RESEARCH PAPER

A role for the sensory neuropeptide calcitonin gene-related peptide in endothelial cell proliferation *in vivo*

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BACKGROUND AND PURPOSE

We have tested the hypothesis that calcitonin gene-related peptide (CGRP) is a mediator of capsaicin-induced angiogenesis *in vivo*.

EXPERIMENTAL APPROACH

In a series of experiments, the knee joints of rats were injected with CGRP, capsaicin or vehicle control. Groups of animals ($n = 6$) were treated with the CGRP receptor antagonist BIBN4096BS and/or the NK_1 receptor antagonist SR140333. Endothelium, proliferating endothelial cell nuclei and macrophages were identified 24 h later in the synovium by immunohistochemistry and quantified by image analysis. mRNA for the receptors for CGRP and adrenomedullin were sought in normal and inflamed rat and human synovia using RT-PCR.

KEY RESULTS

Intra-articular CGRP injection increased the endothelial cell proliferation index, whereas macrophage infiltration and knee joint diameters were similar to saline-injected controls. CGRP-induced endothelial cell proliferation was dose-dependently inhibited by BIBN4096BS. mRNA for adrenomedullin and the CGRP receptor subunits were detected in normal and inflamed human and rat synovia. In capsaicin-induced synovitis, the increased endothelial cell proliferation index was partially blocked by administration of NK_1 or CGRP antagonists individually and was reduced to the level of saline controls by coadministration of both receptor antagonists.

CONCLUSIONS AND IMPLICATIONS

These data support the hypothesis that CGRP stimulates angiogenesis *in vivo* directly by activating CGRP receptors. Capsaicin-induced endothelial cell proliferation was completely blocked by coadministration of CGRP and NK_1 receptor antagonists, indicating that both CGRP and substance P may contribute to angiogenesis in this model of synovitis.

Abbreviations

BIBN4096BS, 1-piperidinecarboxamide-N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl] carbonyl] pentyl]amino]-1-[(3,5dibromo-4-hydroxyphenyl) methyl]-2-oxyethyl]-4-(1,4-dihydro-2-oxo(2*H*)-quinazolinyl)-, [[*R*,(*R**.*S**)]]); BrdU, 5-bromo-2′-deoxyuridine; CGRP, calcitonin gene-related peptide; CI, confidence interval; CRLR, calcitonin receptor-like receptor; DAPI, 4′-6′-diamidino-2-phenylindole hydrochloride; NK_1, neurokinin 1; RAMP, receptor activity modifying protein; SP, substance P; SR140333, 1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidi-*n*-3yl]-4-phenyl-1-azoniabicyclo[2.2.2] octane, chloride; SR140603, *R* enantiomer of SR14033

Introduction

Angiogenesis, the growth of new blood vessels from a pre-existing vasculature, is a common feature of chronic inflammation in most tissues (Walsh and Haywood, 2001), including the synovium (Walsh et al., 1998). Angiogenesis is tightly regulated during normal growth, wound healing and the female reproductive cycle (Walsh, 1999), whereas persistent angiogenesis is characteristic of chronic inflammatory disease. An example of this is the blood vessel growth seen in rheumatoid arthritis (Klareskog et al., 2009). Angiogenesis is also a key component in the transition from acute to persistent inflammation (Ashraf et al., 2010). Thus, an understanding of the factors that can be involved in early angiogenesis may identify targets for treating arthritis.

We have previously shown that exogenous substance P (SP) can increase endothelial cell proliferation in the rat knee joint, and that this can be blocked completely by the competitive nonpeptide NK_1 receptor antagonist SR140333 (nolpitantium) (Malcangio and Bowery, 1994; receptor nomenclature follows Alexander et al., 2011). The specificity of SR140333 cann be summarized by its inhibition constants (K_i) at various receptors. K_i at the NK_1 receptor in rat brain was 0.027 nM, in pig brain was 0.039 nM and in cultured human cells IM9 (lymphoblast), U373 and STTG1 (astrocytoma) cells were 0.019, 0.7 and 0.24 nM respectively. K_i values for binding to NK_2 and NK_3 receptors were >1000 and 500 nM respectively (Emonds-Alt et al., 1993). Endothelial cell proliferation was neither blocked by the inactive enantiomer SR140603 nor by the NK_2 receptor antagonist SR144190 (Seegers et al., 2003). This is consistent with the hypothesis that the proangiogenic action of SP is mediated via the NK_1 receptor. NK_1 receptors have been localized to the vascular endothelium in both rat and human synovium (Walsh et al., 1992; 1993).

Capsaicin, the pungent component in chilli peppers (Szallasi and Blumberg, 1999), can be used to stimulate the release of neuropeptides from sensory nerve fibres, giving rise to neurogenic inflammation. We have shown by immunohistochemistry that intra-articular injection of capsaicin results in the depletion of neuropeptides from nerves within the synovium (Mapp et al., 1996). Thus, this model was chosen as a means to the release of endogenous neuropeptides SP and CGRP.

Enhanced angiogenesis also occurs during this capsaicin-induced synovitis, but this effect is not wholly attributable to actions of SP on NK_1 receptors (Seegers et al., 2003). A dose of 1 μmol SR140333, which completely inhibited the effects of exogenous SP, was found to block only 65% of the capsaicin-induced enhanced endothelial cell proliferation (Seegers et al., 2003). We therefore hypothesized that another mediator may be playing a part in enhanced angiogenesis during capsaicin-induced synovitis.

Calcitonin gene-related peptide (CGRP) is a neuropeptide colocalized with SP in secretory granules at the peripheral terminals of perivascular sensory nerves. It is a potent vasodilator and can enhance endothelial cell proliferation and migration in vitro (Haegerstrand et al., 1990). Thus, CGRP is a candidate for a further neuropeptide involved in capsaicin-enhanced angiogenesis.

The CGRP receptor is a heterodimer consisting of the calcitonin receptor–like receptor (CRLR) and one of three receptor activity modifying proteins (RAMPs). RAMP1 confers specificity for CGRP (Hay et al., 2004), whilst RAMPs 2 and 3 confer specificity for adrenomedullin (McLatchie et al., 1998; see Walker et al. (2010: Recober and Russo (2009).

We used the nonpeptide, competitive, CGRP receptor antagonist BIBN4096BS (Olcegepant) (Doods et al., 2000) to test the contribution of CGRP to in vivo endothelial cell proliferation. The K_i values for BIBN4096BS are 0.018 nM at the human CGRP receptor and 2.1 nM at the rat CGRP receptor. The major determinant of the species selectivity is the amino acid at position 74 in RAMP1 (Mallee et al., 2002). Human RAMP 1 contains tryptophan at this position, whereas this is lysine in rodent RAMP1. Over and above species selectivity, BIBN4096BS shows selectivity for CGRP receptors over adrenomedullin receptors of at least 1000-fold (Hay et al., 2006). BIBN4096BS shows no antagonistic effects on cells expressing CRLR/RAMP2 and CRLR/RAMP3 at concentrations up to 10 μM.

The experiments we have performed have investigated the effects of exogenous CGRP and adrenomedullin on angiogenesis in the rat knee and tested the ability of a CGRP receptor antagonist, both alone and in combination with a NK_1 receptor antagonist to inhibit angiogenesis in capsaicin-induced synovitis. In order to assess the potential relevance of our rat models to human synovitis, we have looked for the presence of mRNAs for CRLR and RAMPs in both normal and inflamed rat and human synovia.

Methods

Animal models

All animal care and experimental proceduress were approved by the University of Nottingham Local Ethics Review Committee and performed under United Kingdom Home Office licence. We used male Wistar rats of approximately 180 g. Animals were given food and water ad libitum and maintained on a 12 h light/dark cycle. CGRP (dose range 0.025–2.5 nmol), adrenomedullin (at the highest practicable dose of 8 nmol), capsaicin (0.5% w/v) or control vehicle was each injected into the right knee ($n = 6$ per group). All the above reagents were obtained from Sigma Aldrich Ltd. (Dorset, UK). Left knees were injected with normal sterile saline alone, which does not increase indices of angiogenesis, macrophage infiltration or knee diameter at 24 h compared with naïve animal knees (Walsh et al., 1998). CGRP and adrenomedullin were dissolved in normal sterile saline. Capsaicin was dissolved in 10% ethanol, 10% Tween 80 and 80% normal sterile saline (v/v/v) (Mapp et al., 1996). Volumes for all intra-articular injections were 100 μL, performed under isofluorane (2%) anaesthesia (Schering Plough, Middlesex, UK). Anaesthesia was assessed by absence of paw withdrawal following foot pinching.

Administration of receptor antagonists

The CGRP receptor antagonist BIBN4096BS (6 mg) (Doods et al., 2000) was dissolved in 200 μL 0.1 M HCl and then diluted to 1 mL with normal sterile saline. The pH was then

adjusted to neutral by the stepwise addition of 0.1 M NaOH. The receptor antagonist was then administered i.v. at a dose of 0.7 or 0.07 µmol per rat immediately before intra-articular injection of 2.5 nmol of CGRP or 0.5% capsaicin. The NK_1 receptor antagonist SR140333 was dissolved in ethanol to a stock concentration of 100 mM and then diluted with normal sterile saline to give a final ethanol concentration of ≤10%. SR140333 was administered by i.p. injection at a dose of 1 µmol per animal (Seegers *et al.*, 2003).

Tissue collection and preparation. Twenty-four hours after intra-articular injection, rats were killed by asphyxiation in carbon dioxide, and the right and left knee synovia with patellae were immediately removed. Synovia for histomorphometry were embedded in Tissue Tek™ frozen in melting isopentane onto cork block mounts and stored at –80°C until use. Synovia for RNA extraction were collected in RNA Later™ (Ambion Inc., Austin, TX, USA) and stored at –20°C before analysis.

Staining procedures. Double sequential immunohistochemistry was used to identify endothelial proliferation using previously published methods (Walsh *et al.*, 1998; Seegers *et al.*, 2003; 2004). Briefly, sections (5 µm) were first immunostained for PCNA using monoclonal antibody clone PC10 and then for endothelium using a monoclonal antibody directed against CD31. PC10 in this setting gives comparable results to 5-bromo-2′-deoxyuridine (BrdU) (Seegers *et al.*, 2004) but does not require tissue fixation, allowing other markers such as ED1 (see below) to be localized in the same tissues. Nuclei were counterstained by immersion of sections in the fluorescent DNA ligand, 4′-6′-diamidino-2-phenylindole hydrochloride (DAPI). Macrophage infiltration was identified by immunoreactivity for the monoclonal antibody clone ED1 (Waseem and Lane, 1990; Male *et al.*, 1995; Seegers *et al.*, 2003; 2004). Proliferation markers and ED1 were developed with diaminobenzidine using the glucose oxidase/nickel-enhanced method (Walsh *et al.*, 1998). Endothelium markers were developed using Fast Red according to manufacturer's instructions. Haematoxylin and eosin stains were performed on sections, after those used for immunohistochemistry, to assist in identification of tissue structures.

Human synovium

Protocols for the use of human tissues had local Research Ethics Committee approval (NNHA/420, NNHA/544, NNHA/673 and 05/Q2403/24). Human synovia were obtained, with informed consent, at total joint replacement surgery from patients fulfilling American College of Rheumatology criteria for rheumatoid arthritis (RA; $n = 5$) (Arnett *et al.*, 1988) or osteoarthritis (OA; $n = 12$) (Altman *et al.*, 1991) or from the knees of patients without known arthritis collected *post mortem* (PM; $n = 7$). OA samples were selected according to the extent of inflammation displayed on a haematoxylin and eosin stained index section from a formalin-fixed tissue block of adjacent synovium to that used for RNA extraction. Inflammation was graded as previously described (Haywood *et al.*, 2003). Samples were graded as inflamed [grade 3 inflammation, OA(i)] or not inflamed [grade 0 inflammation, OA(n)]. Samples from patients with RA (median age 68; range

53–80 years; 2 male) were from knee ($n = 2$), hip ($n = 2$) or elbow ($n = 1$). OA(i) samples were from patients (median age 60; range 41–82 years; 4 male) undergoing arthroplasty of the knee ($n = 5$) or hip ($n = 1$), and OA(n) samples were from patients (median age 65; range 62–81 years; 5 male) undergoing arthroplasty of the knee ($n = 3$) or hip ($n = 3$). Human synovium samples were snap-frozen in liquid nitrogen then stored at –80°C until use.

Measurements made. Quantification was performed by an observer unaware of experimental details, using an Axioskop-50 microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) with a 20× objective lens. Transmitted light and fluorescence images of the same field were each captured using a 3-CCD camera and analysed using a KS300 image analysis system (Imaging Associates Ltd., Abingdon, UK).

Synovium was delineated according to morphology and synovial area was measured. Within this synovial region, a mask of the endothelial area was created that included CD31-positive blood vessels. This endothelial mask was applied over corresponding images of PCNA-positive and DAPI-positive nuclei. Nuclei falling within endothelium were counted as PCNA-positive endothelial nuclei and total endothelial nuclei respectively. Endothelial PCNA index was defined as the percentage of endothelial nuclei positive for PCNA. Vascular density was defined as the percentage of synovial area immunoreactive for endothelium within the frame area measured. Macrophage fractional area was defined as the fraction of synovial area (from 0 to 1) that was ED1-positive. We examined four fields per section and one section per rat to give a minimum coefficient of variation and a standard error of ±12.5% of the mean for the endothelial PCNA index (Walsh *et al.*, 1998) and the same for macrophage fractional area.

Knee diameters were measured (mm) with a digital electronic caliper (Mitutoyo UK Ltd., Andover, UK).

RT-PCR

Total RNA was extracted using Trizol reagent as per manufacturer's instructions. Briefly, synovia were homogenized and shaken with 110 µL chloroform. After centrifugation (12 000× g, 30 min) at 4°C, the upper, aqueous phase was eluted into 700 µL isopropanol and pelleted and washed in 75% ethanol and air-dried.

RNA was suspended in water and split into two halves for rt(+) and rt(–), and reverse transcription reactions similar to previously published methods (Seegers *et al.*, 2004).

A GAPDH PCR was performed on rt(+) and rt(–) samples from each preparation to determine successful RNA extraction and the quality of cDNA produced. Each sample's rt(+) GPADH PCR product was compared with its rt(–) product to ensure amplification of cDNA and not genomic DNA. The best-quality cDNA from each sample was then chosen for further analysis. The 50 µL PCR reaction was the same as previous reported, using 1 unit Taq polymerase, 40 nmol PCR Nucleotide Mix, 10 nmol each of sense and antisense GAPDH primers and an annealing temperature of 54°C for 40 cycles (McWilliams *et al.*, 1998). Samples were then selected for further analysis for the human groups RA ($n = 5$), OA(i) ($n = 6$), OA(n) ($n = 6$) and PM ($n = 7$) and rat groups ($n = 6$ per group).

Table 1

Human PCR primer sequences and product sizes

Human	Primer sequence (5'–3')	Product (bp)
GAPDH sense	GGTGAAGGTCGGAGTCAACGGA	240 (McWilliams *et al.*, 1998)
GAPDH antisense	GAGGGATCTCGCTCCTGGAAGA	
hAdM sense	AAGAAGTGGAATAAGTGGGCT	291 (Matsushita *et al.*, 2004)
hAdM antisense	TGTGAACTGGTAGATCTGGT	
CRLR sense	ACCAGGCCTTAGTAGCCACA	296 (Linscheid *et al.*, 2005)
CRLR antisense	ACAAATTGGGCCATGGATAA	
RAMP1 sense	CTGCCAGGAGGCTAACTACG	298 (Linscheid *et al.*, 2005)
RAMP1 antisense	GACCACGATGAAGGGGTAGA	
RAMP2 sense	GGGGGACGGTGAAGAACTAT	227 (Linscheid *et al.*, 2005)
RAMP2 antisense	GTTGGCAAAGTGGATCTGGT	
RAMP3 sense	AACTTCTCCCGTTGCTGCT	165 (Linscheid *et al.*, 2005)
RAMP3 antisense	GACGGGTATAACGATCAGCG	

Table 2

Rat PCR primer sequences and product sizes

Rat	Primer sequence (5'–3')	Product (bp)
Rat AdM sense	CTCGACACTTCCTCGCAGTT	446 (Pan *et al.*, 2005)
Rat AdM antisense	GCTGGAGCTGAGTGTGTCTG	
Rat CRLR sense	CAACTGCTGGATCAGCTCAG	446 (Pan *et al.*, 2005)
Rat CRLR antisense	CATCGCTGATTGTTGACACC	
Rat RAMP1 sense	GCTGCTGGCTCATCATCTCT	402 (Pan *et al.*, 2005)
Rat RAMP1 antisense	TACACGATGCCCTCTGTGC	
Rat RAMP2 sense	TGAGGACAGCCTTCTGTCA	371 (Pan *et al.*, 2005)
Rat RAMP2 antisense	CATCGCCGTCTTTACTCCTC	
Rat RAMP3 (CRLR-RAMP3-400 sense)	AAGGTGGACGTCTGGAAGTG	352 (Pan *et al.*, 2005)
Rat RAMP3 (CRLR-RAMP3-400 antisense)	CTGGGCGACATTCTTCTAGC	

Forty cycles of PCR amplification was performed with an annealing temperature of 54°C for CRLR, RAMPs-1, -2, and -3, and adrenomedullin in a volume of 50 µL. The PCR reaction mix consisted of 1 unit of Amplitaq Gold in manufacturer's buffer with 1.5 mM $MgCl_2$ (the human CRLR PCR used 2.5 mM $MgCl_2$, and the rat RAMP-1 used 2.0 mM $MgCl_2$), 40 nmol PCR Nucleotide Mix and 10 pmol sense and antisense primers (the rat CRLR and RAMP-1 PCRs used 100 pmol primers). Primer sequences for each reaction are shown in Tables 1 and 2. Each PCR reaction was characterized to ensure that no rt(–) PCR products appeared at similar molecular weights to the rt(+) expected products, and no PCR carry-over contamination was detected in any reaction performed during this study. Rat brain and DU145 (a gift from Dr Neil Cross, Academic Urology, University of Sheffield, UK) or HUVEC (a gift from Julie Corfield, Clinical Oncology, University of Nottingham, UK) cDNAs were used as positive controls for rat and human PCR reactions respectively. The PCR reactions were not tested for sensitivity. PCR products were separated on 2% (w/v) agarose/TBE gels and visualized using ethidium bromide.

Data analyisis

The optimum number of fields per section and sections per case were determined in previous experiments (Walsh *et al.*, 1998). Six cases per group were required to have a 90% chance of detecting a 10% difference in macrophage fractional area and PCNA index. Data (means ± SD unless otherwise stated) are presented on six knees per experimental group and were log-transformed prior to statistical analysis by one-way ANOVA with Bonferroni corrections using GraphPad Prism 4.0 (San Diego, CA, USA).

Materials

Trizol reagent, Supercript II, agarose was from Invitrogen (Paisley, UK). Isopropanol was from Fisher Scientific (Leicestershire, UK). Random hexamer primers, Taq polymerase (Go Taq™) and PCR nucleotide mix were from Promega (Southampton, UK). Amplitaq Gold was from Applera Biosciences (Warrington, UK). Primers were synthesized by Thermo Fisher (Ulm, Germany). Haematoxylin, eosin and Tissue Tek™ were from RA Lamb (Eastbourne, UK). Anti-PCNA antibody clone PC10 was from Dako (Ely, UK). Anti-CD31 (clone TLD-3A12) antibody was from AbD Serotec (Oxford, UK). Horse antimouse-biotinylated antibody was from Vector Labs (Peterborough, UK).

Results

Intra-articular CGRP-injection, synovial angiogenesis and inflammation

The purpose of this experiment was to determine if injection of exogenous CGRP could increase endothelial cell proliferation. Macrophages and joint swelling were monitored to see if CGRP-enhanced endothelial PCNA index was an indirect effect via induction of inflammation. There were no significant differences in joint swelling ($t = 0.55$, $P > 0.05$) between CGRP-treated knees [mean = 11.6 mm; 95% confidence interval (95% CI) 11.5–11.8 mm] or vehicle-treated knees (mean = 11.7 mm; 95% CI 11.5–11.8 mm). Macrophage fractional areas were also not significantly different ($t = -1.2$, $P > 0.05$) between CGRP-treated knees (mean = 3.19%; 95% CI 1.32–3.72%) and vehicle-treated knees (mean = 2.09%; 95% CI 1.26–3.51%).

Intra-articular injection of recombinant CGRP induced an increase over vehicle injection in endothelial PCNA index in rat synovia, which was significant at doses of 0.25 nmol ($t = 4.1$, $P < 0.01$) and 2.5 nmol ($t = 17.3$, $P < 0.001$) (Figure 1A). Figure 1B shows immunohistochemical staining for PCNA and an example of a negative control. Adrenomedullin (8 nmol) did not induce a significant change in endothelial PCNA index over vehicle ($P > 0.05$) [means 2.04 (95% CI1.68-2.60) and 2.11 (95% CI 1.42-3.12) respectively].

To further investigate if exogenous CGRP administration was responsible for the increase in endothelial PCNA index, we employed the specific CGRP receptor antagonist BIBN4096BS. The increase in endothelial PCNA index in synovia that followed intra-articular injection of 2.5 nmol CGRP was reduced to a similar level to naïve knees after i.v. injection of 0.7 μmol per rat of the CGRP receptor antagonist BIBN4096BS ($t = 10.2$, $P < 0.001$ compared with CGRP alone). Administration of 0.07 μmol per rat BIBN4086BS also inhibited the CGRP-induced endothelial PCNA index ($t = 5.4$, $P < 0.001$; Figure 2.

Effects of BIBN4096BS and SR140333 on capsaicin-induced angiogenesis and inflammation

We used 0.5% capsaicin to release the endogenous neuropeptides SP and CGRP from storage granules in the sensory nerves of the synovium. Intra-articular injection of capsaicin
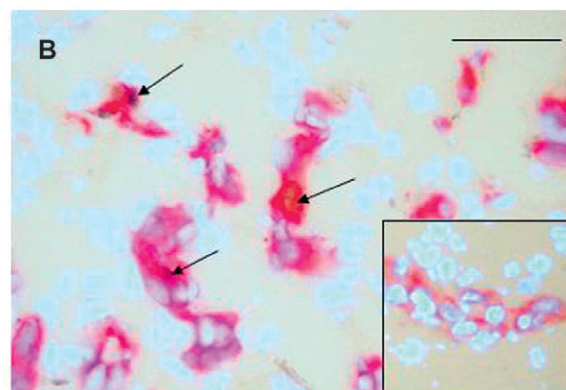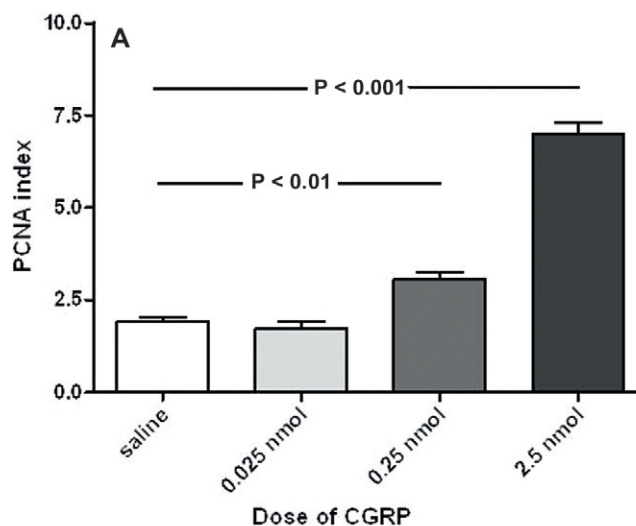
Figure 1

Intra-articular administration of exogenous CGRP causes a dose-dependent increase in the endothelial cell proliferation index in the synovium. (A). PCNA indices in saline-injected controls and 24 h after intra-articular injection of CGRP from 0.025 to 2.5 nmol per knee. The two higher doses of CGRP caused an increase in the PCNA index ($P < 0.01$ and $P < 0.001$, respectively) compared with saline-injected controls. Data shown are means ± SD; $n = 6$ rats per group. (B). Main panel: Synovium from a rat 24 h after intra-articular injection of 2.5 nmol CGRP, showing CD31-positive vascular cells and PCNA-positive proliferating nuclei. Counterstaining with DAPI shows all other nonstained nuclei. Arrows indicate PCNA-positive endothelial cells. This allows determination of the PCNA index as the percentage of vascular nuclei, which were positive for the proliferation marker. Inset shows a blood vessel from a control saline-injected synovium not displaying PCNA-positive endothelial cell nuclei. Bar = 100 μm.

(0.5%) was followed at 24 h by increased joint swelling and endothelial PCNA index within the synovium compared with controls The increased synovial angiogenesis was partially inhibited by the NK_1 receptor antagonist SR140333, 1 μmol per rat ($t = 11.0$, $P < 0.001$), or by the CGRP receptor antagonist BIBN4096BS, 0.7 μmol per rat ($t = 12.6$, $P < 0.001$) (Figure 3A). Coadministration of BIBN4096BS and SR140333 at the same doses completely blocked capsaicin-induced angiogenesis in the rat synovium ($t = 16.7$, $P < 0.001$)
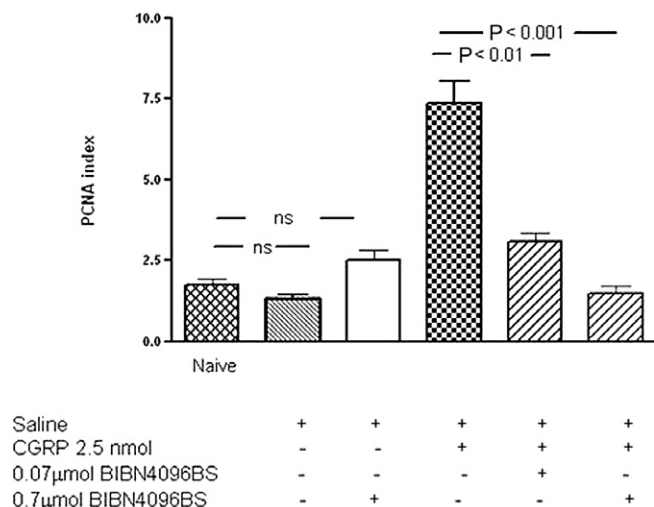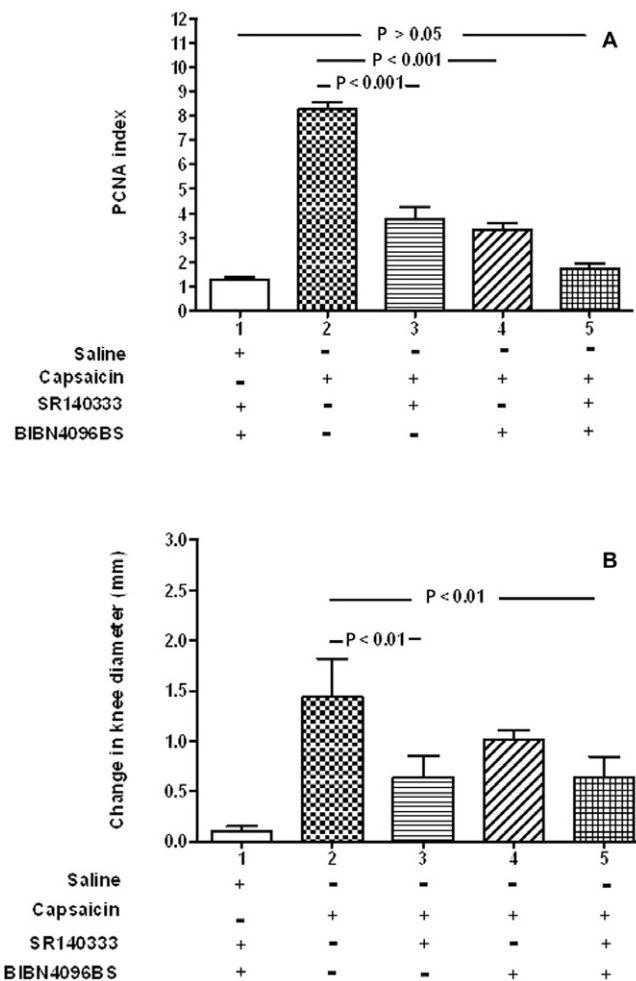
Figure 2

Blockade of the effect of exogenous intra-articular CGRP administration on the endothelial cell proliferation index using the specific CGRP receptor antagonist BIBN4096BS. Bar 1 shows the baseline PCNA index in naïve animals, negative control. Bar 2 shows the response to saline alone injection, which was not significantly different from naïve control. Bar 3 shows the PCNA index in animals intra-articularly injected with saline and i.v. injected with the CGRP receptor antagonist BIBN 4096BS. Intra-articular saline did not significantly increase the PCNA index, negative control. Bar 4 shows the effect of intra-articular administration of 2.5 nmol CGRP (positive control) on the PCNA index. The effect of CGRP on the PCNA index was diminished by the CGRP receptor antagonist BIBN406BS (bars 5 and 6) when compared with CGRP injection alone. Rats were given CGRP 2.5 nmol alone or together with BIBN406BS at either 0.07 μmol per rat (bar 5) 0.7 μmol per rat (bar 6). $P < 0.001$ for both treatments. Data shown are means $\pm$ SD; $n = 6$ rats per group.

(Figure 3A). This effect was greater than each antagonist alone; SR140333 ($t = 5.2$, $P < 0.01$) and BIBN 4096BS ($t = 4.2$, $P < 0.001$).

We monitored knee swelling and macrophage infiltration as measures of inflammation to investigate possible indirect effects of inflammatory mediators on the endothelial cells. Joint swelling 24 h after capsaicin injection was also significantly reduced by administration of SR140333 ($t = 3.6$, $P < 0.01$) and the coadministration of SR140333 and BIBN1096BS ($t = 3.1$, $P < 0.01$), but not by administration of BIBN4096BS alone (Figure 3B). Macrophage fractional area in the synovium was not statistically significantly different between the groups (Figure 4A). Immunocytochemical demonstration of macrophages is shown in capsaicin-treated animals (Figure 4B) and animals treated with both SR140333 and BIBN4096BS (Figure 4C); the quantity and distribution of positive cells appear similar.

RT-PCR

In capsaicin-induced synovitis and naïve rat knees, mRNAs for adrenomedullin and RAMPs-1, -2 and -3 were detected in all samples. mRNA for CRLR was detected in synovia from

Figure 3

Capsaicin-induced angiogenesis was partially inhibited by the administration of each receptor antagonist, and coadministration of BIBN1096BS and SR140333 completely blocked capsaicin-enhanced angiogenesis. (A) Endothelial PCNA indices of rat synovia from knees injected with 0.5% capsaicin were significantly reduced by either the SP receptor antagonist SR140333 (bar 3) or by the CGRP receptor antagonist BIBN4096BS (bar 4) when compared with the positive control capsaicin alone, bar 2; $P < 0.001$ for both treatments. When given in combination, the two receptor antagonists (bar 5) reduced the rise in PCNA index attributable to capsaicin to the same level as saline controls; $P > 0.05$. PCNA index was defined as the percentage of endothelial cell nuclei positive for PCNA. Data shown are means $\pm$ SD; $n = 6$ rats per group. (B). Knee joint diameters corresponding to the same groups in (A), showing significant inhibition of joint swelling by SR140333, either alone (bar 2, $P < 0.01$) or in combination with BIBN4096BS (bar 5, $P < 0.01$). The change in knee diameter from baseline to 24 h is shown in mm, as means $\pm$ SD; $n = 6$ rats per group.. In each panel, the negative control (bar 1) represents data from synovia 24 h after intra-articular saline injection accompanied by i.v. injection of the CGRP receptor antagonist BIBN4096BS and i.p. injection of the SP receptor antagonist SR140333. The positive control (bar 2) represents data from synovia 24 h after intra-articular injection of capsaicin in the absence of either antagonist. Capsaicin is believed to release endogenous CGRP and SP from sensory nerves in the synovium. All groups of rats (bars 2–5), with the exception of saline controls (bar 1), were treated with capsaicin.
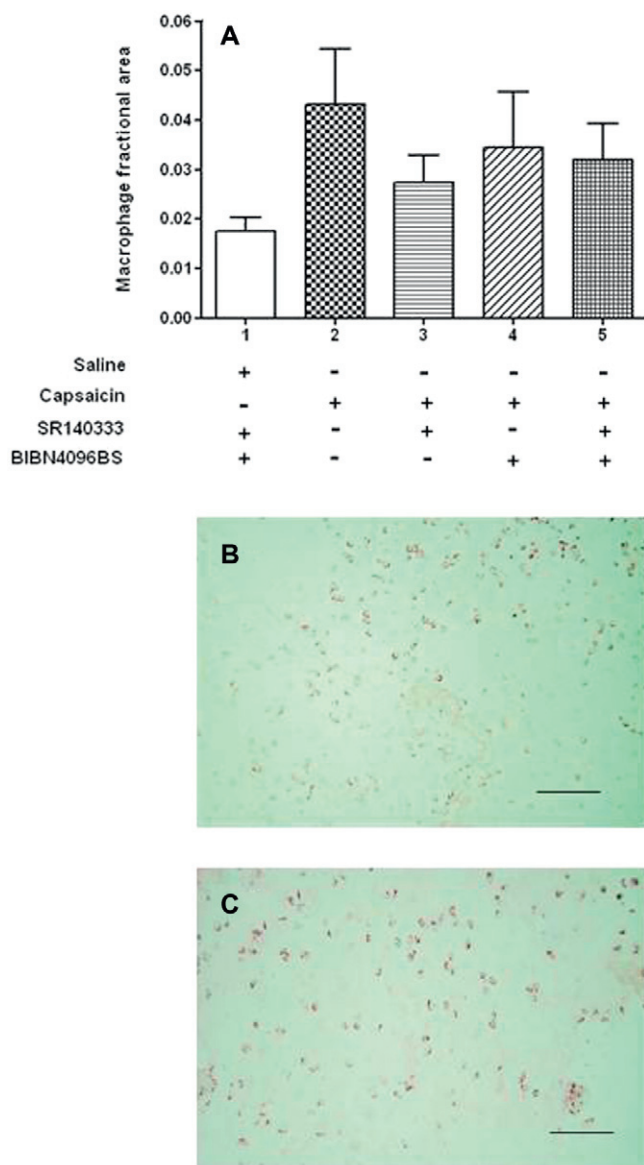
Figure 4

Detection of macrophages in the synovium. (A). Low levels of macrophage infiltration in all groups. There were no statistically significant differences in macrophage fractional areas for each group of rats, indicating that any small cellular inflammatory response present was below the detection threshold. Data shown are means ± SD; $n = 6$ rats per group Macrophages in the synovium were detected by immunohistochemistry using the primary antibody ED1 and were stained with nickel/DAB and appear as black. (B) Synovium from a capsaicin-treated animal. (C) Synovium from a saline-injected knee of an animal treated with the CGRP receptor antagonist BIBN4096BS i.v. and the SP receptor antagonist SR140333 i.p. The number of positively staining cells in each panel appears similar. When quantified, there were no statistically significant differences in the macrophage fractional area between any of the groups. Bar = 100 μm.

three out of six capsaicin-injected and four out of six naïve knees (Figure 5A). In human synovia, RAMPs, CRLR and adrenomedullin were detectable in samples from all disease groups (Figure 5B,C).

Discussion

We have previously shown (Seegers *et al.*, 2003) that the angiogenesis induced by intra-articular injection of 0.5% capsaicin could be incompletely inhibited by an NK_1 receptor antagonist, SR140333 (nolpitantium). Capsaicin stimulates the release of both SP and CGRP from storage granules in the peripheral terminals of fine unmyelinated sensory nerves (Mapp *et al.*, 1996; Szallasi and Blumberg, 1999). SP can stimulate synovial angiogenesis (Seegers *et al.*, 2003), and we now show that CGRP is also able to stimulate angiogenesis in the rat knee. Until relatively recently, CGRP receptor antagonists have been small peptides (e.g. CGRP 8–37) with short half-lives, which has made the contribution of CGRP to arthritis difficult to determine. Our study is one of the first in this field to employ a nonpeptide CGRP receptor antagonist BIBN4096BS. Inhibition of the angiogenic effect of CGRP by administration of BIBN4096BS indicates mediation by the CGRP receptor. Inhibition of capsaicin-induced endothelial cell proliferation by either the NK_1 receptor antagonist SR140333, or the CGRP receptor antagonist BIBN4096BS suggests that both these neuropeptides mediate capsaicin-induced angiogenesis in the rat knee. Complete inhibition of capsaicin-induced endothelial cell proliferation by a combination of these two receptor antagonists suggests that neuropeptide release is a prerequisite for capsaicin-induced angiogenesis. This supports the concept of 'neurogenic angiogenesis'.

Capsaicin (Min *et al.*, 2004) and a related compound capsiate (Pyun *et al.*, 2008) can inhibit angiogenesis both *in vivo* and *in vitro*. *In vivo*, this is true of test systems such as the Matrigel plug assay or the chick chorioallantoic membrane assay. Both of these test systems and *in vitro* tests do not contain sensory nerve fibres, which we believe to be the source of the angiogenic stimulus.

Capsaicin acts through the transient receptor potential vanilloid (TRPV1) receptor on the peripheral nerve. TRPV1 receptors have also been localized to mast cells and other cell types that may be present within the synovium (Stander *et al.*, 2004), raising the possibility that there may be a small contribution of other TRPV1-expressing cells to the angiogenic effect. However, immunoreactivities for SP and CGRP are localized to sensory nerves in both human and rat synovia (Mapp *et al.*, 1990; 1994), and blockade of capsaicin-induced angiogenesis by neuropeptide receptor antagonists suggests that this effect is due to neuropeptide release rather than actions on other cell types. The low levels of macrophage infiltration in the current study contrast with observations 24 h after intra-articular injection of carrageenan, when macrophage infiltration can reach levels of 20% of the synovial area (Walsh *et al.*, 1998). Thus, whilst we cannot rule out the possibility of a contribution of low levels of inflammation to the endothelial cell proliferation induced by CGRP or capsaicin, direct effects of neuropeptides on their receptors appear to predominate.

Adrenomedullin is another angiogenic peptide that may interact with the CRLR receptor (Zhao *et al.*, 1998). We detected mRNAs for adrenomedullin in rat and human synovia. Adrenomedullin has been shown to cause endothelial cell proliferation in culture at concentrations of 1×10^{-9} M (Miyashita *et al.*, 2003) and may stimulate prolif-
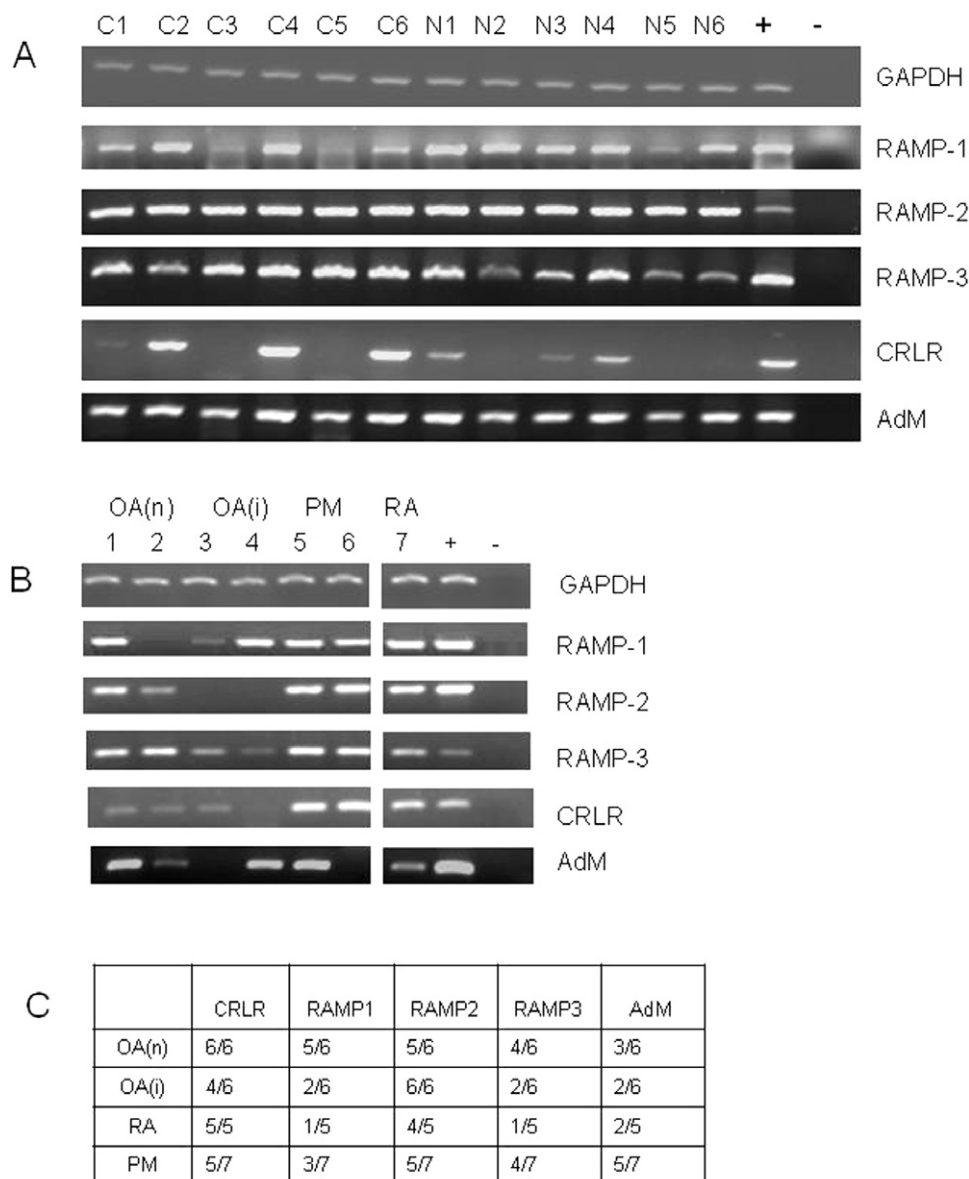
Figure 5

Components of the CGRP and adrenomedullin receptor-ligand complex are expressed in rat and human synovia. (A). Products of RT-PCR for GAPDH, adrenomedullin (AdM), CRLR, RAMP-1, RAMP-2 and RAMP-3 are shown after separation on agarose gels. Samples from synovia taken 24 h after intra-articular injection with capsaicin (C1–C6, lanes 1–6) and naïve knees (N1–N6, lanes 7–12) were analysed. Positive controls (+, lane 13) were rat brain cDNA. Negative controls (–, lane 14) were PCR's without any template DNA. (B). Examples of PCR products from RT-PCR on human synovia for CRLR, RAMP-1, RAMP-2, RAMP-3 and adrenomedullin are shown after separation on agarose gels. Samples from OA without inflammation (OA(n), lanes 1, 2), severely inflamed OA (OA(i), lanes 3, 4), normal *post mortem* (PM; lanes 5, 6) and RA (lane 7) are shown. Positive controls (+, lane 9) were HUVEC cells for CRLR and DU145 cells for RAMPs-1, -2 and -3. Negative controls (–, lane 10) were PCRs without any template DNA. (C). Table showing detection rates for PCR products in human synovia.

eration of vascular smooth muscle cells (Shichiri *et al.*, 2003). However, we were unable to detect angiogenic or pro-inflammatory effects of recombinant adrenomedullin in the rat knee. We used the maximum economical dose of adrenomedullin (8 nmol per animal) and saw no response. The dose used was four times higher than the highest dose of CGRP that we used. There are a variety of reasons why adrenomedullin may have been ineffective perhaps through a lack of potency or synovial fluid protein/mucopolysaccharide

binding. However, adrenomedullin in this context has served as a control showing that CGRP has not induced synovial angiogenesis through nonselective irritant properties. However, possible roles of adrenomedullin in synovitis, particularly through non-angiogenic pathways, deserve further study.

A neurogenic component to inflammation has been extensively described (see Walsh and McWilliams, 2006). Intra-articular injection of capsaicin induces synovitis, as

indicated by joint swelling and infiltration of the synovium by inflammatory cells demonstrable at later time points (Mapp *et al.*, 1996). NK_1 receptor antagonists have been shown to attenuate carrageenan-induced plasma extravasation in the rat knee joint (Lam and Ferrell, 1991). The involvement of the nervous system in this model was demonstrated by prior denervation, which caused a 37% reduction in plasma extravasation, and pretreatment with 1% capsaicin, which produced a 44% decrease in plasma extravasation. Pretreatment of the knee with a peptide antagonist of SP, d-Pro[4],d-Trp[7,9,10]-SP(4–11), resulted in a 93% reduction in plasma extravasation. SP stimulates plasma extravasation in the synovium, acting through the NK_1 receptor (Lam and Ferrell, 1991). Our finding that the NK_1 receptor antagonist SR401333 inhibited joint swelling after intra-articular capsaicin injection is consistent with this being a model of neurogenic inflammation.

CGRP is a potent vasodilator, although, consistent with previous reports (Buckley *et al.*, 1991), the lack of joint swelling after intra-articular injection of CGRP in our study suggests that it does not on its own induce oedema formation. CGRP can potentiate IL-1α-induced oedema formation in the skin (Buckley *et al.*, 1991) and also potentiates SP-induced oedema in the synovium (Cruwys *et al.*, 1992). However, the lack of significant effect of BIBN4096BS on capsaicin-induced joint swelling suggests that CGRP is not a major facilitator of oedema 24 h after intra-articular injection of capsaicin.

This study, plus others (Seegers *et al.*, 2003; 2004), shows that neuropeptides stimulate angiogenesis as well as synovitis. SP (Ziche *et al.*, 1990) and CGRP (Haegerstrand *et al.*, 1990, Zheng *et al.*, 2010) can stimulate angiogenic activities in cultured endothelial cells. Administration of SP caused a concentration-dependent increase in proliferation of cultured human endothelial cells, with 0.1 nM SP giving the maximal response. An NK_1 agonist induced similar proliferation, and two different NK_1 antagonists blocked the response to SP. Administration of 10 nM CGRP to human endothelial cells in culture caused a rise in intracellular cAMP, a 40% increase in tritiated thymidine incorporation and a 50% increase in cell numbers, all as compared with controls, over a stimulation period of 24 h. More recently (Toda *et al.*, 2008), CGRP has been shown to increase tube formation by endothelial cells *in vitro* and to enhance sponge-induced angiogenesis *in vivo*. Furthermore, growth of implanted tumours and tumour-associated angiogenesis were decreased in CGRP knockout mice compared with wild-type mice. The peptide CGRP 8–37, a CGRP receptor antagonist, was found to block tumour growth and tumour-associated angiogenesis in wild-type mice. Taken together, these data indicate that CGRP facilitates tumour-associated angiogenesis.

Increased synovial endothelial cell proliferation was detected early (24 h) after intra-articular injection of capsaicin, SP (Seegers *et al.*, 2003), or CGRP. These data suggest induction of angiogenesis through direct actions of neuropeptides on the vasculature. Infiltration of the synovium and production of other angiogenic factors by macrophages may help maintain angiogenesis and synovitis at later time points in chronic synovitis, whereas neuropeptides may play key roles in the initiation of angiogenesis in acute synovitis. The current studies were designed to investigate neuropeptide effects on endothelial cell proliferation and further work,

for example, studying later time points in models of chronic synovitis would be required to investigate possible consequences of neurogenic angiogenesis on persistent synovitis.

Human synovia, like rat synovia, express NK_1 receptors for SP, localized to vascular endothelium (Walsh *et al.*, 1992). Human and rat synovia have also been found to express CRLR (Uzan *et al.*, 2006) or receptors for CGRP (McMurdo *et al.*, 1997). We now show expression of both CRLR and RAMP1 mRNA in both human and rat synovia. The PCRs show widespread expression of CRLR/RAMPs across two species in diseased and nondiseased states. They support our hypothesis that CRLR/RAMP1 complexes contribute to the angiogenesis but do not rule out other receptors or CRLR/RAMP complexes. Although mRNA for CRLR and RAMPs were not detected in all samples, this may be due to limited sensitivity, or to samples containing variable proportions of synovial lining and sublining fatty tissues. Other studies of human joint tissue (Uzan *et al.*, 2006) have failed to find mRNA for CRLR in all samples. In a study of cultured joint tissue from osteoarthritic and rheumatoid patients, CRLR mRNA was detected in five of five RA sample and two of four OA samples. Western blot analysis for the CRLR protein showed protein in two RA samples and a faint band in one of two OA samples tested. Although densitometric comparisons may show some differences in the levels of mRNA expression, for instance, in RAMP3 or adrenomedullin, further studies of larger numbers of cases using quantitative real-time PCR would be required to determine whether expression of these genes is associated with inflammation.

CRLR and RAMP1 combine to form specific cell surface receptors through which CGRP mediates its biological effects. BIBN4096BS is a competitive antagonist of CGRP at its receptor (Verheggen *et al.*, 2002). Inhibition of synovial angiogenesis by BIBN4096BS therefore implicates CGRP and its receptor in blood vessel growth. The presence in human synovia of CGRP-immunoreactive nerves (Mapp *et al.*, 1990) and mRNA for CGRP receptors raises the possibility that, as in rats, CGRP receptor antagonists may have potential to inhibit synovial angiogenesis in man. The presence of CGRP receptors, as well as CGRP-immunoreactive nerves (Mapp *et al.*, 1990) in both normal and diseased synovium, indicates potential roles of neurogenic angiogenesis and inflammation in very early synovitis. In a broader context, the nervous system has been implicated in human arthritis. Neurological lesions such as poliomyelitis and strokes are protective of both RA and OA. The nervous system clearly regulates the classic signs of acute inflammation: calor, rubor, tumor and dolor. These signs can be induced in man by the i.d. injection of capsaicin (Shenker *et al.*, 2008). Thus, the mechanisms for neurogenic inflammation are in place, but the overall contribution is not known.

In conclusion, our data indicate that CGRP-induced endothelial cell proliferation within the synovium was both dose-dependent and sensitive to a specific CGRP receptor antagonist. The data are consistent with the hypothesis that CGRP can stimulate angiogenesis *in vivo*, and that this is a direct effect mediated by CGRP receptors. Capsaicin-induced endothelial cell proliferation was completely blocked by coadministration of both NK_1 and CGRP receptor antagonists, an indication that both peptides may contribute to angiogenesis in this model of synovitis. Initiation of angio-

genesis by neuropeptides released from the peripheral terminals of sensory nerves (neurogenic angiogenesis) occurred in parallel to neurogenic inflammation. The contribution of neurogenic angiogenesis to human synovitis and the therapeutic potential of combined NK_1 and CGRP-receptor antagonism deserve further study.

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