Demonstration of the neurotransmitter role of calcitonin gene-related peptides (CGRP) by immunoblockade with anti-CGRP monoclonal antibodies

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1 Monoclonal antibodies (MAbs) against rat α -calcitonin gene-related peptide (α CGRP) were produced. Those which bound CGRP in a radioimmunoassay and inhibited the binding of 2-[¹²⁵I]-iodohistidyl¹⁰-CGRP in a receptor binding assay were selected for immunoblockade experiments.

2 The effect of MAbs on CGRP inhibition of electrically stimulated contractions of the rat isolated vas deferens was characterized. Four out of 11 MAbs tested shifted the concentration-response curve of CGRP to the right compared with vehicle or irrelevant MAb control. MAb C4.19 produced equipotent blockade of rat α CGRP and rat β CGRP and was chosen for further studies. MAb C4.19 had no pharmacologically significant effect on the concentration-response relationship of isoprenaline, rat β -endorphin or somatostatin.

3 We demonstrated that the pharmacological response to CGRP in the presence of MAb C4.19 could be predicted when the dissociation constant and concentration of binding sites of the antibody were known. Comparison of experimental and computer simulated data showed good agreement for EC₅₀ and maximum effect of CGRP in the presence of MAb C4.19.

4 Capsaicin at 1 μ M inhibited the electrically stimulated contractions by 60.8% (95% confidence interval 51.8% to 69.9%). This effect was significantly attenuated by MAb C4.19 to 26.0% (95% confidence interval 15.2% to 36.8%; $P < 0.0003$).

5 The immunoblockade of exogenous and endogenous CGRP described here, together with complementary evidence from other studies, strongly suggest that CGRP has a major neurotransmitter role at the neuroeffector junction of the rat vas deferens.

Keywords: Calcitonin gene-related peptide; monoclonal antibodies, rat isolated vas deferens; immunoblockade; neurotransmission; capsaicin

Introduction

Calcitonin gene-related peptide (CGRP) is produced by alternative processing of the primary mRNA transcripts of the calcitonin gene (Rosenfeld *et al.*, 1983). A second CGRP gene encoding another 37-amino acid peptide was subsequently identified (Amara *et al.*, 1985; Steenbergh *et al.*, 1985). This peptide (β CGRP) differs from the originally discovered CGRP (α CGRP) by only one amino acid at position 35 in the rat. Unlike calcitonin, CGRP is primarily localized in the brain and peripheral nervous tissue. Diverse biological effects have been attributed to CGRP but its physiological importance remains to be established in many organ systems. The localization of CGRP-like immunoreactivity in primary afferent neurones innervating many different tissues and the wide distribution of CGRP binding sites suggest that CGRP may be a physiologically important neurotransmitter.

One important criterion that must be fulfilled for any neurotransmitter is that modulation of the effects of the exogenous putative neurotransmitter by drugs should have corresponding effects on responses to nerve stimulation. Pharmacological blockade is normally achieved through the use of specific receptor antagonists. A number of C-terminal fragments of CGRP have been demonstrated to behave as receptor antagonists (Mimeault *et al.*, 1991). The C-terminal (8–37) fragment of human α CGRP has been well characterized and is commercially available. However, CGRP (8–37) demonstrates variable antagonistic potency in different tissues and is a relatively poor antagonist in the rat isolated vas deferens preparation (Dennis *et al.*, 1990). This has led to the postulation that multiple receptor subtypes exist for

CGRP. An alternative approach to pharmacological blockade is the use of antibodies which bind to and inhibit the biological activities of putative neurotransmitter peptides. Immunoblockade may be a more general approach to the elucidation of the physiological roles of neuropeptides since no assumptions have to be made concerning receptor multiplicity and the relative selectivity of receptor antagonists.

The major objective of the present study was to investigate the role of CGRP as a principal neurotransmitter, using the neuroeffector junction of the rat isolated vas deferens as a model for neurotransmission. The release of endogenous CGRP from nerves was achieved through the use of capsaicin. Capsaicin is the pungent ingredient of hot peppers of the genus *Capsicum* which selectively stimulates a sub-population of primary afferent neurones (reviewed by Holzer, 1991). It has been widely used as a pharmacological tool to investigate the 'efferent' function of primary afferent neurones (reviewed by Maggi & Meli, 1988). Stimulation of capsaicin-sensitive sensory neurones leads to release of neuropeptides stored in nerve terminals which may, in turn, mediate various efferent functions. Both capsaicin and CGRP inhibit the nerve-mediated contractions of the isolated vas deferens. It is therefore reasonable to hypothesize that CGRP may be involved in neurotransmission at the neuroeffector junction of the rat vas deferens.

Analysis of the effects of individual endogenous neuropeptides is often difficult because of the co-release of several neuropeptides by capsaicin in many tissues. In particular, tachykinins co-released with CGRP often produce a similar biological response (e.g. dilatation of arteries). In the electrically stimulated isolated vas deferens, neurokinin A and substance P enhance contractions (Moritoki *et al.*, 1987) in

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contrast to the inhibitory effect of CGRP (Goto *et al.*, 1987). Tissue concentrations of tachykinins are low in the rat vas deferens and substance P-like and neurokinin-like immunoreactivity were undetectable by immunocytochemistry in this tissue (Saito *et al.*, 1987). In this respect, the rat vas deferens is a particularly useful model for the investigation of CGRP as a major neurotransmitter.

Immunoblockade of biologically-active peptides is a relative new pharmacological tool which has not been subject to rigorous scientific evaluation. Therefore a further objective of the present investigations was to validate the use of immunoblockade as an experimental tool for probing the physiological role of CGRP. We have done this by the use of monoclonal antibodies (MAbs) and appropriate experimental designs to confirm the specificity of blockade. In addition, we have fitted appropriate models to the observed data and performed limited simulations in order to compare observations with theoretical predictions.

Methods

Production of monoclonal antibodies

Rat α CGRP was conjugated to bovine serum albumin using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (O'Shaughnessy, 1982). The conjugate was mixed with Freund's adjuvant to produce a water-in-oil emulsion. Two hundred μ l of the mixture, containing 35 μ g of the immunogen, was injected into each mouse by the intraperitoneal route. Ten female Balb/C mice were immunized. Freund's complete adjuvant was used for the priming dose and Freund's incomplete adjuvant used for the first and second booster injections. The first and second booster injections were given at 4 weekly intervals. A final aqueous injection was given 3 days prior to fusion.

Sera collected 8 days following the second booster injection were screened by indirect enzyme-linked immunoadsorbent assay (ELISA; described below) and were found to be positive for antibodies against CGRP. Fusion between Sp2 myeloma cells and spleen cells from mice was performed by a modification of the method described by Galfre & Milstein (1981). Hybridoma cells were co-cultured with feeder layer cells (irradiated MRC-5 human lung diploid fibroblasts; Long *et al.*, 1986). Supernatants from the fusion were screened by indirect ELISA. Prior to cloning, 100 positive hybridomas detected by the primary ELISA screen were subjected to secondary screening assays which included further ELISAs, radioimmunoassay (RIA) and receptor binding assay using a rat liver membrane preparation (Nakamuta *et al.*, 1986). These assays are described fully below. Four hybridomas (coded C4.19, C4.6, R1.50, R2.73) were strongly positive in the RIA and the receptor binding assay. The hybridoma lines were cloned twice by limiting dilution (Goding, 1986).

MAbs were produced in bulk by growing hybridoma cells as ascitic tumours (Galfre & Milstein, 1981) in Balb/C mice which had been previously primed with a 100 μ l intraperitoneal injection of pristane (2,6,10,14-tetramethyl-pentadecane). MAbs were purified by ammonium sulphate precipitation (Goding, 1986) followed by re-suspension in phosphate-buffered saline (PBS). Protein concentrations were estimated using a Coomassie brilliant blue dye binding assay (Bio-Rad) with bovine γ -globulin as standard.

Indirect enzyme-linked immunoadsorbent assay (ELISA)

Ninety-six well microtitre plates (Linbro EIA plus titration plates; Flow) were coated with 50 μ l of 400 nM CGRP solution or PBS vehicle per well, sealed with film, and left overnight at 4°C. Further steps were performed at room temperature. Plates were washed three times with 300 μ l PBS/

Tween 20 0.05% between each step of the ELISA. Wells were incubated with 100 μ l porcine gelatin 0.25% in PBS pH 7.4 (blocking step) for 2 h. Fifty μ l serum or hybridoma culture supernatant, diluted as appropriate, was added to each well and incubated for 2 h. Serial dilutions were made in PBS/Tween 20 0.05%/gelatin 0.25%. Controls used were normal mouse immunoglobulin (10 μ g ml⁻¹), culture medium and Sp2 myeloma cell culture supernatant. Wells were incubated with 50 μ l horseradish peroxidase-conjugated rabbit anti-mouse antibody (1 in 1000 in PBS/Tween 20 0.05%/gelatin 0.25%) for 1 h. One hundred μ l substrate reagent (0.006% hydrogen peroxide, 0.1 M acetate-citrate buffer pH 6) containing tetramethylbenzidine (100 μ g ml⁻¹) as chromogen (Bos *et al.*, 1981) was added to each well. Positive wells developed a blue colour when 25 μ l of 2 M sulphuric acid was added to give a yellow colour with greater intensity. Absorbance was measured at 450 nm using an ELISA plate reader (Titertek Multiskan Plus Mk II, Flow Laboratories).

Selectivity of antibodies for different CGRPs was assessed by coating plates with the α and β forms of rat and human CGRP. Relative affinity of antibodies for CGRP was assessed by serial dilution of hybridoma supernatants. The validity of this simple method of determining relative affinity (van Heyningen *et al.*, 1983) depends on prior knowledge that the variation of immunoglobulin concentration in supernatants from confluent hybridomas is generally less than ten fold.

Receptor binding assay

Livers dissected from Wistar rats were homogenized in 50 mM Tris HCl pH 7.4 containing 0.1 mM phenyl-methylsulphonyl-fluoride (PMSF). The homogenate was centrifuged at 15000 g for 20 min at 4°C. Supernatant was decanted and centrifuged at 48000 g for 60 min at 4°C. The pellet was resuspended in buffer and centrifuged at 48000 g for 60 min at 4°C. The supernatant was discarded and pellet resuspended in binding assay buffer containing aprotinin (20 U ml⁻¹), bacitracin (0.1%), PMSF (0.1 mM), MgCl₂ (5 mM), bovine serum albumin (0.5%) in 50 mM Tris HCl pH 7.4 (at 4°C).

The binding assay incubation mixture consisted of 50 μ l hybridoma supernatant (diluted as appropriate), 50 μ l binding buffer, 50 μ l (2-[¹²⁵I]-iodohistidyl¹⁰-human α CGRP (40,000 c.p.m.) and 100 μ l liver membrane suspension (3 mg ml⁻¹). Non-specific binding was assessed using excess rat α CGRP (10⁻⁶ M). Cell culture medium, Sp2 myeloma supernatant and normal mouse immunoglobulin (10 μ g ml⁻¹) were used as controls. The mixture was incubated at 4°C for 2 h, vacuum filtered through Whatman GF/C filter paper pre-soaked in polyethyleneimine (250 μ l in 200 ml 50 mM Tris HCl pH 7.4), and washed thrice with 3 ml ice-cold buffer (50 mM Tris HCl pH 7.4 containing bovine serum albumin 0.5%). Radioactivity on the filter paper was counted in a Beckman Gamma 5500 counter.

Radioimmunoassay (RIA)

For screening procedures, the RIA incubation mixture consisted of 50 μ l hybridoma supernatant, 50 μ l 2-[¹²⁵I]-iodohistidyl¹⁰-human α CGRP (40,000 c.p.m.) and 400 μ l of assay buffer (50 mM sodium phosphate buffer pH 7.4 containing 10 mM EDTA and 0.3% bovine serum albumin). Tissue culture medium, myeloma (Sp2) culture supernatant, normal mouse immunoglobulin (10 μ g ml⁻¹) and a blank without supernatant were used as controls. The assay was performed at 4°C with a 4 day incubation period. Bound and free CGRP was separated by charcoal precipitation. One dextran-coated charcoal tablet (Steranti Separex; Steranti Research) was stirred in 15 ml separation buffer (assay buffer containing 0.25% gelatin) for 30 min at 4°C. Two hundred and fifty μ l of the dextran/charcoal suspension was added to each assay tube followed by centrifugation at 2000 g for 20 min.

Supernatant and charcoal were separated and the pellet counted in a Beckman Gamma 5500 counter.

RIA was also performed to estimate the dissociation constants (K_d) of MABs and the maximum binding capacity (B_{max}) of purified ascites. The displacement of 2-[¹²⁵I]-iodohistidyl¹⁰-human α CGRP binding to MAB C4.19 by rat α and β CGRP was investigated. No difference in binding was found whether assay buffer or Krebs buffer was used in the RIA. MAB C4.19 did not cross-react with substance P at 1 μ M.

Tissue bath experiments

Approximately 1.5 cm sections of the prostatic portion of the vas deferens were obtained from male Sprague-Dawley rats (220–300 g). Left and right vas deferens from the same animal were used as test and control tissues. The tissues were mounted for isometric tension recordings in 3 ml siliconized (Sigmacote, Sigma) tissue baths containing Krebs physiological salt solution (PSS) maintained at 37°C, pH 7.4, oxygenated with 95% O₂ and 5% CO₂. The Krebs PSS had the following composition (mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, and glucose 11.1. A resting load of 0.5 g tension was applied. Isometric tension changes along the longitudinal axis were recorded with a force transducer connected to a Grass Model 7D Polygraph recorder. Platinum wire electrodes were placed at the top and bottom of the organ bath for electrical field stimulation. The tissues were electrically stimulated using a Multistim System-D330 (Digitimer Limited) stimulator. The following stimulation parameters were used: frequency 0.2 Hz; pulse width 0.5 ms; voltage 60–80 V.

Following a 90 min equilibration period, over which the tissues were electrically stimulated, anti-CGRP MABs or controls (see below) were added to the tissue baths. Experiments were performed to determine whether pre-incubation with MAB for different periods affected the response to 10 nM rat α CGRP (Table 1). In all other experiments, tissues were pre-incubated with MAB or control for 10 min prior to the determination of cumulative concentration-response relationships to the appropriate agonists (either CGRP, isoprenaline, rat β -endorphin, or somatostatin). Preliminary experiments with CGRP showed that a second concentration-response determination produced a lower maximal effect. Therefore, only one cumulative concentration-response relationship was obtained for CGRP in each tissue. Since tachyphylaxis develops rapidly to somatostatin (Vizi *et al.*, 1984), the response to a single concentration of somatostatin was tested only once in each tissue. Responses to isoprenaline and rat β -endorphin did not desensitize. Cross-over experiments were therefore performed in which the same tissue was pre-incubated for 10 min with anti-CGRP MAB or control.

Time course experiments were performed to investigate the influence of MAB incubation time on the response to capsaicin (Table 1). The effect of MAB C4.19 on the response to capsaicin was compared with controls following a 45 min incubation. A concentration of capsaicin producing maximal response (1 μ M) was used (Maggi *et al.*, 1993). Individual tissues were exposed to a single concentration of capsaicin.

MABs were added in the form of crude ascites or MABs

purified as described above. Seven MABs against human CGRP C- and N-terminal fragments (from C. Plumpton) were tested along with the four MABs obtained from immunization of mice with rat α CGRP described above. The MAB vehicle (PBS) was used as a control. A mouse anti-rat MAB MAR 18.5 or a mouse anti-thyroid-stimulating hormone (TSH) MAB was also used as irrelevant antibody controls at the same protein concentration as anti-CGRP MABs.

Data analysis

The K_d and B_{max} of the MAB C4.19 for rat α CGRP and rat β CGRP were estimated by fitting the following model to binding data from RIA displacement experiments:

$$B(C) = \frac{B_{max} \times C}{K_d \times (1 + D/K_{dD}) + C} + NS \times C$$

where $B(C)$ is the binding B of ligand (2-[¹²⁵I]-iodohistidyl¹⁰-human α CGRP) concentration C in the presence of displacer (rat α CGRP or β CGRP) concentration D . K_d and K_{dD} are the dissociation constants of ligand and displacer respectively. B_{max} is the concentration of binding sites and NS is the non-specific binding parameter.

For a given total concentration of ligand, the concentration of ligand bound was predicted from the following binding model for simulation purposes:

$$B(C) = \frac{B_{max} \times C}{K_d + C}$$

The concentration of unbound CGRP was simulated for the range of concentrations used in organ bath experiments. The pharmacological response in the presence of MAB could then be simulated with the pharmacodynamic model described below.

The inhibitory effect of CGRP in organ bath experiments was expressed as % inhibition relative to stable pre-dosing contractions. The following sigmoidal E_{max} (logistic) model was fitted to concentration-response data obtained from individual tissues:

$$E = E_0 + \frac{E_{max} \times C^N}{EC_{50}^N + C^N}$$

Where E_0 is the baseline effect, E_{max} , the maximum effect, EC_{50} the concentration which produces half-maximal effect and N the steepness factor (Hill coefficient).

Parameter estimation and simulation were performed by use of the non-linear extended least-squares regression package MKMODEL version 4.72 (Biosoft, Cambridge). EC_{50} was assumed to be log-normally distributed (Carpenter, 1986). The paired t test was performed for comparison of test and control data from paired tissues. One-way analysis of variance followed by Tukey's test for multiple comparisons were performed to assess the influence of incubation time on immunoblockade of CGRP and capsaicin responses in unpaired tissues. Ninety-five percent confidence intervals were used to indicate the precision of estimates (Gardner & Altman, 1989). Differences discernible at the 0.05 level were

Table 1 Effect of incubation time of MAB C4.19 on the mean responses (95% confidence interval) to rat α CGRP and capsaicin (% inhibition: tissues from 6 animals at each time point)

Incubation time (min)	Response to rat α CGRP (10 nM)	Response to capsaicin (1 μ M)
10	14.3 (7.6 to 21.0)	55.0 (46.9 to 63.2)*
45	17.8 (9.4 to 26.2)	26.0 (19.0 to 33.1)
90	20.2 (11.8 to 28.6)	29.4 (21.2 to 37.5)

* $P < 0.05$ compared with responses at 45 or 90 min.

Inhibition of electrically stimulated contractions of the rat isolated vas deferens is expressed as % inhibition relative to stable contractions recorded immediately before addition of rat α CGRP or capsaicin.

considered statistically significant. Statistical analysis was performed by use of the Statgraphics-Plus package (STSC, Inc., Rockville, U.S.A.).

Materials

Rat α CGRP, rat β CGRP, human α CGRP, human β CGRP and rat β -endorphin were obtained from Peninsula. Bacitracin, polyethyleneimine, PMSF, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride, pristane, capsaicin, (–)-isoprenaline hydrochloride and somatostatin were purchased from Sigma. 2-[125 I]-iodohistidyl 10 -human α CGRP was supplied by Amersham. Aprotinin was obtained from Bayer. Tetramethylbenzidine and horseradish peroxidase-conjugated rabbit anti-mouse antibody were from ICN Flow. Anti-thyroid stimulating hormone MAb (Code MA/732.162) and mouse anti-rat MAb 18.5 (ATCC No. TIB 216) were from Serono and the European Collection of Animal Cell Cultures, Porton Down, respectively. A 10 mg ml $^{-1}$ stock solution of capsaicin was prepared in 70% ethanol and further diluted with Krebs solution for addition to organ baths. All other drugs were diluted in Krebs solution.

Results

The four MABs C4.19, C4.6, R1.50, R2.73 bound CGRP by ELISA and RIA. All four MABs cross-reacted with the α and β forms of rat and human CGRP by ELISA. CGRP binding was inhibited by these antibodies in the receptor binding assay which served as the screening system for blocking MABs.

Rat α CGRP and rat β CGRP produced concentration-dependent inhibition of electrically stimulated contractions of

Figure 1 Effect of MAb C4.19 at various protein concentrations on the inhibitory effect of 10 nM rat α CGRP. The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses from 2 experiments are shown.

Figure 2 Concentration-response curves observed for rat α CGRP (a) or β CGRP (b) in the presence of PBS vehicle (●) or purified MAB C4.19 ascites at 60 μ g ml $^{-1}$ protein (▲). The effect of CGRP is expressed as % inhibition relative to stable pre-dosing contractions. Mean responses ($n = 6$ to 8) are plotted with standard error bars. (c) Shows simulated concentration-response curves to rat α CGRP in the presence of PBS vehicle control (○) or MAB C4.19 (Δ) at 60 μ g ml $^{-1}$ protein using estimated K_d of 1.9 nM and B_{max} of 79 nM.

Table 2 Estimated geometric mean EC_{50} (95% confidence interval) of drugs following pre-incubation of the electrically stimulated rat isolated vas deferens with MAB C4.19 or control (nm: $n = 6$ to 8)

Drug	EC_{50} in presence of MAB C4.19 at 60 μ g ml $^{-1}$	Control EC_{50} (PBS vehicle ^a or irrelevant MAB MAR 18.5 at 60 μ g ml $^{-1b}$)	Ratio of MAB: Control EC_{50} (95% confidence interval)
Rat α CGRP	52 (27 to 98)	2.3 (1.5 to 3.6) ^a	21.9 (13.3 to 36.2)
Rat α CGRP	38 (26 to 54)	4.4 (2.8 to 7.1) ^b	8.5 (7.5 to 9.6)
Rat β CGRP	32 (21 to 49)	2.4 (1.3 to 4.4) ^a	13.3 (6.2 to 28.4)
Isoprenaline	25 (7.9 to 77)	19 (6.4 to 58) ^a	1.3 (0.3 to 4.8)
Rat β -endorphin	56 (32 to 98)	44 (25 to 76) ^a	1.3 (1.0 to 1.6)

the rat isolated vas deferens. The two forms of CGRP were equipotent (Table 2). Effective blocking concentrations of MABs were found by assessing the effect of various concentrations of MABs on the response to 10 nM rat α CGRP. For example, it was found that 60 $\mu\text{g ml}^{-1}$ of purified MAB C4.19 ascites was required to block most of the response to 10 nM rat α CGRP (Figure 1). The effect of a single concentration of MAB on the concentration-response relationship of CGRP was then examined to assess whether an approximate ten fold $\log_{10}$ shift to the right could be obtained. None of the MABs tested had any effect on baseline contractions. Four out of 11 MABs tested, including MAB C4.19, C4.6 and R2.73 described above, shifted the concentration-response curve of CGRP to the right compared with vehicle or irrelevant MAB control. The use of RIA and a receptor binding assay as biochemical screens was generally successful in predicting blocking MABs. An interesting exception was MAB R1.50 which clearly showed the greatest activity in these assays and in the ELISA. Although raised in mice immunized with rat α CGRP, it was highly potent at blocking human α CGRP but blocked rat α CGRP poorly. The unexpected selective blocking activity of MAB R1.50 highlighted the need to perform positive control experiments with exogenous peptide of the form known to be present in the tissue. MAB R2.73 was effective at blocking rat α CGRP but not β CGRP despite cross-reaction with both forms of CGRP in the ELISA.

MAB C4.19 was found to block rat α CGRP (Figure 2a) and rat β CGRP (Figure 2b) effectively and was chosen for detailed studies at a protein concentration of 60 $\mu\text{g ml}^{-1}$ (8.5 μl of a stock solution of 21.1 mg ml^{-1} added to tissue bath). Time-course experiments showed that a 10 min incubation period with MAB C4.19 was sufficient for optimal immunoblockade (Table 1). The onset of effect of CGRP was more gradual in tissues incubated with MAB C4.19 than in tissues incubated with PBS vehicle or irrelevant MAB MAR 18.5 (Figure 3). The magnitude of the rightward shift (ratio of MAB: control EC_{50} ; Table 2) of the concentration-response curves for rat α CGRP and β CGRP due to MAB C4.19 were not significantly different. The dissociation constants (K_d) of MAB C4.19 for rat α CGRP and β CGRP were very similar (1.9 and 2.5 nM respectively). Irrelevant mouse MAB MAR 18.5 did not significantly affect the concentration-response relationship of α CGRP compared with PBS

vehicle (Table 2). The MAB was purified from ascites fluid by ammonium sulphate precipitation in the same manner as anti-CGRP MABs. MAB C4.19 had no pharmacologically significant effect on the concentration-response relationship of isoprenaline, rat β -endorphin or somatostatin (Table 2, Figure 4).

The pharmacological response to CGRP in the presence of MAB C4.19 could be predicted when the dissociation constant and concentration of binding sites of the antibody were known. Comparison of experimental and computer simulated data showed good agreement for EC_{50} and E_{max} of CGRP in the presence of MAB C4.19 (Figure 2c). The EC_{50} derived from simulated data was 44 nM. This result was close to the mean EC_{50} of 52 nM (95% confidence interval 27 to 98 nM) estimated from data observed in the presence of MAB C4.19. However, the steepness factor (N) of the theoretical concentration-response curve in the presence of C4.19, 1.81, was

Figure 4 Concentration-response curves observed for isoprenaline (a), rat β -endorphin (b) and somatostatin (c) in the presence of PBS vehicle (●) or MAB C4.19 at 60 $\mu\text{g ml}^{-1}$ protein (▲). Mean responses ($n = 6$) are plotted $\pm$ s.e.mean.

Figure 3 Original traces showing the inhibitory effect of rat α CGRP on electrically stimulated contractions of the rat isolated vas deferens in the presence of irrelevant control MAB MAR 18.5 (a) or MAB C4.19 at 60 $\mu\text{g ml}^{-1}$ protein (b). The examples are from an experiment using paired tissues from a single animal.

Table 3 Mean inhibitory effect of 1 μ M capsaicin (95% confidence interval) in the presence of MAb C4.19 or control ($n = 6$ to 8)

% inhibition in presence of MAb C4.19 at 60 μ g ml ⁻¹	Control % inhibition (PBS vehicle ^a or irrelevant anti-TSH MAb at 60 μ g ml ^{-1b})	Mean MAb-Control difference (95% confidence interval)
26.0 (15.2 to 36.8)	60.8 (51.8 to 69.9)*	- 34.8 (- 22.6 to - 47.0)*
36.1 (16.6 to 55.5)	67.0 (50.0 to 83.9) ^b	- 30.9 (- 14.3 to - 47.5)**

* $P = 0.0003$; ** $P = 0.005$

Inhibition of electrically stimulated contractions of the rat isolated vas deferens was expressed as % inhibition relative to stable contractions recorded immediately before addition of capsaicin.

Figure 5 Original traces showing the inhibitory effect of 1 μ M capsaicin on the electrically stimulated contractions of the rat isolated vas deferens in the presence of irrelevant control MAb directed against thyroid-stimulating hormone (a) or MAb C4.19 at 60 μ g ml⁻¹ protein (b and c). The degree of immunoblockade was variable, with partial (b) or almost complete (c) blockade of the capsaicin effect observed in different tissues compared with control.

significantly higher than the steepness factor of 1.12 (95% confidence interval 0.75 to 1.51) estimated from experimental data.

Capsaicin inhibited the electrically stimulated contractions of the isolated vas deferens (Figure 5, Table 3). The response to 1 μ M capsaicin was completely desensitized on second administration to tissues. This suggested that the response to capsaicin was due to a specific action on sensory nerves (Maggi *et al.*, 1987; Maggi & Meli, 1988). The effect of capsaicin was significantly attenuated by a 45 min pre-incubation with MAb C4.19. Time-course experiments showed that an antibody incubation period of 45 min produced optimal immunoblockade (Table 1). The peak effect of capsaicin was observed rapidly in the presence of vehicle, followed by a gradual recovery. The time to peak effect was

slower and recovery more gradual in the presence of MAb C4.19. Blockade was evident when the response in every pair of tissue obtained from the same animal was compared. The blockade of the effect of capsaicin by MAb C4.19 was also significant compared with an irrelevant mouse anti-TSH MAb (Table 3). The mean responses to capsaicin in the presence of MAb C4.19 or control were in reasonable agreement with the magnitude of immunoblockade of exogenous CGRP responses.

Discussion

We have identified several MABs which block a biological effect of CGRP. The effects of MAb C4.19 have been characterized in detail, with particular reference to specificity of immunoblockade. Good agreement of observed and predicted immunoblockade of exogenous CGRP with MAb C4.19 provided the basis for attempts to block endogenous CGRP released by capsaicin. Our findings suggest that endogenous CGRP released from primary afferent neurones is partly responsible for inhibition of nerve-mediated contractions of the rat vas deferens.

Immunoblockade has been used to investigate the role of endogenous CGRP in mediating the effect of capsaicin in the rat vas deferens (Maggi *et al.*, 1987). However, a rabbit antiserum was used, the blockade of exogenous rat β CGRP was not characterized, and no attempt was made to model the dynamics of immunoblockade. The low dilution of antiserum (1 in 25) required for immunoblockade in this study raised issues of specificity. The authors proposed immunoblockade as a pharmacological tool to study the possible physiological roles of CGRP as a neurotransmitter. We have performed a further evaluation of immunoblockade as an experimental tool.

The binding of an antibody to a peptide does not always result in blockade of biological activity. Indeed, some workers have reported potentiation of the biological effects of peptides by anti-peptide antibodies (Tilders *et al.*, 1990; Shaw *et al.*, 1992). This was not observed in the present investigation. Our pharmacological screening studies with a panel of anti-CGRP MABs allowed selection of those that blocked both species of rat CGRP effectively. A MAB such as C4.19 which equipotently blocks the biological activity for both forms is likely to be more generally useful than MABs which are selective for either form (e.g. MAB R2.73). It is unclear at present whether α CGRP or β CGRP is the major form found in the vas deferens. α CGRP appears to be the major form found in peripheral sensory nerves whereas β CGRP appears to predominate in enteric nerves (Mulder *et al.*, 1988; Sternini & Anderson, 1992). However, both forms of CGRP are expressed in peripheral sensory nerves innervating many tissues (Noguchi *et al.*, 1990; Sternini & Anderson, 1992).

The time-course experiments showed that due consideration must be paid to antibody incubation periods. However, a distinction should be drawn between requirements for the blockade of exogenous CGRP added to the tissue bath and endogenous CGRP released into the synaptic cleft compart-

ment. Antibodies which are effective in immunoblockade should achieve maximum binding of antigen within a time frame of seconds (van Oers & Tilders, 1991). Therefore it was not surprising that incubation of MAb C4.19 for longer than 10 min produced no enhancement of blockade of exogenous CGRP. No difference is expected because 10 min should be sufficient for the antibody to distribute homogeneously within the tissue bath and antibody concentration did not change with time. In contrast, an incubation time of 10 min was clearly inadequate to achieve blockade of the capsaicin response, indicating that more time must be allowed for the MAb C4.19 to diffuse into the synaptic cleft. The results of the time-course experiment suggested that the concentration of the antibody had reached equilibrium in the synaptic cleft after 45 min since incubation with MAb C4.19 for 90 min did not enhance blockade of the capsaicin response.

The use of MAbs does not obviate the need to demonstrate specificity. The very specificity of an MAb could result in cross-reaction with other peptides or proteins bearing the same epitope. The potential non-specific binding of the MAb C4.19 to CGRP was characterized by controlled experiments with a MAb directed against an irrelevant epitope (MAb MAR 18.5). The potential of the anti-CGRP MAb C4.19 to cross-react with other drugs and peptides producing similar pharmacological responses was assessed by experiments with isoprenaline, rat β -endorphin and somatostatin. Similarly, a non-specific blockade of endogenous CGRP released by capsaicin was excluded by an experiment using an irrelevant anti-peptide (TSH) MAb as a control. We were also able to confirm the specificity of MAb C4.19 in immunocytochemistry experiments. Pre-incubation of the MAb C4.19 with 1 μ M rat α CGRP for 90 min at room temperature completely abolished immunostaining in rat spinal cord sections (K. Tan, unpublished observations).

Apparent parallel rightward shifts in the CGRP concentration-response curves were observed in the presence of MAb C4.19. The magnitude of the rightward shift was predicted well by the binding and pharmacodynamic models. However, the theoretical curve was steeper because of the non-linear relationship between total and bound concentration of CGRP predicted by the antibody binding model. One reason for the discrepancy between observed data and theoretical prediction might be experimental error. Another explanation is the assumption of equilibrium conditions for antibody binding prior to consideration of receptor binding. However, the antibody has to compete with receptor for binding, initially under non-equilibrium conditions, when CGRP is added to the tissue bath. A steady-state, corresponding to stable pharmacodynamic response observed, is eventually achieved. Under non-saturating conditions, the proportion of CGRP bound to antibody or receptor is likely to depend on the relative on- and off-rates of antibody and receptor binding and the relative concentrations of antibody and receptor. Modelling of the competitive binding of CGRP

to antibody or receptor clearly requires more complex mathematical treatment. However, the present analysis has shown that the assumption of an equilibrium antibody binding model is sufficient to explain the magnitude of the rightward shift in the CGRP concentration-response relationship in the presence of MAb C4.19. It is interesting to note that a steeper non-parallel rightward shift of the CGRP concentration-response relationship, similar to the one simulated here, has been observed in the presence of an anti-CGRP antiserum in the guinea-pig isolated bladder base (Maggi *et al.*, 1988). However, a near-parallel shift of the CGRP concentration-response relationship was observed with the antiserum in the rat isolated vas deferens preparation (Maggi *et al.*, 1987).

Immunoblockade should be considered as a complementary tool to immunocytochemical, electrophysiological and pharmacological studies using receptor antagonists. CGRP-like immunoreactive outflow from the rat vas deferens is increased 4.5 fold by 10 μ M capsaicin (Santicoli *et al.*, 1988). This effect was desensitized on second application of capsaicin. Desensitization is a feature of the specific action of capsaicin on sensory nerves (Maggi & Meli, 1988). CGRP immunoreactive nerves are found around the deferential artery, in the smooth muscle layer and in the lamina propria (Goto *et al.*, 1978). Pre-incubation with capsaicin *in vitro* causes disappearance of CGRP-like immunostaining in the rat vas deferens (Saito *et al.*, 1987). The capsaicin antagonist, capsazepine, blocks the effect of capsaicin on the electrically-evoked contractions of rat isolated vas deferens but not the inhibitory effect of CGRP (Maggi *et al.*, 1993).

Several lines of evidence point to a direct effect of CGRP on smooth muscle rather than a prejunctional effect on sympathetic nerves. Rat α CGRP does not alter the uptake or release of tritiated noradrenaline in mouse vas deferens (Al-Kazwini *et al.*, 1987). In electrophysiological studies of transmural nerve stimulation in the rat isolated vas deferens, CGRP did not affect the amplitude of excitatory junction potentials which are associated with nerve excitation or transmitter release. In contrast, CGRP inhibited the contraction of rat vas deferens induced by direct stimulation of smooth muscles (Goto *et al.*, 1987). The C-terminal fragment human α CGRP (8–37) blocked the effect produced by a low concentration of capsaicin (30 nM) in the rat isolated vas deferens (Maggi *et al.*, 1991). The results of our immunoblockade experiments, together with the complementary evidence reviewed, provide strong evidence that CGRP partly mediates the efferent function of capsaicin-sensitive primary afferent neurons in the rat vas deferens.

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