Biosciences, San Jose, USA) in carbonate coating buffer (pH 9.6), washed three times with wash buffer (0.02 M Tris pH 7.4, 0.15 M NaCl, 0.1% Tween-20) and blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in wash buffer. Plates were washed again prior to the addition of plasma samples in duplicates. The samples were tested at 1:100, 1:400, 1:1600 and 1:6400 dilutions. The captured antibody was detected using an anti-mouse kappa-horseradish peroxidase (BD Biosciences, San Jose, USA), followed by application of *o*-phenylene-diamine (Sigma–Aldrich, St Louis, USA) for 6 min; the reaction was stopped by adding 1 N HCl. A standard curve of the purified anti-CGRP antibody was used to calculate the plasma concentration.