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Calcitonin gene-related peptide increases blood flow and potentiates plasma protein extravasation in the rat knee joint

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1 The effects of calcitonin gene-related peptide (CGRP) and other vasoactive mediators of inflammation on blood flow in the synovial vessels and plasma protein extravasation into the knee (femoro-tibial) joint of the pentobarbitone-anaesthetized rat were measured.

2 Changes in synovial blood flow were estimated by ^{133}xenon clearance from the synovial cavity. CGRP (0.1 pmol and 10 pmol) and prostaglandin E_1 (PGE_1; 3 pmol and 300 pmol) significantly increased clearance from the knee joint measured 5 min after intra-articular injection. Substance P (10 pmol) had no effect on synovial blood flow.

3 Intra-articular perfusion of the rat knee with CGRP at concentrations up to 0.1 mM, or PGE_1 at concentrations up to 10 μM, did not increase plasma extravasation into the synovial cavity measured by accumulation of intravenously injected ^{125}I-albumin in the perfusate.

4 Plasma extravasation into the knee was significantly increased by infusion of bradykinin (0.1 μM), 5-hydroxytryptamine (1 μM) and histamine (0.1 mM), compared with the contralateral joints in the same animals which were perfused with Tyrode solution.

5 Perfusion of the knee joint with substance P did not specifically induce ^{125}I-labelled albumin accumulation in the synovial cavity even at doses that had systemic effects as observed by marked plasma extravasation into other tissues.

6 The increase in plasma extravasation induced by histamine (0.1 mM) was potentiated by co-infusion with CGRP (0.1 μM) and PGE_1 (3 μM). However the response to a submaximal dose (0.1 μM) of bradykinin, which induced similar plasma extravasation to histamine (0.1 mM), was not increased by co-infusion with CGRP or PGE_1.

7 These results show that CGRP is a potent vasodilator in the rat knee. CGRP released from sensory nerves may act synergistically with mediators of increased vascular permeability to modify the inflammatory response in this site.

Keywords: CGRP; calcitonin gene-related peptide; knee joint, synovium; blood flow; plasma protein extravasation; substance P

Introduction

Small diameter primary afferent, or C-fibre, nerves containing the vasoactive peptides, calcitonin gene-related peptide (CGRP) and substance P, have been identified in rat synovium, (Konttinen *et al.*, 1990). Release of peptides by antidromic stimulation may contribute to inflammation by increasing blood flow and vascular permeability. The nerves also have a close anatomical relationship to mast cells in normal tissue (Hukkanen *et al.*, 1991) and may modulate mast cell function in this site.

Extravasation of plasma protein into the synovial cavity or joint capsule is a commonly used marker of acute inflammation in the joint although the neural mechanisms and mediators which control this process are not well defined and may be species-dependent. In the cat knee joint, Ferrell & Russell (1986) showed plasma extravasation was decreased by stimulation of sympathetic efferents and increased by stimulation of C-fibre afferent nerves. Based on intra-articular perfusion of mediators in surgically or chemically sympathectamised rats, the possibility of an interaction between the sympathetic nerves and C-fibre nerves has been proposed by Coderre *et al.* (1989). They consider C-fibre evoked plasma extravasation to be at least partly dependent on intact sympathetic postganglionic nerves and mast cells. Specific roles for individual neuropeptides in the joint have not been defined, but there is some evidence that substance P may exacerbate adjuvant arthritis (Colpaert *et al.*, 1983; Weihe *et al.*, 1988). Elevated levels of substance P have also been found in rabbit knee joints after injection of inflammatory cytokines (O'Byrne *et al.*, 1990). A recent study showed intra-articular injection of substance P at high doses increased vascular permeability in the rat joint by releasing histamine and 5-hydroxytryptamine (5-HT) from mast cells and possibly by acting on vascular receptors (Lam & Ferrell, 1990).

CGRP is established as a potent and long-lasting vasodilator in the skin (Brain *et al.*, 1985). In this tissue CGRP also potentiates oedema formation by mediators of increased vascular permeability (Brain & Williams, 1985; 1989). The activity of CGRP has not been extensively studied in the joint although Kidd *et al.* (1990) have demonstrated modulation of vascular permeability changes in this site and Lam & Ferrell (1991) have shown a prolonged vasodilator response following topical application to the rat knee.

The aim of this study was to establish the effects of CGRP and other mediators of inflammation on synovial blood flow and plasma extravasation in the rat knee joint. Interactive effects of CGRP and two mediators of vascular permeability, histamine and bradykinin were also investigated. We have adapted and validated a ^{133}xenon clearance method, previously described in skin (Williams, 1979), for measuring synovial blood flow. Plasma extravasation was studied by a modification of the joint perfusion method described by Coderre *et al.* (1989).

Methods

Blood flow and oedema formation were measured in the knee joint of male Wistar rats (300–400 g) anaesthetized with 50 mg kg^{-1} pentobarbitone by intraperitoneal injection.

Measurement of blood flow changes by ^{133}xenon clearance

One ml of test agent in Tyrode solution or Tyrode solution alone (for control) was mixed with 10 MBq of ^{133}Xe and then 100 µl rapidly injected into the synovial cavity with a 30 G needle. The animals received Tyrode solution in one knee and test agent in the opposite joint so that each acted as its own control. Five minutes after completion of the intra-articular injections the animals were killed by anaesthetic overdose and the knee joints removed and counted for radioactivity. Changes in blood flow were calculated by comparing ^{133}Xe clearance over 5 min from both knees in the same animal.

Measurement of plasma extravasation by ^{125}I-albumin accumulation

Each rat received a mixture of Evans Blue (25 mg kg^{-1}) and ^{125}I-labelled human serum albumin (50 kBq) by injection via the tail vein. A 1 cm incision was made in the skin over the anterior aspect of both femoro-tibial joints. Two 27 G needles were placed in the synovial cavity approximately 3 mm apart and connected via flexible cannulae to a roller pump (Watson-Marlow). Tyrode solution was infused and withdrawn at a constant rate of 100 µl min^{-1} into both joints for 30 min. Placement of the needles within the synovial space was verified by lack of swelling of peri-articular tissues and a clear perfusate which could be continuously withdrawn at 100 µl min^{-1}. Mediators were then perfused for 4 min into one joint whilst the opposite joint acted as a control and was perfused with Tyrode solution. The solutions were left in the joint for a further 10 min after which perfusion was recommenced and 1 ml of perfusate collected from each joint. At the end of the experiment, blood (1 ml) was withdrawn by cardiac puncture and then the animal killed by anaesthetic overdose. Radioactivity was counted in 100 µl of plasma and in the 1 ml perfusate samples to quantify extravasation into the joint as µl of plasma. To measure potentiation of oedema, histamine or bradykinin were perfused with CGRP or prostaglandin E_1 (PGE_1) into one joint whilst histamine or bradykinin alone was perfused into the opposite joint.

^{125}I-labelled albumin accumulation was also measured in the skin of the hind paws in some experiments to assess systemic effects of test agents on vascular permeability. Immediately after death, approximately 100 mg of skin was removed from the dorsal aspect of each of the hind paws, weighed, counted for radioactivity and extravasation calculated as for the joint perfusates.

The effect of cyclo-oxygenase inhibition on plasma extravasation was investigated in animals treated with indomethacin (20 mg kg^{-1}, subcutaneously) or vehicle 30 min before the start of joint perfusion.

Statistical analysis

Results are expressed as mean ± s.e.mean and each data point represents one rat. The significance of the blood flow data was analyzed by Bonferroni's modified t test using the s.e. estimate for the analysis of variance to account for multiple comparisons to the control value. Student's paired t test was used to compare the treated and control limbs in the plasma extravasation studies.

Materials

The following drugs were used: histamine, 5-hydroxytryptamine, bradykinin, indomethacin, substance P, prostaglandin E_1 (PGE_1), all from Sigma Chemical Company, Poole, Dorset; human alpha calcitonin gene-related peptide, a gift from Dr U. Ney, Celltech, Slough, Berks; human endothelin-1 (ET-1) from Bachem Ltd, Saffron Walden, Essex. Each was dissolved in Tyrode solution except for PGE_1 and bradykinin, which were initially prepared in stock solutions of 1 mg ml^{-1} in absolute ethanol, and indomethacin which was dissolved in 5% NaH_2CO_3. ^{133}Xenon and ^{125}I-labelled human serum albumin (^{125}I-HSA) were obtained from Amersham International, Aylesbury, Bucks. The composition of the Tyrode solution was as follows (mM): NaCl 136.89, KCl 2.68, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.05, glucose 5.55.

Results

Blood flow

Changes in blood flow were calculated by comparing ^{133}Xe clearance in test joints with that observed in control joints of the same animal. In initial experiments (designed to test the validity of the assay) the difference in ^{133}Xe clearance between joints when both were injected with equal amounts of ^{133}Xe in 0.1 ml Tyrode solution was small (Figure 1). To establish whether a decrease in synovial blood flow could be detected, the response to the potent vasoconstrictor peptide endothelin-1 was measured. There was virtually no clearance (3.9 ± 6.0% of total radioactivity injected, mean ± s.d., $n = 3$) from the joint after injection of 100 pmol endothelin-1. In joints which received 10 pmol of endothelin-1 18.2 ± 22.7% of total radioactivity injected (mean ± s.d., $n = 3$) was cleared. CGRP and PGE_1 produced a dose-dependent increase in blood flow of up to 300% over the 5 min clearance period when compared with contralateral Tyrode injected knee (Figure 1). A significant effect was seen with a dose of 100 fmol CGRP but, by contrast, substance P did not increase blood flow at doses of 0.1 and 10 pmol.

Plasma extravasation

In animals where both joints were perfused with Tyrode solution there was minimal plasma extravasation into the synovial cavity (0.76 µl ± 0.22, mean ± s.e.mean, $n = 6$). Plasma extravasation into the treated joint was increased in a dose-dependent manner by bradykinin, histamine and 5-hydroxytryptamine (Figure 2a), but not by CGRP or PGE_1 (Figure 2b). Substance P induced plasma extravasation only at the highest dose tested (0.1 mM) when systemic effects, resulting in the death of 3 out of 8 animals, were also seen.

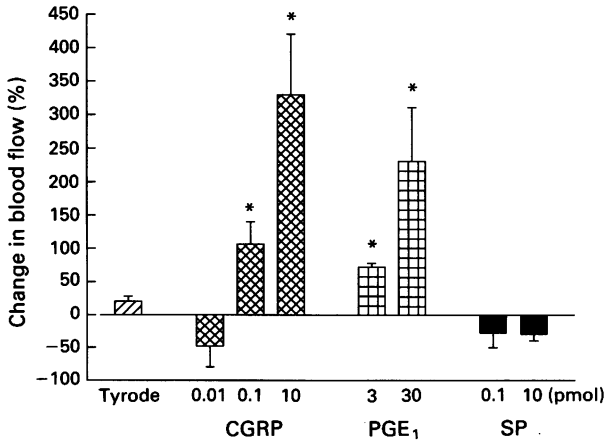

Figure 1 The effect of intra-articular injection of 0.1 ml of calcitonin gene-related peptide (CGRP), prostaglandin E_1 (PGE_1) or substance P (SP) on blood flow in the rat knee joint. Clearance of ^{133}Xe from test knees is compared, over 5 min, with the clearance of Tyrode alone (solid line at 0%) in the opposite knee of each rat ($n = 5$–8 animals). The hatched column represents the effect of Tyrode solution into the test joint. Results are mean values (s.e.mean indicated by vertical bars) and * indicates a significant change ($P < 0.05$, Bonferroni's t test) in blood flow compared with control.

At the highest doses of the mediators, plasma extravasation into the control joint was not increased by any mediator (Figure 3a). Infusion of 0.1 mM substance P, but none of the other mediators into the treated joint produced significant plasma extravasation into the skin of both the treated and control limbs (Figure 3b).

In further experiments interactions between the vasodilators (CGRP and PGE_1) and mediators of increased vascular permeability were studied. Plasma extravasation produced by submaximal doses of histamine and bradykinin is shown in Figure 4. The response to histamine was potentiated by co-perfusion with doses of CGRP and PGE_1 previously shown to have significant vasodilator activity in the ^{133}Xe clearance assay. Co-perfusion of the vasodilators with a submaximal dose of bradykinin, however, did not result in potentiation of oedema formation (Figure 4).

The response to a high dose of histamine (0.2 mM) appeared to be reduced in indomethacin-treated rats, presumably due to the inhibition of vasodilator prostaglandin production, but this was not significant ($P = 0.0593$, $n = 12$). Co-perfusion with CGRP (0.1 μM, $n = 7$) or PGE_1 (3 μM, $n = 4$) attenuated the inhibitory trend of indomethacin on histamine-induced plasma extravasation. Indomethacin pretreatment had no effect on the response to bradykinin (0.1 μM) or 5-HT (1 μM) in other experiments, (results not shown).

Discussion

These results show CGRP to be a potent vasodilator in the rat knee joint, as intra-articular injection of 100 fmol caused a significant increase in synovial blood flow. Although endogenous release of CGRP into rat joints has not been studied, our results show that physiological amounts of this peptide could modulate blood flow (a fundamental component of the inflammatory response), at this site. CGRP alone did not stimulate oedema formation in our model, however co-infusion of CGRP potentiated the effects of histamine, probably by increasing blood flow to leaky microvessels. Thus CGRP may be important in modulating oedema formation induced by amines released from activated mast cells. Synergy between CGRP and a range of mediators of increased vascular permeability have been demonstrated in skin (Brain & Williams, 1985; Gamse & Saria, 1985). There is also evidence that CGRP may also potentiate acute inflammation by increasing neutrophil accumulation induced by mediators that include interleukin-1, in skin (Buckley *et al.*, 1991a,b).

In our study bradykinin was the most potent mediator of plasma extravasation into the joint but the effects of a submaximal dose were not increased by the vasodilators, possibly because bradykinin alone was able to stimulate blood flow in addition to increasing vascular permeability. Interestingly, using a different protocol Kidd *et al.* (1990) were able to show potentiation of bradykinin by CGRP in the rat knee. In our study 5-HT was also a potent mediator of joint oedema and, if released in to the joint from mast cells, could contributed to acute inflammation in rodents. Sensory C-fibre

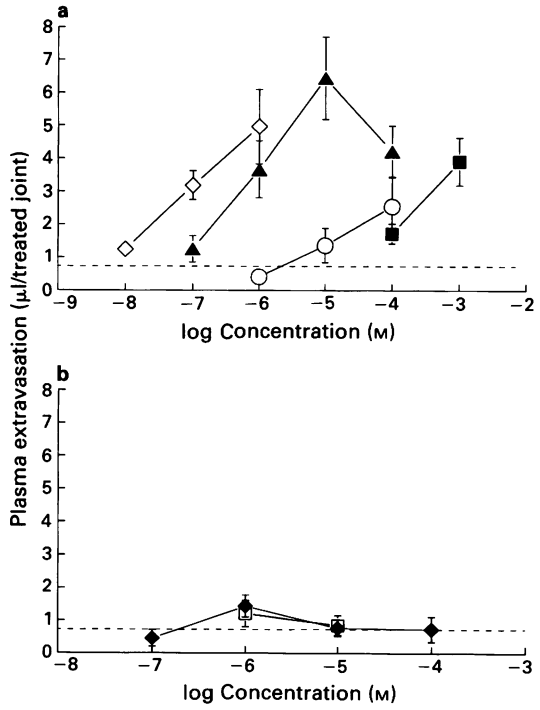

Figure 2 The effect of mediators on plasma extravasation into the rat knee. Plasma extravasation induced by increasing doses of bradykinin (◇), 5-hydroxytryptamine (▲), substance P (○) and histamine (■) is shown in (a) and by calcitonin gene-related peptide (◆) and prostaglandin E_1 (□) in (b) Plasma extravasation was measured by comparing the accumulation of intravenously injected ^{125}I-albumin in the joint perfusate to activity in plasma, as detailed in the methods section. The dashed line refers to plasma extravasation in joints infused with Tyrode solution alone. Results are mean values (s.e.mean indicted by vertical bars) of $n = 6-12$ rats.

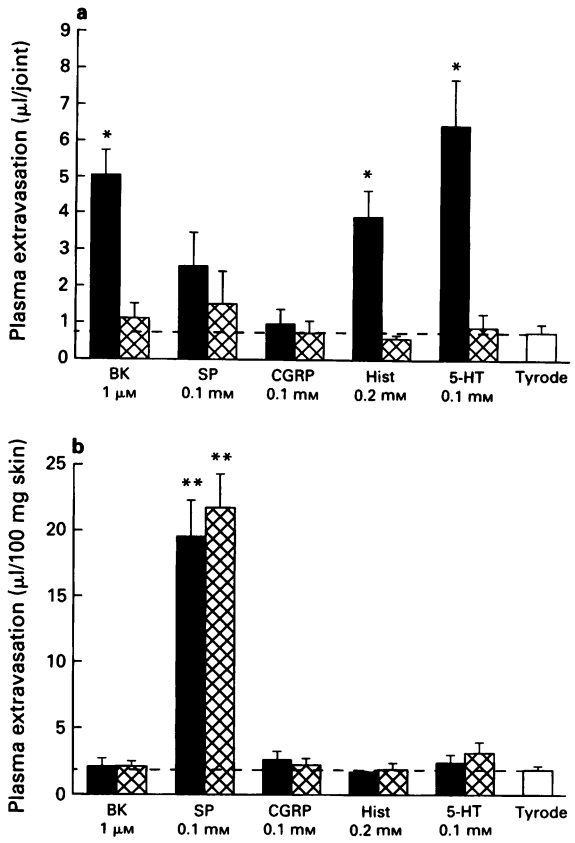

Figure 3 Effect of high doses of mediators on plasma extravasation into (a) knee joints and (b) skin of both hind paws. Results in (a) show plasma exudation induced by high doses of mediators infused into the treated knee (solid columns), whilst the control knee received Tyrode solution alone (cross-hatched columns). Plasma exudation into the skin in these experiments is shown in (b). The open column, and dashed line, refers to 3 rats which received Tyrode solution into both knees. BK: bradykinin; SP: substance P; CGRP: calcitonin gene-related peptide; Hist: histamine; 5-HT: 5-hydroxytryptamine. Results are the mean values (s.e.mean indicated by vertical bars) for $n = 6-10$ rats. A significant effect of mediator in injected knee compared to the opposite knee in the same animal is indicated *($P < 0.05$, paired t test). Significant extravasation into the skin compared to Tyrode solution is indicated **($P < 0.01$, unpaired t test).

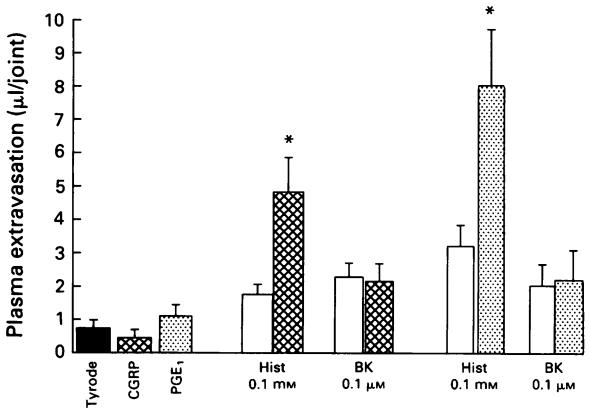

Figure 4 The effect of calcitonin gene-related peptide (CGRP) and prostaglandin E_1 (PGE_1) on plasma extravasation induced by histamine (Hist) and bradykinin (BK). Extravasation induced by Tyrode solution, CGRP (0.1 μM) and PGE_1 (3 μM) is shown by the solid, cross-hatched and stippled columns respectively. The response to histamine and BK alone is shown in the open columns. The effect of co-administration of CGRP and PGE_1 with histamine and BK is shown by the adjacent cross-hatched and striped columns. Results are mean values (s.e.mean indicated by vertical bars) for $n = 6-12$ rats. A significant potentiating effect of CGRP and PGE_1 compared to histamine alone in the opposite joint is indicated * ($P < 0.05$, paired t test.

nerves in the rat ankle are activated by 5-HT (Grubb *et al.*, 1988) and it is possible that some of the actions of both 5-HT and bradykinin may be related to release of neuropeptides, including CGRP. Alternatively it is possible that vasodilator prostanoids contribute to the responses, although this is not supported by the results of our study where indomethacin had no effect on bradykinin or 5-HT-induced plasma extravasation. Plasma extravasation induced by a high dose of histamine was attenuated by indomethacin (although this did not reach significance).

The importance of substance P in producing joint inflammation is difficult to assess. Lam & Ferrell (1989) showed that intra-articular injection of 20 μg substance P caused significant plasma extravasation into the joint capsule of the rat knee. In our study, minimal plasma extravasation was found in the joint even at doses sufficient to cause generalized plasma extravasation into most tissues, as demonstrated by accumulation of radioactivity in the skin. At lower concentrations, substance P had no significant effect on blood flow or plasma extravasation. O'Byrne and colleagues (1990) found levels of substance P up to 0.5 pmol in lavage fluid from rabbit knees after intra-articular injection of interleukin-1. Our results indicate that this amount would not contribute to oedema and we consider it unlikely that sufficient substance P can be released in the joint to cause local vasoactive effects. There is indirect evidence, however, for a pro-inflammatory role of capsaicin-sensitive nerves in chronic arthritis in the rat. Capsaicin selectively activates then desensitizes sensory nerves and systemic capsaicin pretreatment decreased substance P content of peripheral nerves in arthritic rats (Colpaert *et al.*, 1983). Paw swelling and degree of arthritis was also decreased in these animals although it is difficult to establish a direct relationship between these findings. Capsaicin also depletes CGRP and this may also contribute to its long-term anti-inflammatory effects. Levine *et al.* (1984) showed that intra-articular injections of substance P exacerbated arthritis in rats and more severe arthritis was found in joints, such as the ankle, which are densely innervated with substance P-containing fibres. Substance P may play a more important role in activating cellular rather than vascular mechanisms, as small doses of substance P stimulate proliferation of rheumatoid synoviocytes in culture and cause release of collagenase and PGE_2 (Lotz *et al.*, 1987). Recently, however, O'Byrne *et al.* (1991) have shown interleukin-1-induced cartilage degradation to be independent of substance P.

The specific contribution of CGRP to arthritis is not clear although the chronic stages of adjuvant arthritis in rats may be attentuated by immunization with CGRP prior to induction of disease (Louis *et al.*, 1990). Immunohistochemical methods have demonstrated substance P- and CGRP-containing nerves throughout the synovium of normal rats but inflammatory arthritis produces complex changes in peripheral innervation. In rats with chronic adjuvant arthritis there is increased staining for CGRP and substance P in the sciatic nerve, dorsal root ganglia and tissue overlying the joints (Weihe *et al.*, 1988) suggesting increased synthesis of these neuropeptides. In the proliferating synovium, however, the amount of nervous tissue is reduced and neuropeptide containing nerves are found only at the junction of the bone and the synovial membrane (Konttinenn *et al.*, 1990). This may be due to increased turnover and release of neuropeptides but it is also possible that release of CGRP is decreased in severe inflammatory arthritis. The relevance of these findings to disease progression is as yet unknown. Raud *et al.* (1991) have recently reported that pretreatment with either CGRP or capsaicin leads to an inhibition of oedema induced by inflammatory mediators in human skin, rat paw and hamster cheek pouch. These findings illustrate significant species differences but also raise the possibility that loss of sensory nerves in severe arthritis may contribute to, rather than inhibit, synovial inflammation. The role of neuropeptides may therefore differ in acute or chronic inflammation as the distribution of sensory nerves is altered by the ongoing inflammatory response.

In conclusion, our results show that CGRP is a potent vasodilator in the rat knee and can potentiate oedema formation induced by histamine. CGRP is significantly more potent as a vasodilator than substance P with which it is often co-localized in sensory nerves. We consider that CGRP may modulate blood flow in the synovium and hence contribute to the inflammatory response in the joint.

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