The substance P fragment SP-(7–11) increases prostaglandin E_2, intracellular Ca^{2+} and collagenase production in bovine articular chondrocytes

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Substance P (SP) is found in increased concentrations in inflamed joints and is believed to play a role in joint pathology. Culture of bovine articular chondrocytes with SP or with the related mammalian tachykinins neurokinin A or B (NKA or NKB) produced no effect on prostaglandin E_2 (PGE_2) or collagenase production. However, the C-terminal fragment of SP, SP-(7–11), increased PGE_2 and collagenase production at concentrations greater than 1 μM. The N-terminal fragments SP-(1–4) and SP-(1–6) had no effect on PGE_2 or collagenase production. In addition, SP-(7–11), but not intact SP, SP-(1–4), SP-(1–6), SP-(8–11) or SP-(9–11), nor the tachykinins NKA and NKB, caused an increase in the intracellular calcium concentration as measured by the fluorescent dye Fura-2. The maximal change in intracellular calcium induced by 10 μM SP-(7–11) was 140 ± 30 nM. We postulate that cleavage of SP by neutral endopeptidases which are present in the synovial fluid and which yield SP-(7–11) may be of biological importance in chondrocyte-mediated cartilage pathology.

INTRODUCTION

Tachykinins are peptide neurotransmitters in sensory nerves. They are defined by their ability to rapidly contract a variety of smooth muscles and they have the common C-terminal amino acid sequence Phe-Xaa-Gly-Leu-Met-NH_2, where Xaa is an aromatic or aliphatic amino acid. Three mammalian tachykinins have been isolated so far: substance P (SP), neurokinin A (NKA) and neurokinin B (NKB).

Approx. 90% of the SP produced in the neurons of the dorsal root ganglia is transported peripherally, where it can be released by antidromic stimulation [1]. These sensory nerves serve a dual sensory and effector function, and SP has been shown to be a mediator of nocioception [2] and inflammation [3–5].

There is evidence that SP-induced neurogenic inflammation contributes to the pathophysiology of arthritis. Studies in rats with adjuvant arthritis have shown that the SP content is increased in peripheral nerves innervating the inflamed joints [6]. In the same model, infusion of SP into the knee joint increased cartilage loss and soft tissue swelling [7]. Depletion of SP in sensory nerves, by the administration of capsaicin, resulted in attenuation of paw swelling and tenderness [8]. SP has also been detected in the synovial fluid of patients with inflammatory arthropathies [9]. The amount of SP present in the joint appears in part to be modulated by cytokines, including interleukin 1α (IL-1α) and tumour necrosis factor α [10].

SP may contribute to the pathogenesis of arthritis by virtue of its ability to activate a variety of inflammatory cells, including neutrophils [11,12,13], lymphocytes [14,15], mast cells [16], macrophages and monocytes [17,18]. SP has also been reported to stimulate rheumatoid synovial cell collagenase and PGE_2 production [19]. We have demonstrated that SP, NKA and NKB do not alter chondrocyte total protein or proteoglycan biosynthesis [20]. The lack of effect of SP on chondrocyte function does not preclude a role for tachykinins in inflammatory arthritis. It is possible that, *in vivo*, SP is rapidly hydrolysed by the metalloendoproteinase, neutral endopeptidase (NEP) (EC 3.4.24.11) [21] which has 94% sequence similarity with the integral membrane protein CD10 [22], also known as CALLA (common acute lymphoblastic leukaemia antigen). NEP is elevated in the synovial fluid of patients with rheumatoid arthritis but not osteoarthritis [23]. Incubation of SP with this enzyme generates a number of fragments, including the SP-(1–4), SP-(1–6) and SP-(7–11) fragments [24] which may themselves have significant biological effects and contribute to neurogenic inflammation. To test this hypothesis, we studied the effects of intact mammalian tachykinins and SP fragments on bovine articular chondrocyte collagenase and PGE_2 production. Furthermore, we have used the fluorescent indicator Fura-2 to determine whether tachykinins or SP fragments can alter intracellular free calcium concentration ($[Ca^{2+}]_i$).

MATERIALS AND METHODS

Cell culture

Bovine articular chondrocytes were isolated as follows. Articular cartilage shavings were digested at 37 °C for 18 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Cytosystems), 1.5 mg/ml bacterial collagenase (Sigma), 1.0 mg/ml bovine testicular hyaluronidase (Sigma) and 3% penicillin/streptomycin (Cytosystems). Chondrocytes were pelleted by centrifugation and washed twice in serum-free DMEM before being seeded into 24-well tissue culture plates in medium containing 10% heat-inactivated FCS, 1% penicillin/

Abbreviations used: $[Ca^{2+}]_i$, free intracellular calcium concentration; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's minimal essential medium; FCS, foetal calf serum; PGE_2, prostaglandin E_2; SP, substance P; NKA, neurokinin A; NKB, neurokinin B; (hr)IL-1α, (human recombinant) interleukin-1α; NEP, neutral endopeptidase; APMA, 4-aminophenylmercuriacetate; MMP-1, matrix metalloproteinase 1; TIMP, tissue inhibitor of metalloproteinases.

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streptomycin, 1% L-glutamine and 20 mM Hepes buffer. High-density (5×10^5 cells/ml) cultures containing only chondrocytes were usually confluent within 7 days. For measurement of intracellular Ca^{2+}, the chondrocytes were resuspended in Hanks' balanced salt solution (HBSS) before loading with Fura-2.

PGE_2 assay

Chondrocytes were cultured in serum-free medium for 18 h with various concentrations of neuropeptides or 100 units/ml human recombinant (hr) IL-1α. PGE_2 was measured in the culture medium by radio-immunoassay [24] using anti-PGE_2 serum and standard PGE_2 obtained from Sigma. Briefly, following a 2 h incubation of ^{3}H-labelled PGE_2 (Amersham) and anti-PGE_2 antibody with the culture medium at 37 °C, bound and unbound PGE_2 fractions were separated using cold dextran-coated charcoal.

Collagenase assay

Acid-soluble type I collagen was prepared from rat tails using the method of Bazin and Delaunay [25]. Collagen (20 μg) was plated into microwell modules (Nunc) on ice in the following buffers. Collagen [stock concentration 2 mg/ml in 0.2% (v/v) acetic acid] in neutralizing buffer (100 mM Tris/HCl, 200 mM NaCl, 0.04% NaN_3, pH 7.8) was gelled to the microwells by incubation for 16 h at 30 °C under humidified conditions, followed by a further 24 h incubation under dry conditions. The wells were washed in distilled water and allowed to dry at room temperature. Collagenase activity, i.e. matrix metalloproteinase 1 (MMP-1), in the culture medium was assayed using the spectrophotometric method of Nethery et al. [26]. Samples were mixed with a one-tenth volume of 1.0 M Tris and 0.2% NaN_3, pH 7.5. Latent collagenase was activated by incubation at 35 °C for 10 min with either 25 μg/ml trypsin in 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$ and 0.2% NaN_3, pH 7.5 (assay buffer), or with 1 mM 4-aminophenylmercuriacetate (APMA). Trypsin activity was inhibited with a 5-fold molar excess of soy bean trypsin inhibitor. Assays were performed for 18 h at 35 °C, after which wells were washed with deionized water and allowed to dry. The wells were stained with 100 μl/well Coomassie Brilliant Blue R250 (0.25 mg/ml in 50% methanol/10% acetic acid/40% water) for 25 min at room temperature. Wells were rinsed, allowed to dry and the absorbance was read at 590 nm on a spectrophotometer (Titertek Multiskan). Each assay contained the following controls: 25 μg/ml trypsin (measure of native collagen) in assay buffer, assay buffer alone (zero digestion) and conditioned medium from BC-1 cells, a rat mammary carcinoma cell line with high spontaneous tissue collagenase (MMP-1) activity (to act as a positive control for collagenolytic activity). The collagenolytic activity of each sample was expressed as units/ml, with 1 unit of activity being defined as that amount of enzyme required to degrade 1 μg of collagen/min per ml of sample at 35 °C.

To determine whether neuropeptides could induce the production of tissue inhibitor of metalloproteinases (TIMP), we inhibited TIMP activity in cell supernatants by the method of Dean and Woesner [27]. In brief, cell supernatants were incubated with 2 mM dithiothreitol at 37 °C for 30 min followed by a further treatment with 5 mM iodoacetamide for 30 min at 37 °C. Samples were dialysed against a buffer containing 50 mM Tris/HCl, 10 mM $CaCl_2$, 0.2 M NaCl, 0.05% Brij 35 and 0.02% NaN_3, pH 7.4, at 40 °C for 18 h. Control samples were treated with PBS under the same conditions as samples which had been TIMP-inactivated. Samples were then assayed for collagenase activity.

Measurement of $[Ca^{2+}]_i$

Bovine chondrocytes isolated by collagenase digestion were washed and incubated in 7 mM HBSS containing 1.3 mM $CaCl_2$, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4.0 mM $NaHCO_3$ and 0.3 mM Na_2HPO_4, pH 7.3. Chondrocytes were loaded with 1 μM Fura 2/AM for 30 min at 37 °C. After incubation, excess and non-hydrolysed Fura-2/AM was removed by washing twice with HBSS. Chondrocytes were resuspended in HBSS at 1×10^6 cells/ml and kept in a water-bath at 37 °C. Chondrocytes in HBSS were placed into glass cuvettes and placed in a Perkin–Elmer LS 50 fluorospectrophotometer using excitation and emission wavelengths of 340 nm and 510 nm respectively; slit-widths were both 10 mm [28]. Maximal fluorescence ($F_{max.}$) was determined by the addition of 0.1% Triton-100. Minimum fluorescence ($F_{min.}$) was determined by the simultaneous addition of 2 mM EGTA and 25 mM Tris/HCl. The change in $[Ca^{2+}]_i$ was calculated by the formula described by Grynkiewicz et al. [28] using a dissociation constant (K_d) for Fura-2 of 220 nM.

Neuropeptides

Tachykinins and fragments were purchased from AUSPEP and were dissolved in 0.01 M acetic acid and kept under N_2 at −70 °C until used experimentally. Control medium contained the highest concentration of acetic acid (0.01 M) in PBS. Acetic acid at this concentration was shown to have no effect on $[Ca^{2+}]_i$.

Statistics

Data are expressed as means ± S.E.M. of 3–4 separate experiments. Student's *t*-test was used to test for significance differences between means.

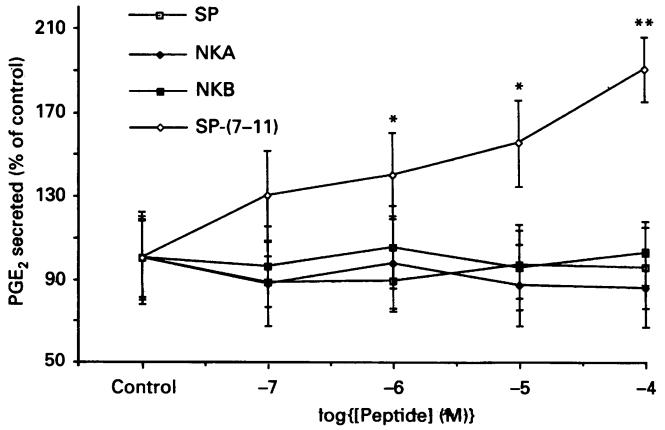

Figure 1 Effect of SP, NKA, NKB and the SP fragment SP-(7–11) on bovine chondrocyte PGE_2 secretion

Confluent chondrocytes were incubated with neuropeptide (0.1–100 μM) for 18 h. PGE_2 secreted into the medium was determined by radioimmunoassay. Values represent means ± S.E.M. for four separate experiments each performed in quadruplicate. $^*P < 0.05$; $^{**}P < 0.01$ compared with control. Data are expressed as percentages of the control value, which was 16.9 ± 3.2 ng/ml. The effect of SP-(7–11) could be inhibited by the presence of 15 μM indomethacin.

RESULTS

PGE_2 production is increased by SP-(7–11) in bovine chondrocytes

Confluent chondrocytes were incubated in serum-free medium containing 0.1–100 μM neuropeptide for 18 h. Incubation with hrIL-1α (100 units/ml) was used as a positive control, since it is a known biological stimulator of PGE_2 synthesis in chondrocytes. Typically 100 units/ml hrIL-1α increased PGE_2 secretion by 2–3-fold over control levels (results not shown). The C-terminal pentapeptide fragment SP-(7–11) at concentrations greater than 1 μM significantly increased PGE_2 secretion into the medium (Figure 1; $P < 0.05$ compared with control). The maximum effect was noted at 100 μM SP-(7–11), at which PGE_2 increased from control levels of 16.9 ± 3.2 to 34.8 ± 4.2 ng/ml ($P < 0.01$; $n = 4$). In contrast, SP, NKA and NKB had no effect on PGE_2 production. We found that spontaneous PGE_2 secretion was blocked by 93% in the presence of the cyclo-oxygenase inhibitor indomethacin (15 μM) and that the effect of SP-(7–11) (50 μM) was completely inhibited in the presence of indomethacin [control, 89.7 ± 15.0 ng/ml; 15 μM indomethacin, 6.2 ± 0.8 ng/ml; 15 μM indomethacin plus 10 μM SP-(7–11), 5.86 ± 0.73 ng/ml]. Spontaneous PGE_2 secretion by chondrocyts was unaffected by 100 μM or 2 mM EGTA. However, when chondrocytes were incubated with SP-(7–11) (10 μM) in the presence of EGTA (100 μM), PGE_2 secretion was reduced to control levels [control, 17.5 ± 2.1 ng/ml; SP-(7–11), 32.5 ± 3.2 ng/ml; 100 μM EGTA $\pm$ SP-(7–11), 16.3 ± 4.1 ng/ml; $n = 4$].

To determine the length of the C-terminal fragment necessary to elevate PGE_2, we studied the effects of fragments SP-(8–11) and -(9–11). Neither of these fragments stimulated PGE_2 production (results not shown). In a series of separate experiments the effects of the N-terminal fragments SP-(1–4) and SP-(1–6) were compared with those of SP-(7–11). Only the latter increased PGE_2 synthesis; the N-terminal peptides had no effect (Figure 2).

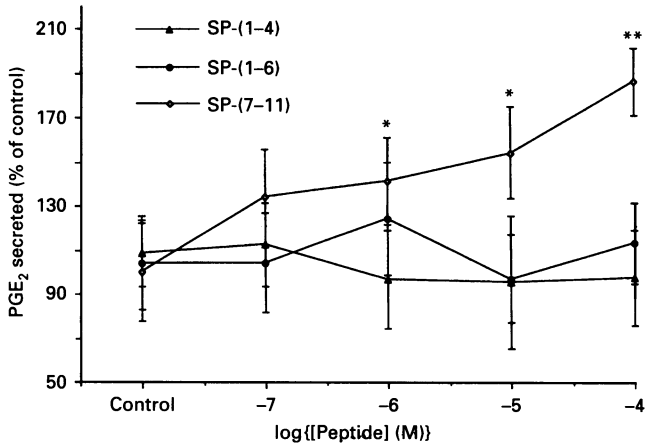

Figure 2 Effect of N-terminal and C-terminal SP fragments on PGE_2 secretion in bovine chondrocytes

The experimental protocol was as in Figure 1. Values represent means $\pm$ S.E.M. for four separate experiments each performed in quadruplicate, and are expressed as percentages of control. Control values were 15.8 ± 3.6 ng/ml (*$P < 0.05$; **$P < 0.01$ compared with control; $n = 4$).

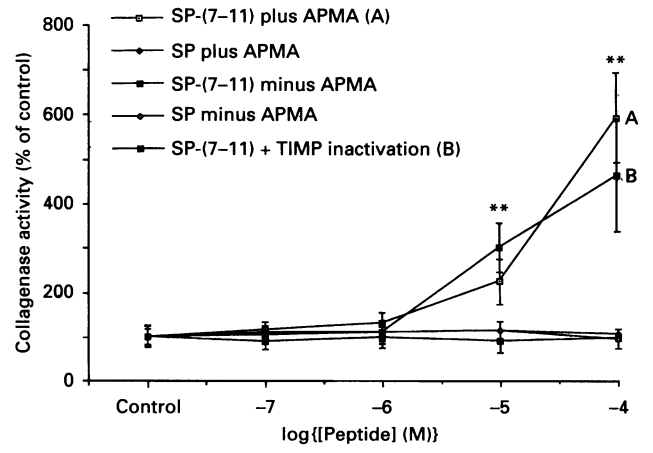

Figure 3 Effect of SP and SP-(7–11) on collagenase secretion by bovine chondrocytes

Confluent chondrocytes were incubated for 24 h with SP or SP-(7–11). TIMP activity was inactivated following reduction and alkylation with 2 mM dithiothreitol and 5 mM iodoacetamide for 30 min at 37 °C. Samples were subsequently dialysed as outlined in the Materials and methods section. Latent collagenase was activated with 1 mM APMA or 25 μg/ml trypsin followed by inactivation with soy bean trypsin inhibitor. Collagenase activity was measured as outlined in the Materials and methods section. Values represent means $\pm$ S.E.M. for four experiments and are expressed as percentages of the control, which was 13.8 ± 4.1 munits/ml (*$P < 0.05$, **$P < 0.01$ compared with control).

SP-(7–11) increases collagenase activity in bovine chondrocytes

Chondrocytes were incubated with neuropeptides (0.1–100 μM) in serum-free medium for 24 h, after which the medium was assayed for collagenolytic activity. SP had no effect on collagenase production in bovine articular chondrocytes at any of the concentrations tested (Figure 3). However, SP-(7–11) at concentrations greater than 1 μM significantly increased ($P < 0.05$; $n = 4$) collagenase levels in a dose-dependent manner. The response was maximal at 100 μM and was approx. 4 fold higher than control values [control and 100 μM SP-(7–11), 13.8 ± 4.1 and 60.8 ± 6.2 munits/ml respectively; $P < 0.01$, $n = 4$]. Only samples which had been activated with APMA or trypsin contained activity, suggesting that collagenase was secreted in a latent form (see Figure 3).

Following TIMP inactivation, we found that both treated and untreated media which had been exposed to SP-(7–11) contained increased collagenolytic activity. These results indicate that the effect of SP-(7–11) was due to an increase in collagenase synthesis and not to decreased TIMP production. Furthermore, treatment with cycloheximide completely inhibited the effect of SP-(7–11), suggesting that the increase in collagenase production was due to *de novo* synthesis, whereas treatment with indomethacin had no effect on collagenase production induced by SP-(7–11) (results not shown).

SP-(7–11) elevates $[Ca^{2+}]_i$ in isolated bovine articular chondrocytes

The Ca^{2+} ionophore A23187 (1 μM) induced an immediate elevation of $[Ca^{2+}]_i$, reaching maximal levels within 10 s of the initial stimulus (Figure 4a). The change in $[Ca^{2+}]_i$ was calculated as 295 ± 24 nM ($n = 4$) [28]. This response was maintained over a period of at least 120 s and had not returned to baseline levels before the addition of Triton X-100. Addition of hrIL-1α (1–1000 units/ml) had no effect on $[Ca^{2+}]_i$ (results not shown).

Addition of SP-(7–11) to isolated bovine chondrocytes at concentrations of 1–100 μM resulted in a gradual elevation in

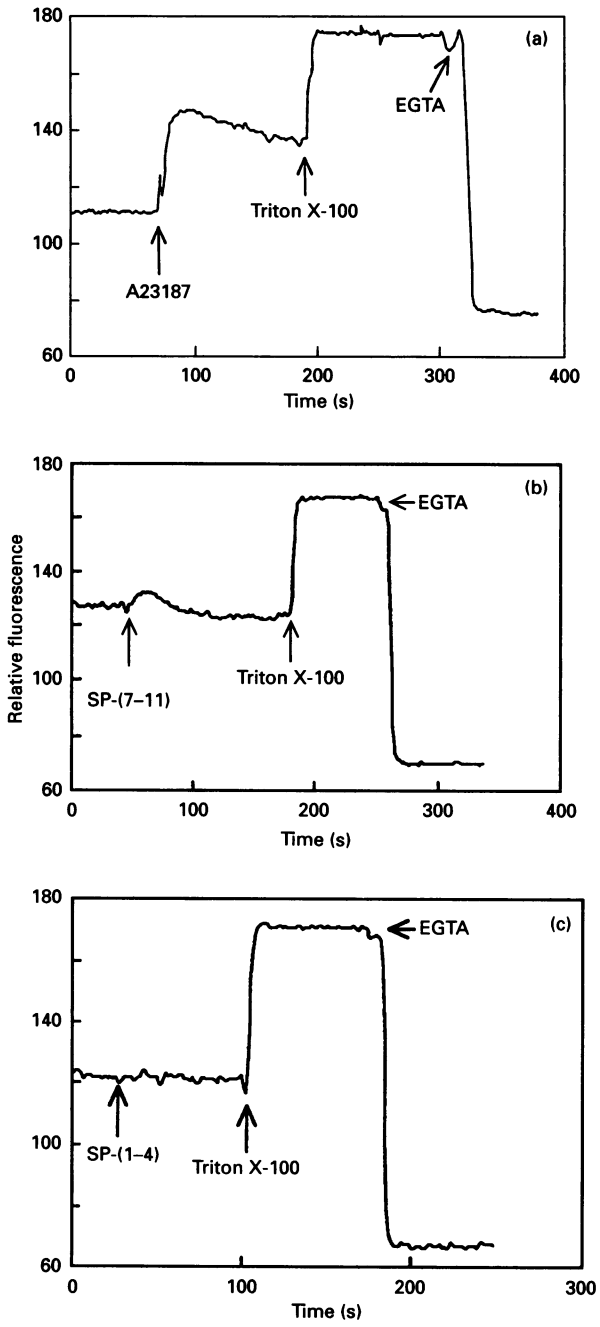

Figure 4 Changes in $[Ca^{2+}]_i$ in bovine articular chondrocytes

(**a**) Sustained increase in $[Ca^{2+}]_i$ in response to Ca^{2+} ionophore A23187. Fura-2-loaded chondrocytes were stimulated with 1 μM A23187. $F_{max.}$ was obtained by the addition of 0.1% Triton-X100; $F_{min.}$ was obtained by chelation of extracellular Ca^{2+} with 2 mM EGTA. The change in $[Ca^{2+}]_i$ was determined as described in the Materials and methods section. (**b**) and (**c**) Transient increases in $[Ca^{2+}]_i$ in response to S-(7–11) (**b**) and lack of response to the N-terminal fragment SP-(1–4) (**c**). Representative experiment showing the transient (40 s) increase in $[Ca^{2+}]_i$ in response to SP-(7–11) but not to SP-(1–4) (10 μM).

$[Ca^{2+}]_i$ from baseline levels (94 $\pm$ 3.3 nM) (n = 4) which peaked 20–25 s after the initial stimulus and returned to baseline values after 40–50 s. The maximal change in $[Ca^{2+}]_i$ was calculated as 140 $\pm$ 30 nM (n = 4) with 10 μM SP-(7–11) (Figure 4b). Furthermore, the effect of SP-(7–11) was refractory to a second stimulus following recovery intervals of between 1 and 2 min (results not shown). Neither SP nor the N-terminal peptides SP-(1–4) and SP-(1–6) altered $[Ca^{2+}]_i$ (Figure 4c). NKA, NKB and the SP fragments SP-(8–11) and SP-(9–11) also failed to induce a change in $[Ca^{2+}]_i$ (results not shown).

The addition of either 1 mM or 2 mM EGTA to chelate the extracellular Ca^{2+} abolished the increase in intracellular $[Ca^{2+}]_i$ induced by 10 μM SP-(7–11), i.e. $[Ca^{2+}]_i$ levels did not change from the baseline levels of 96.4 $\pm$ 7.3 nM.

DISCUSSION

We have studied the effects of the mammalian tachykinins SP, NKA and NKB and N- and C-terminal fragments of SP on bovine articular cartilage chondrocyte function. We found that the C-terminal fragment SP-(7–11) (Phe-Phe-Gly-Leu-Met-NH_2), but not SP, NKA, NKB nor the N-terminal fragments SP-(1–4) or SP-(1–6), stimulated collagenase and PGE_2 secretion and increased $[Ca^{2+}]_i$ (see Figure 5).

The mechanism of this activation has not been fully determined. One possibility is that PGE_2 and collagenase levels are enhanced by the increase in $[Ca^{2+}]_i$ which is induced by SP-(7–11). The source of increased intracellular Ca^{2+} appears to be of extracellular origin as chelation with EGTA prior to the addition of SP-(7–11) greatly reduced the effect of the peptide fragment on both PGE_2 and $[Ca^{2+}]_i$. Mobilization of intracellular Ca^{2+} from microsomal stores therefore does not appear to contribute to this effect. However, the intracellular processes by which PGE_2 and collagenase production increase appear to be independent of each other, as incubation with cyclo-oxygenase inhibitors did not influence collagenase secretion in response to SP-(7–11).

Phospholipase A_2, the enzyme which releases arachidonic acid from phospholipids, is regulated by calmodulin [29], which is activated and undergoes a considerable conformational change after binding intracellular Ca^{2+}. In rabbit articular chondrocytes, the Ca^{2+} channel blockers nifedipine, verapamil and diltiazem had no effect on collagenase synthesis. However, inhibitors of internal Ca^{2+} movement do inhibit collagenase synthesis [30], suggesting that $[Ca^{2+}]_i$ is involved in the regulation of collagenase biosynthesis in chondrocytes.

The receptor-mediated effects of SP are dependent on the C-terminal sequence of the undecapeptide. This requirement has been demonstrated in the central nervous system, for T lymphocyte proliferation and for release of interleukins from human monocytes [31,32]. Receptor-mediated activation requires low concentrations (10^{-9}–10^{-6} M) of SP. However, far higher concentrations (micromolar) of SP are required to release histamine from rat peritoneal mast cells *in vitro* [33], an effect mediated by the N-terminal domain sequence of SP (Arg-Pro-Lys-Pro) [33,34]. The existence of a receptor for the N-terminal domain has been questioned, since radioligand binding studies have failed to demonstrate a specific SP receptor on mast cells. It has been postulated by Devillier et al. [33] and also by Mousli et al. [35,36] that SP can directly activate G proteins independently of a specific SP receptor. Since SP-(7–11) activates PGE_2 and collagenase secretion at concentrations greater than 1 μM, it is possible that it may also directly activate the α subunit of G proteins, resulting in stimulation of PGE_2 and collagenase synthesis.

The observation that SP has no effect on chondrocyte function, and yet SP-(7–11) does, suggests that catabolism of SP in the joint may regulate the biological activity of SP on connective tissue and other cells of the joint. In several tissues, including airways, SP actions are regulated by peptidases which hydrolyse

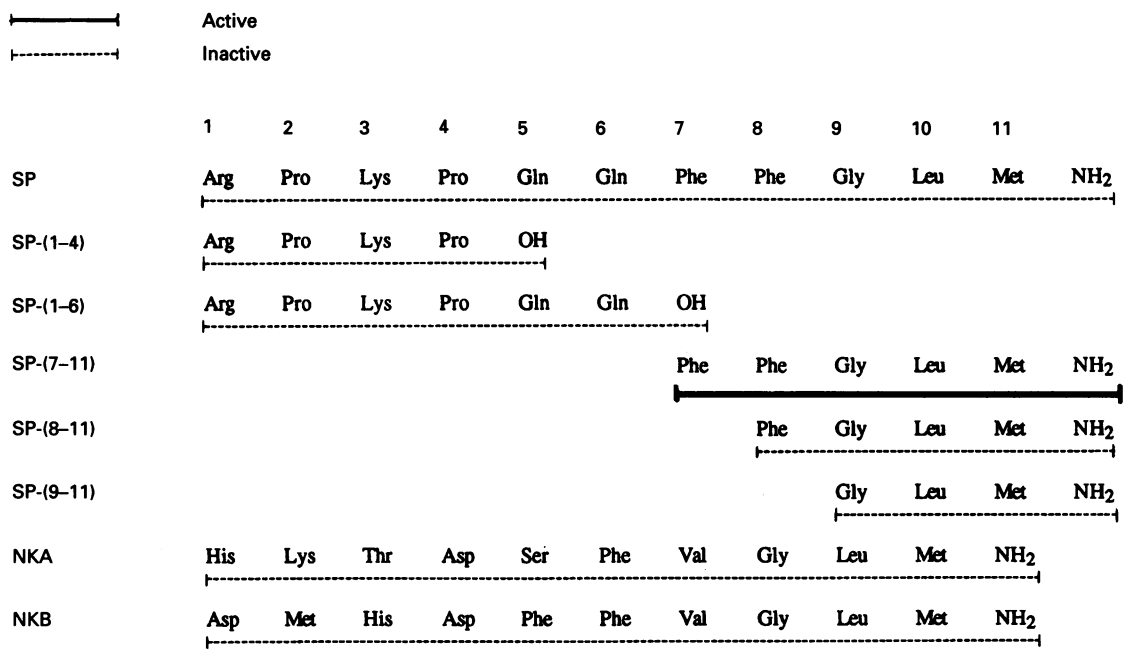

Figure 5 Amino acid sequences of SP and related tachykinins and SP fragments achieved by NEP cleavage, indicating their ability to induce chondrocyte PGE_2 and collagenase secretion

this peptide. In the joint it is likely that SP is rapidly hydrolysed by NEP, which is present on the cell membrane of human neutrophils [37–39]. NEP hydrolyses proteins on the N-terminal side of phenylalanine and there are two such sites in SP. In particular, a cleavage site between Gln-6 and Phe-7 would generate the SP-(7–11) fragment [40], although this fragment has not been quantified in synovial fluid. Human neutrophils contain high concentrations of intracellular cathepsin G [41]. SP is also cleaved by cathepsin G, but the primary site of cleavage is between Phe-7 and Phe-8, generating the SP-(1–7) and SP-(8–11) fragments, neither of which are biologically active in bovine chondrocytes. Therefore we suggest that the regulation of NEP may be relevant for SP metabolism and the subsequent induction of joint pathology by the resultant SP-(7–11) fragment.

NEP is a membrane-bound zinc-metallopeptidase which cleaves opioid peptides and enkephalins *in vitro* [42] and *in vivo* [43]. These metal-ion-dependent enzymes are induced by exposure to growth factors, tumour promoters and cytokines [44]. In the inflamed joints, levels of NEP in the synovial fluid are increased [23], possibly in response to cytokines, resulting in the likely generation of SP-(7–11). This fragment stimulates articular chondrocyte collagenase production and increases PGE_2 synthesis, both of which contribute to joint destruction.

In conclusion, SP does not appear to be directly involved in chondrocyte-mediated joint destruction via any effect on collagenase and PGE_2 synthesis. In this regard only the C-terminal pentapeptide sequence SP-(7–11) is biologically active in bovine articular chondrocytes. Furthermore, the induction of both collagenase and PGE_2 synthesis appear to be related in part to an increase in $[Ca^{2+}]_i$.

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