Production and characterization of a monoclonal antibody against human calcitonin gene-related peptide (CGRP) and its immunohistochemical application to salivary glands

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Summary

A monoclonal antibody (mAb), 129CD8 was raised against a C-terminal fragment (aa28-37) of α-human calcitonin gene-related peptide (CGRP) coupled to bovine serum albumin. The specificity of the monoclonal antibody 129CD8 was corroborated by dot immunobinding experiments, enzyme-linked immunosassay and immunostaining of tissue sections. In vitro studies showed that the mAb 129CD8 readily recognized the fragment 28-37 of α-human CGRP and to a slightly lesser degree whole α-human CGRP and the fragments containing the C-terminal part of the molecule. The mAb 129CD8 also recognized the β-human CGRP but not the α-rat CGRP. The mAb 129CD8 did not react with substance P, katacalcin, calcitonin, amylin or fragments of α-human CGRP lacking the C-terminal part of the molecule.

Immunocytochemical staining was performed on human skin, guinea-pig thyroid and salivary glands and the trigeminal ganglion, and rat thyroid gland. Our findings demonstrate, in keeping with previous studies, that in human skin, nerve fibres containing CGRP immunoreactivity are found in both epidermis and dermis. In accordance with previous investigators, the Merkel cells were immunoreactive for CGRP. In the guinea-pig and rat thyroid gland CGRP immunoreactivity was localized in the C-cells. The distribution of CGRP immunoreactivity in the guinea-pig salivary glands is different from that previously reported for rat salivary glands. In the guinea-pig trigeminal ganglion, CGRP immunoreactivity was localized mainly in small-sized neurons and fibres traversing the ganglion. Double staining with substance P performed on guinea-pig trigeminal ganglion revealed four types of sensory neurons, those containing both peptides, those containing only substance P or CGRP and those lacking both peptides. Guinea-pig parotid gland, but not the submandibular or sublingual glands, contained periacinar fibres exhibiting both immunoreactivities. Substance P-positive, CGRP-positive fibres were also seen around parotid and submandibular, but not around sublingual, gland ducts. All glands received perivascular innervation showing immunoreactivities for both peptides. The present results support the idea that in the peripheral nervous system only a subpopulation of sensory neurons contains both substance P and CGRP. Consequently, colocalization of substance P and CGRP indicates a sensory nerve, while those containing either substance P or CGRP may be sensory or parasympathetic.

Introduction

The existence of calcitonin gene-related peptide (CGRP) was first predicted by Amara and the others based on studies of medullary thyroid carcinoma cell lines (Amara et al., 1982; Rosenfeld et al., 1983). In the rat, this 37-amino-acid peptide exists in two forms, α- and β-rat CGRP (α- and β-rCGRP) which differ from each other only for one amino acid residue. Later on, the human form of CGRP was isolated and characterized by Morris et al. (1984). α-Human CGRP (α-hCGRP) and β-human CGRP (β-hCGRP) differ from each other by three amino acid residues. There is approximately 90% homology between α-hCGRP and α-rCGRP (Amara et al., 1985; Steenberg et al., 1985; Höppener et al., 1985). CGRP immunoreactivity has been reported in several endocrine cells, such as the C-cells of normal thyroid gland (Grunditz et al., 1986; Nitta et al., 1986; Zabel et al., 1987), adrenal medullary cells, and some cells of pancreatic islets (Rosenfeld et al., 1983). Furthermore, CGRP has been detected...
immunochemically in human pituitary gland (Tschoopp et al., 1984). Immunohistochemical and biochemical studies show the presence of CGRP in medullary thyroid carcinoma (Morris et al., 1984; Tschoopp et al., 1984; Gkonos et al., 1986; Poston et al., 1987; Williams et al., 1987), and mRNA encoding for CGRP has been identified in human lung tumour cell-lines (Edbrooke et al., 1985).

CGRP is widely expressed in the central and peripheral nervous systems, including motor, sensory and autonomic systems (Rosenfeld et al., 1983; Gibson et al., 1984; Lee et al., 1985; Skofitsch & Jacobowitz, 1985; for reviews see Brain et al., 1985; Girgis et al., 1985; Goodman & Iversen, 1986; Holman et al., 1986; Brain & Williams, 1988; Dockray, 1988). CGRP is released from cultured trigeminal ganglion cells, supporting the idea that it acts as an extracellular transmitter or modulator (Mason et al., 1984). By inhibiting endopeptidases CGRP may enhance the action of other peptides, as suggested for substance P in the spinal cord (Nyberg et al., 1988). CGRP contributes to the innervation of salivary glands (Soinila et al., 1989), a widely used model tissue for studies of neurotransmitter–neuropeptide interactions, probably by modulating the response to secretory neurotransmitters (Ekström et al., 1988).

Although CGRP is co-localized with SP in several sensory neurons (Lee et al., 1985; Skofitsch & Jacobowitz, 1985; Brain & Williams, 1988) it is obvious that neither substance P nor CGRP is a specific marker for sensory neurons (Lee et al., 1985; Gibbins et al., 1987). For identification of the functional nature of CGRP-immunoreactive neurons or nerve fibres it is necessary to visualize other neurotransmitters or neuropeptides co-localized with CGRP. For this purpose, we have raised and characterized a monoclonal antibody against the C-terminal fragment of α-hCGRP (aa 28–37). We report that this antibody recognizes both human and guinea-pig CGRP, and demonstrate that co-localization of CGRP and substance P marks sensory nerve fibres in the guinea-pig salivary glands.

Materials and methods

Monoclonal antibody production

The monoclonal antibodies (mAbs) were raised against a C-terminal fragment of α-hCGRP (amino acids 28–37) (Peninsula, Belmont, CA, USA). For immunization, the CGRP fragment (1 mg ml⁻¹ in distilled water) was conjugated to bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) using water-soluble 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) (Sigma, St Louis, MO, USA; final concentration 10 mg ml⁻¹) (Harlow & Lane, 1988). The reaction was continued at room temperature for 4 h. The conjugate was dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4°C for 72 h and aliquots of the conjugate were stored at −20°C. Protein concentration was estimated according to Lowry et al. (1981) using BSA as a standard. Antibodies were raised by immunizing BALB/c mice with the α-hCGRP (aa 28–37)–BSA conjugate in Freund’s complete adjuvant. The subsequent two immunizations were made at 2-week intervals using Freund’s incomplete adjuvant. The last immunization was made by injecting the antigen in PBS i.p., 3 days before the fusion. Hybridomas were established by standard techniques (Köhler & Milstein, 1975) using P3 × Ag 8.653 myeloma cells, maintained in RPMI 1640 medium, supplemented with 20% fetal calf serum (Gibco, Paisley, Scotland) and antibiotics. The hybridomas were screened using the α-hCGRP (aa 28–37)–BSA conjugate in enzyme-linked immunoassay (ELA) and in dot immunobinding assay. The hybridoma 129CD8 was selected for detailed studies. Cloning of the hybridoma culture was performed twice manually by picking up single cells with a micropipette. The clones were propagated with the help of peritoneal macrophages. The mAb 129CD8 was of IgG₁ isotype, as revealed by Ouchterlony immunodiffusion technique using a commercial kit (Sigma).

Dot immunobinding experiments

Dot immunobinding experiments were performed according to Herbrink et al. (1982) by spotting 1 μl α-CGRP-BSA (aa 28–37) conjugate onto nitrocellulose at concentrations of 100 μg ml⁻¹, 10 μg ml⁻¹ and 1 μg ml⁻¹. The slips were incubated with 3% BSA in PBS containing 0.02% NaN₃ to block non-specific protein-binding sites followed by the primary antibody for 1 h at room temperature. The slips were washed in PBS and exposed to peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark), diluted 1:100 in 3% BSA–PBS containing 0.4% Triton X-100, for 1 h. The peroxidase reaction was developed with 3,3’-diaminobenzidine (0.25 mg ml⁻¹ in Tris-HCl buffer, pH 7.6) containing 0.03% H₂O₂. For pre-absorption experiments, the mAb 129CD8 was diluted 1:1 in 5% BSA in PBS containing 0.4% Triton X-100 and buffer, or one of the following peptides was added at 0.1 μM, 1 μM or 10 μM concentration: katacalcin, calcitonin, substance P (SP), amylin, α-hCGRP, β-hCGRP, α-αCGRP, fragments of α-hCGRP; aa 8–37, aa 15–22, aa 15–24, 15–37, aa 28–37. All peptides were obtained from Peninsula. Pre-absorption was allowed for overnight at 4°C.

Enzyme immunoassay

Competitive inhibition ELA was used to quantitate the inhibition of the reactivity of the mAb 129CD8 with the α-hCGRP (aa 28–37)–BSA conjugate by various peptides and α-hCGRP fragments (Bosworth et al., 1983). Briefly, microtitre plates were coated with the α-hCGRP (aa 28–37)–BSA conjugate (1 μg ml⁻¹) in 50 mM sodium bicarbonate buffer, pH 9.6, overnight incubation at 4°C. Unoccupied binding sites were blocked with 3% BSA–PBS. Solutions of katacalcin, calcitonin, SP, amylin, α-hCGRP, β-hCGRP, α-αCGRP, fragments of α-hCGRP; aa 1–10, aa 8–37, aa 15–22, aa 15–24, aa 15–37, aa 28–37, as 3% BSA–PBS, were incubated with the mAb 129CD8 (diluted 1:10) overnight at 4°C. All peptides were tested at 10, 5.0, 2.5, 1.25, 0.625, 0.313, 0.156 or 0.078 μM concentrations. The pre-absorbed mAb 129CD8 was added to the wells and the incubation was allowed to proceed overnight at 4°C. The plates were washed and incubated with peroxidase-conjugated rabbit anti-mouse IgG antiserum diluted 1:1000 (Dakopatts) at 37°C for 1 h. For visualization of the enzyme activity, tetramethylbenzidine (0.5 mg/I00 ml dimethyl-sulphoxide; Sigma) was used as a chromogenic substrate. Absorbance was measured at 450 nm on an automatic ELA