Br. J. Pharmacol. (1989), **96**, 823–828

The relaxant properties of human calcitonin gene-related peptide on vascular and extravascular (capsular) smooth muscle of the isolated blood-perfused spleen of the anaesthetized dog

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1 The 37 amino acid human calcitonin gene-related peptide (CGRP), was injected intra-arterially into the isolated, blood perfused spleen of the dog.

2 The only vascular response observed to CGRP, once threshold had been reached (10–20 fmol), was a dose-dependent splenic arterial vasodilatation.

3 The mean intra-arterial bolus dose of CGRP to reduce the splenic arterial vascular resistance by 50% of maximum response was 0.52 ± 0.12 pmol. This value was significantly lower than the ED_{50} for the non-selective β-adrenoceptor agonist isoprenaline ($P < 0.01$) in the same experiments. CGRP is the most potent splenic vasodilator yet tested.

4 The mean maximum vasodilator response to CGRP was significantly less ($P < 0.01$) than that achieved with isoprenaline.

5 The time course of the splenic arterial vascular response to CGRP was substantially longer than that to isoprenaline.

6 The splenic vasodilator response to CGRP was not altered by the prior administration of the selective β_2-adrenoceptor antagonist, ICI 118,551.

7 At all doses of CGRP that caused splenic vasodilatation there were substantial increases in spleen volume. The time course of the response and slope of the regression line suggested an active capsular relaxation component.

8 In view of its location within the spleen and high molar potency, CGRP may be considered as a potential factor in the local control of the circulation through the spleen.

Introduction

A consequence of the alternative processing of RNA from the calcitonin gene within neural tissue is a mRNA which codes for a 37 amino acid peptide termed calcitonin gene-related peptide (CGRP). CGRP-like immunoreactivity has been identified in nerves supplying many tissues including the heart, systemic blood vessels and gastrointestinal tract (Mulderry *et al.*, 1985a,b). CGRP is a potent relaxant of isolated preparations of vascular smooth muscle (Hanko *et al.*, 1985; Uddman *et al.*, 1986) and causes peripheral vasodilatation and hypotension when infused intravenously into man (Franco-Cereceda *et al.*, 1987a) or into spontaneously hypertensive rats (Lappe *et al.*, 1987). In many vascular preparations its vasodilator properties have been shown to be dependent upon the presence of an intact endothelial lining (Brain *et al.*, 1985; Grace *et al.*, 1987). However, the actions of CGRP on extravascular smooth muscle are less well defined.

The aim of the present series of experiments was to establish the primary actions of human CGRP on the vascular and extravascular (capsular) smooth muscle of a single blood perfused organ, the spleen of the dog. This is particularly relevant since CGRP binding sites have been reported in the red pulp of the rat spleen (Sigrist *et al.*, 1986) although the precise histological location has not been established. In addition, the molar potency of CGRP was

assessed and its relative potency to other peptides was examined so that any potential physiological role could be evaluated.

A preliminary account of these results has been published (Withrington, 1986).

Methods

The experiments were performed on 4 dogs (mean weight 25.5 ± 0.54 kg; range 24.5–27.0 kg) anaesthetized with an intravenous mixture of chloralose and urethane (50 and 500 mg kg^{-1} respectively) after induction with methohexitone sodium (Brietal, 6.0 mg kg^{-1}).

The surgical procedures for isolation and perfusion of the spleen and continuous recording of splenic arterial blood, perfusion pressure and changes in spleen volume were as described previously (Corder *et al.*, 1987). Essentially, after careful preparation and isolation of the major splenic blood vessels the spleen was removed from the donor and, after the splenic artery and vein had been cannulated, placed in a perspex plethysmograph. The spleen was then perfused with arterial blood derived from the femoral circuit whilst the splenic venous blood drained passively into the femoral vein. An electromagnetic flow probe and strain gauge transducer was incorporated into the splenic arterial circuit to measure splenic arterial mean blood flow (SABF) and splenic arterial mean perfusion pressure (SAPP) respectively. These averaged signals were fed into an IBM personal computer programmed to calculate absolute values and changes in splenic arterial vascular resistance (SAVR). The plethysmograph was filled with liquid paraffin and sealed; the displacement of liquid paraffin from a weighed reservoir, connected to the plethysmograph contents, by changes in spleen volume allowed an accurate measurement of alterations in organ size (dSV) to be evaluated. A 'T' piece inserted into the arterial circuit permitted the close arterial administration of low doses of vasoactive substances. The majority of the dose-response relationship of the splenic smooth muscle systems could therefore be constructed without evoking general cardiovascular responses in the donor animal and altering the conditions of the perfusion. The temperature of the spleen and of the donor dog was maintained at approx 37°C. Hourly arterial blood samples allowed the monitoring of arterial blood P_{CO_2},P_{O_2} and pH and, if appropriate, correction to normal values was made by the intravenous infusion of 1 mmol $NaHCO_3$. The flow probe was calibrated with whole blood at the end of each experiment at which stage the spleen was also weighed after clamping of the artery and vein.

Drugs and vehicles

Isoprenaline and CGRP were injected directly into the splenic arterial line through a 'T' piece and were then washed in with saline (0.9% w/v NaCl solution) to give a constant injection volume of 2.0 ml. Human α-CGRP was purchased from Bachem and made up in sterile saline which contained human serum albumin (10 mg ml^{-1}; Elstree) and Polypep (2.5 mg ml^{-1}; low viscosity; Sigma). Close-arterial injection of this vehicle produced no change in either splenic arterial blood flow or spleen volume. The human serum albumin and Polypep were used to reduce non-specific binding of CGRP on to plastic surfaces. Isoprenaline hydrochloride (Pharmax Limited) was diluted immediately prior to injection in normal saline. All solutions were stored in ice. ICI 118,551 (erythro-DL-1-(7-methylindan-4-yloxyl) 3-isopropylamino-butan-2-ol) was prepared in saline at a concentration of 1.0 mg ml^{-1}.

Statistics

Results are presented as means ± standard errors of mean (s.e.mean). Tests for significance refer to Student's *t* test.

Results

Control values

The spleen weight was 288 ± 50.3 g representing 1.12 ± 0.18% of the body weight. The initial splenic arterial mean blood flow was 153 ± 16 ml min^{-1}. The initial splenic arterial mean perfusion pressure was 140 ± 11 mmHg giving a calculated splenic arterial mean vascular resistance of 0.95 ± 0.10 mmHg ml^{-1} min. These initial control values approximate to those recently reported from this laboratory for similar perfused spleen preparations (Corder *et al.*, 1987).

Splenic vascular smooth muscle responses

Isoprenaline Isoprenaline (Iso) has been shown previously to produce, following close arterial bolus injection, a characteristic splenic vascular response consisting of an increase in blood flow of rapid onset and short duration (Figure 1). This increase in splenic arterial blood flow represents, at constant arterial perfusion pressure, a reduction in splenic arterial vascular resistance and vasodilatation. This splenic vasodilatation is predominantly due to β_2-adrenoceptor activation since it is diminished by prior administration of the selective β_2-adrenoceptor antagonist ICI 118,551 (Corder & Withrington,

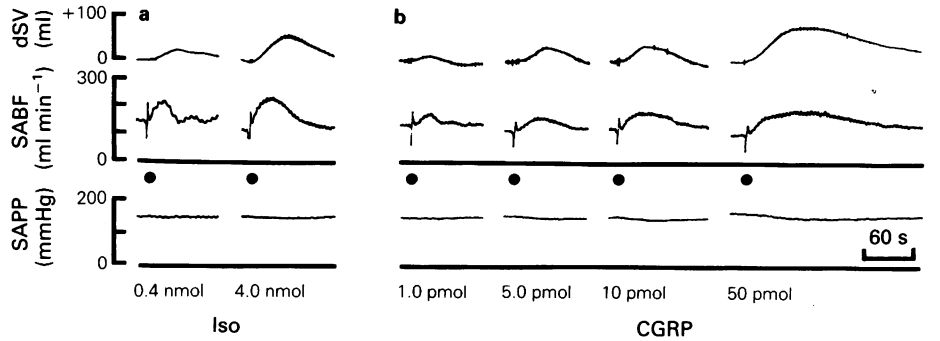

Figure 1 Responses of splenic vascular and capsular smooth muscle to intra-arterial bolus injections of isoprenaline and calcitonin gene-related peptide (CGRP). Records are from the top; dSV, increase in spleen volume; SABF, splenic arterial blood flow; SAPP, splenic arterial perfusion pressure. The two panels (a) and (b) illustrate the changes in response to intra-arterial injections of 2 doses of isoprenaline (Iso, 0.4 and 4.0 nmol) and 4 doses of CGRP (1.0, 5.0, 10 and 50 pmol). Spleen 152 g. Note the increased duration of the CGRP responses and also the greater increases in spleen volume to CGRP for approximately the same maximum vasodilator response (compare 0.4 nmol isoprenaline and 10 pmol CGRP).

1988). In the present series of experiments isoprenaline was injected over the dose-range 0.5 pmol–10 nmol and the only vascular response observed, once the threshold dose (usually 5–10 pmol) had been reached, was splenic vasodilatation. The mean maximum vasodilator effect of isoprenaline was to increase splenic arterial mean blood flow by 82.3 ± 7.4% of the control flow before the injection. The mean ED_{50}, the mean molar dose of isoprenaline to reduce the splenic arterial vascular resistance by 50% of the maximum in each experiment, was 109 ± 26.8 pmol.

Calcitonin gene-related peptide The peptide CGRP was injected as a bolus directly into the splenic artery over the dose range 1.0 fmol to 100 pmol. The only vascular response to CGRP, once the threshold (10–20 fmol) had been reached, was an increase in splenic arterial blood flow in the absence of any changes in the systemic perfusion pressure. This vasodilator response was graded with dose (Figure 1) and, characteristically, had a longer duration of action than isoprenaline when responses were compared which reached the same maximum change. Vasoconstriction was never observed. However, in any individual experiment, the maximum vasodilator effect to CGRP was less than that to isoprenaline. In this series the mean maximum increase in blood flow to CGRP was 53.8 ± 3.01% of control; this was significantly less ($P < 0.01$) than the mean maximum, in the same experiments to isoprenaline. The mean maximum increase in blood flow to CGRP was 67.3 ± 8.4% of the maximum observed to isoprenaline in the same experiments. In this series the mean molar dose-response curve relating the vasodilator activity, i.e. the reduction in splenic arterial vascular resistance, to the molar intra-arterial bolus dose of CGRP lay well to the left of the dose-response curve for isoprenaline (Figure 2). The mean ED_{50} for CGRP was 0.52 ± 0.12 pmol; a value highly significantly less ($P < 0.01$) than the ED_{50} for isoprenaline.

There was no obvious change in the splenic vasodilator response to CGRP or in the position of its dose-response curve following the i.v. administration

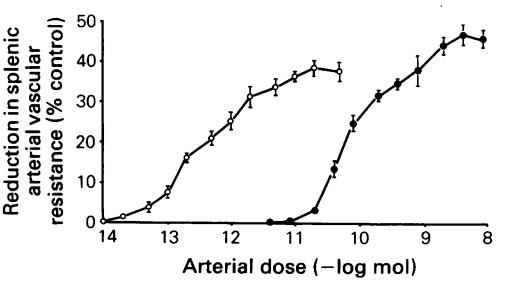

Figure 2 The relationship obtained in 4 separate isolated blood-perfused preparations, between the vasodilator activity (reduction in splenic arterial vascular resistance as a percentage of the preinjection control) and the intra-arterial molar dose of isoprenaline (●) and calcitonin gene-related peptide (CGRP, ○). The points represent the means of between 4 and 6 observations with the bars indicating the s.e.mean. There is a significant difference in the position of the curves within the co-ordinates and between the maximum change induced by the two substances.

of a dose of ICI 118,551, a selective β_2-adrenoceptor antagonist, which previous observations (Corder & Withrington, 1988) have shown to reduce significantly the splenic vasodilator response to intra-arterial injections of isoprenaline.

Splenic extravascular (capsular) smooth muscle responses

Accompanying the graded splenic arterial vasodilator responses to both intra-arterial isoprenaline and CGRP were increases in spleen volume (Figure 1). These were analysed to assess the quantitative relationship to the concomitant increases in splenic arterial blood flow. A distinction may be drawn between an active relaxation of the splenic capsule and an increase in volume that results passively from the primary relaxation of vascular smooth muscle leading to vasodilatation.

Isoprenaline There was a high correlation (0.94) between the maximum vasodilator action of isoprenaline and the maximum increase in spleen volume to any bolus dose. The slope of the regression line (Figure 3) was significantly different from zero (0.50; $P < 0.05$) although there was no significant intercept on either axis. That is, no increase in spleen volume occurred in the absence of an increase in splenic arterial blood flow. This result confirms, in the present series of experiments, previous observations (Corder & Withrington, 1988), that β-adrenoceptors are not present in the splenic capsule to induce active relaxation.

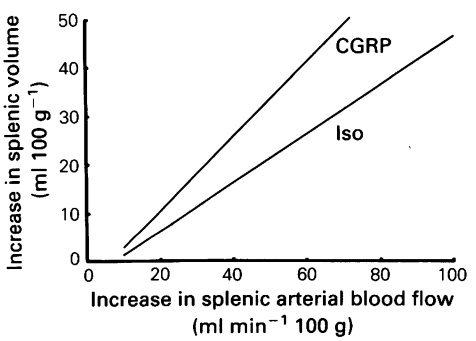

Figure 3 Computer plotted regression lines relating the increase in spleen volume (per 100 g) to the increase in splenic arterial blood flow (per 100 g) for close arterial bolus injections of isoprenaline (Iso, 45 points) and calcitonin gene-related peptide (CGRP, 40 points) in the 4 isolated, blood-perfused spleen preparations. The slopes of both lines are significantly different from zero and from each other. None of the intercepts are significantly different from zero. Individual points not included for clarity.

Calcitonin gene-related peptide Again there was a high positive correlation (0.90) between the increase in spleen volume/100 g weight and the increase in splenic arterial blood flow/100 g weight due to CGRP injections. However the slope of the regression line (0.75) was significantly greater than zero and also significantly greater than that for isoprenaline ($P < 0.01$; 0.05 respectively). These results suggest a direct relaxant action of CGRP on splenic capsular smooth muscle. There was no significant intercept on either axis, indicating the close relationship between the two parameters flow and volume for the peptide (Figure 3).

Discussion

The present experiments on the isolated, blood-perfused spleen of the anaesthetized dog reveal that the 37 amino acid calcitonin gene-related peptide (CGRP) is one of the most potent vasodilator substances yet examined in this particular organ. The mean intra-arterial bolus dose required to reduce splenic arterial vascular resistance to 50% of the control value was found to be 0.52 pmol; in similar experiments the ED_{50} for vasoactive intestinal peptide (VIP), an established vasodilator neuropeptide, was 9.9 pmol (Corder & Withrington, 1988). The two values are significantly different ($P < 0.05$). These observations in the spleen confirm the high vasodilator potency of CGRP reported for other vascular preparations either blood-perfused (dog liver, Withrington, 1987; rat renal, mesenteric and hindquarter vascular, Lappe *et al.*, 1987), *in vivo* (skin, Brain *et al.*, 1985) or isolated preparations (rat and rabbit mesenteric vasculature perfused with Krebs solution containing noradrenaline, Marshall *et al.*, 1986). In some isolated preparations e.g. rat aortic rings, the relaxant potency of the peptide has been found to be dependent upon an intact endothelium (Brain *et al.*, 1985; Grace *et al.*, 1987) the removal of which significantly lowers the sensitivity of the preparation to CGRP. However, this is not true of bovine isolated coronary vessels (Greenberg *et al.*, 1987), suggesting the peptide acts directly, at this site, on the vascular smooth muscle.

There is little available information on the histochemical distribution of CGRP-like immunoreactivity within the spleen. In the guinea-pig CGRP-LI was found in the gastroepiloic and splenic arteries (Uddman *et al.*, 1986) although very few fibres were found in the small arteries within the splenic parenchyma. Autoradiography of the rat spleen has revealed [^{125}I]-iodo-CGRP binding associated with the red pulp, but not white pulp (Sigrist *et al.*, 1986). The distribution within other tissues of the cardiovascular system (Mulderry *et al.*, 1985b) and gastro-

intestinal tract (Mulderry *et al.*, 1985a) would suggest that the peptide is located within the rich sensory innervation of the spleen. The role of the sensory innervation of visceral structures and the major blood vessels has been the subject of much constructive discussion recently since many potent vasoactive peptides, in addition to CGRP, appear to be located within defined afferent terminals. Substance P (SP), a very potent vasodilator is, in many tissues including the spleen (Franco-Cereceda *et al.*, 1987b), co-located with CGRP within terminal afferent neurones. It is necessary to reconsider the role of the sensory innervation to provide a more local function for afferent terminals in terms of a fine sensing of the environment within an organ or tissue. If appropriate, regulation of local blood flow could be achieved by the release of vasoactive peptides within the same neurone by a mechanism similar to the 'axon-reflex'. Such a local mechanism would operate in addition to the conventional projection of afferent information to the CNS.

In the present experiments the vasodilator activity of CGRP was, unlike that of isoprenaline, unaffected by the prior administration of the selective β_2-adrenoceptor antagonist ICI 118,551. This confirms, in the spleen vasculature, previous observations in other vascular beds that activation of smooth muscle β-adrenoceptors is not involved in the relaxant properties of CGRP.

There is conflicting information available about the actions of CGRP on extravascular smooth muscle. In human isolated bronchi CGRP causes dose-dependent contraction of smooth muscle (Palmer *et al.*, 1987). The extravascular smooth muscle of the dog's spleen forms the capsular and trabecular system by means of which the spleen exerts a significant capacitative function (Davies & Withrington, 1973). In the present experiments substantial increases in spleen volume accompanied the vasodilator responses to intra-arterial CGRP and there was a close quantitative relationship between the maximum increase in splenic arterial blood flow and the maximum increase in spleen volume. The similarity and interdependence of these two splenic responses may suggest a causal relationship in that the increase in volume to CGRP is the passive result of a primary active increase in arterial blood flow similar to the mechanisms proposed to occur to isoprenaline. However the increased slope of the regression line of CGRP compared to isoprenaline and vasoactive intestinal peptide (Corder & Withrington, 1988) indicates the involvement of another mechanism. This may be the active relaxation of the splenic capsule following activation of CGRP receptors on the smooth muscle cells forming the enveloping structure. It is difficult to be more precise in analysing this component since any spleen volume change represents the integral of the instantaneous arterial-venous flow difference. In the present experiments, splenic venous flow was not continuously measured and, with electromagnetic flow probes, would be difficult since the calibrations of these instruments are haematocrit-sensitive. In the dog the splenic venous haemotocrit may vary considerably from over 80% during contraction to below 30% during splenic enlargement, for example, as the result of splenic venous pressure elevation which results in selective erythrocyte sequestration (Withrington *et al.*, 1980). Nevertheless these observations confirm in the whole organ the previous accounts (Sigrist *et al.*, 1986) of an active relaxation of rat spleen strips to CGRP although only when contracted by prior addition of noradrenaline.

CGRP appears to possess unique properties of a potent vasodilator together with active relaxation of splenic capsular smooth muscle. This profile of vasodilatation and active increase in splenic capacitance is in contrast to other neuropeptides such as NPY and VIP. Its presence within the sensory innervation to the spleen indicates that its function, in contrast to the other peptides, may be to modify splenic microcirculation and cell sequestration functions as the results of local factors.

The author sincerely thanks Mr Ray Croxton for his expertise during the experiments and Miss Carol Brown for her efficiency and help in the preparation of the manuscript. Many thanks are also due to Miss Karin Fernandes and Mr. Gary Davis for preparation and photography of the figures.

The study was supported by the Joint Research Board of St. Bartholomew's Hospital.

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(Received August 9, 1988
Revised November 30, 1988
Accepted December 5, 1988)