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Regulation of hyaluronan secretion into rabbit synovial joints *in vivo* by protein kinase C

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Hyaluronan (HA) is important for joint cavitation, lubrication, volume regulation and synovial fluid drainage but little is known about the regulation of joint HA synthesis/secretion *in vivo*. We investigated whether HA secretion into joints *in vivo* can be regulated by protein kinase C (PKC). Secretion into the knee joint cavity of anaesthetised rabbits was measured over 6 h by washout and chromatography. Joints received intra-articular injections of Ringer vehicle (control) or an activator of classical PKC isoforms, phorbol-12-myristate-13-acetate (PMA), at 20–2000 ng ml^{-1}. The effects of PKC inhibition by bisindolylmaleimide (BIM) and protein synthesis inhibition by cycloheximide (CX) on basal and stimulated HA secretion were also studied. The endogenous HA mass, 181 ± 8 μg (n = 26, mean ± S.E.M.), and basal secretion rate, 4.4 ± 0.4 μg h^{-1}, indicated a turnover time of 41 h. Secretion rate showed a dose-dependent response to PMA (n = 30), rising 5-fold to 21.7 ± 5.0 μg h^{-1} (n = 5) at 2000 ng ml^{-1} PMA (P < 0.0001, one-way ANOVA). PMA-induced stimulation was partially suppressed by CX (HA secretion: 5.8 ± 1.7 μg h^{-1}, n = 8, P < 0.01) and totally blocked by BIM (HA secretion: 3.2 ± 0.6 μg h^{-1}, n = 9, P < 0.001). Basal HA secretion was unaffected by CX over 6 h (4.2 ± 0.7 μg h^{-1}, n = 8) but was reduced by 29 % by BIM (3.1 ± 0.6 μg h^{-1}, n = 10, P = 0.03). It is concluded that: (1) PKC can stimulate HA secretion into joints *in vivo* through mechanisms involving protein synthesis *de novo* as well as phosphorylation; (2) basal HA secretion is only partially PKC dependent; and (3) hyaluronan synthase turnover time is > 6 h *in vivo*, which is slower than *in vitro* (< 2–3 h).

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Hyaluronan, a long-chain polysaccharide of $>10^6$ Da composed of repeating *N*-acetylglucosamine-D-glucuronic acid disaccharides, is a multifunctional extracellular matrix component with key roles in cardiac embryogenesis, cell motility and migration in wound healing and cancer, cartilage biomechanics, tissue hydraulic permeability and joint function (Tammi *et al.* 2002). Joint hyaluronan is crucial both for cavitation during embryogenesis (Ward *et al.* 1999) and for mature joint function. The hyaluronan in the synovial fluid of a mature joint (~3.6 mg ml^{-1}) has two main physiological roles. First, as long recognised, hyaluronan confers hydrodynamic viscous lubrication. Second, and only recently recognised, it buffers fluid loss from joints during flexion, which raises synovial fluid pressure. The hyaluronan acts via an osmotic, concentration polarisation mechanism that depends on the partial reflection of hyaluronan by the synovial lining during fluid escape. This process prevents a joint from wringing itself dry during a sustained flexion. Synovial fluid conservation is also supported by hyaluronan within the synovial lining matrix, where the presence of anchored hyaluronan causes a high hydraulic resistance (Levick *et al.* 1999).

Hyaluronan is secreted into joints by B-type lining cells or 'synoviocytes', which are distinguished from fibroblasts by high levels of uridine diphosphoglucose dehydrogenase, *N*-acetyl-β-glucosaminidase, oxidative enzymes and a 6-fold greater production of soluble hyaluronan (Pitsillides *et al.* 1993; Iwanaga *et al.* 2000). Two observations indicate that the secretory process *in vivo* is subject to a relatively rapid physiological regulation. First, acute hydration and/or stretch stimulates hyaluronan secretion into synovial interstitium over a few hours (Price *et al.* 1996), as may also be the case in skin, lung and intestine (e.g. Townsley *et al.* 1994). Second, joint distension increases hyaluronan secretion into the cavity within 4 h (Coleman *et al.* 1997). The physiological significance of these secretory responses is thought to be homeostasis, namely the preservation of high, functional hyaluronan concentrations in the face of dilutional influences such as increased capillary filtration into the joint cavity.

The pathways responsible for regulating the rate of hyaluronan secretion into joints *in vivo* have never been investigated. Such evidence as exists comes from work on

cultured cell lines *in vitro*. Biochemical studies show that hyaluronan is synthesised at the inner face of the plasma membrane by a membrane-spanning 63 kDa protein, hyaluronan synthase (HAS). A cytoplasmic loop catalyses the alternate addition of uridine diphosphoglucose dehydrogenase (UDP)-glucuronate and UDP-*N*-acetyl-glucosamine to the internal, growing end of the hyaluronan chain, while the opposite end is continuously extruded into the extracellular space through a channel formed by transmembrane domains (Weigel *et al.* 1997; Itano *et al.* 1999; Recklies *et al.* 2001). The physiological significance of this unique process is that synthesis and secretion are intimately coupled; there is no vesicle storage and release phase. Any increase in hyaluronan secretion rate is a direct consequence of increased hyaluronan synthesis by HAS.

How might HAS activity be regulated *in vivo*? One mechanism operating over a period of hours could be altered genomic transcription and synthesis of HAS *de novo*, a process dependent on protein synthesis. HAS turnover can be rapid *in vitro*, as indicated by a HAS half-life of ~100 min in cultured chondrocytes (Bansal & Mason, 1986) and 120–180 min in Swiss 3T3 fibroblasts (Kitchen & Cysyk, 1995). A second potential regulatory mechanism is the phosphorylation of pre-existing HAS and/or a regulatory protein (Klewes & Prehm, 1994; Suzuki *et al.* 1995). HAS may exist as a complex with other regulatory factors (Asplund *et al.* 1998) but the nature of such factors is unclear in eukaryotic cells. A phosphorylation step at some point along the regulatory pathway (whether pre- or postgenomic) is indicated by the finding that hyaluronan secretion by cultured mesothelial cells, B6 cells and fibroblasts is stimulated by growth factors (e.g. platelet-derived growth factor) and some cytokines (e.g. interleukin β_1) (Heldin *et al.* 1992; Klewes & Prehm, 1994), which activate classical and novel isoforms of protein kinase C (PKC) via the phospholipase C-γ–diacylglycerol (DAG) pathway. Direct activation of PKC by phorbol esters likewise stimulates hyaluronan secretion *in vitro*.

The phenotype and metabolic response of cultured cells can in some instances differ substantially from cells *in situ*, due to their altered cellular micro-environment. Mature synoviocytes *in situ* are non-confluent cells anchored to a complex interstitial matrix by heterogeneous receptors, whereas cultured fibroblasts etc. are usually examined at confluence on a mono-anchor such as fibronectin or collagen. The aims of the present study were, therefore, first to determine whether PKC influences hyaluronan secretion into joints by synoviocytes of normal, physiological phenotype *in situ*; and second to assess whether *de novo* protein synthesis is necessary for the stimulation of hyaluronan secretion *in vivo*. To this end synovial PKC was activated using intra-articular phorbol-12-myristate-13-acetate (PMA) in the presence and absence of the protein synthesis inhibitor cycloheximide. Some of the findings have been published in abstract form (Anggiansah *et al.* 2001).

METHODS

Hyaluronan secretion into the knee joint cavity was measured in a total of 45 rabbits over 6 h *in vivo*, using the washout method of Coleman *et al.* (1997). One knee received intra-articular injections of active agent (PMA +/− inhibitors) and the opposite knee received an equal volume of vehicle or PMA without inhibitor as appropriate. The hyaluronan recovered in the washes was analysed by high performance gel exclusion chromatography.

Materials

Phorbol-12-myristate-13-acetate (PMA, 617 Da, Calbiochem, Nottingham, UK) was dissolved in a 50 : 50 dimethyl sulphoxide (DMSO)–ethanol at 1 mg ml^{-1} and diluted with Ringer solution to its final concentration. Solutions of 200–2000 ng ml^{-1} PMA contained 0.01–0.10 % DMSO by volume and the same concentration of ethanol. PMA at these concentrations acts at the DAG binding site to activate the classical Ca^{2+}-dependent isoforms α, βI, βII and γ and the Ca^{2+}-independent novel isoforms δ, ϵ, η and θ, but not the atypical isoforms ζ, λ and ι (Castagna *et al.* 1982; Mellor & Parker, 1998). Bisindolylmaleimide I (BIM, 412 Da, Calbiochem) is a highly selective, cell-permeable inhibitor of all of the PKC isoforms tested to date, namely α, βI, βII, γ, δ, ϵ. It competitively inhibits the ATP binding site of the PKC catalytic domain (Toullec *et al.* 1991). Cycloheximide (CX, 281 Da, Sigma, Poole, UK) is an inhibitor of ribosomal translation that is widely used to block protein synthesis. The Ringer solution was a commercial, sterile, nonpyrogenic product comprising (mM): 147 Na^+, 4 K^+, 2 Ca^{2+} and 156 Cl^- (Baxter Healthcare Ltd, Thetford, UK).

Animal preparation

New Zealand white rabbits of 2.5–3.0 kg were anaesthetised by I.V. sodium pentobarbitone (30 mg kg^{-1}) and ethyl carbamate (500 mg kg^{-1}). Corneal blink reflex inhibition was maintained by repeat doses at 30 min intervals. After tracheostomy and pump ventilation with air at 700 ml min^{-1} both knee joints were placed in 100–130 deg extension. Each cavity was cannulated lateral to the patellar ligament by an intravenous, 20 gauge, sterile, nonpyrogenic Medicut cannula (Sherwood Medical, Tullamore, Ireland), secured in place by a purse-string suture. The experiments conformed to UK 1986 animal legislation. Animals were killed by an overdose of I.V. sodium pentobarbitone (2 ml 'Euthatal', Rhône Mérieux Ltd, Harlow, UK) at the end of the experiment (~6 h).

Hyaluronan mass recovery

A 1 ml volume of Ringer solution was injected slowly into the synovial cavity and the joint flexed and extended five times to mix the intra-articular fluid. The fluid was then aspirated, aided by gentle pressure over the popliteal fossa. The aspirate was stored at −80 °C for later analysis. Washout with 1 ml fresh Ringer solution was repeated 15 times. Hyaluronan recovery is essentially complete by the 12th to 14th wash (Coleman *et al.* 1997; confirmed here). The washout was performed on both knee joints simultaneously by two operators.

After the 15th wash each joint received a 1 ml bolus of test solution, namely PMA +/− inhibitor on one side and PMA alone or vehicle (Ringer solution with a matching concentration of DMSO and ethanol) on the contralateral side, depending on the

particular protocol. The joints were then left for 6 h to secrete fresh hyaluronan. Higher drug concentrations were injected *in vivo* (see later) than is customary in cell culture because small, rapidly diffusible solutes are cleared rapidly by the synovial microcirculation (clearance ~55 μl min^{-1}; Poli *et al.* 2002). To maintain the concentration of active agent in the face of continuous microvascular clearance, ~0.3 ml intra-articular fluid was aspirated every 20 min and replaced with 0.5 ml fresh solution; trans-synovial drainage over 20 min accounted for the volume difference. The adopted replacement interval was roughly equal to the intra-articular half-life, namely 14–24 min for solutes of 370–582 Da (Poli *et al.* 2001), so that intra-articular concentration fell by no more than a half before replenishment. Aspirates were saved for analysis. After 6 h each joint was washed out with a further 15 × 1 ml Ringer solution to harvest the hyaluronan secreted *de novo*.

Previous studies established that basal hyaluronan secretion rate is independent of time over 2–4 h (Coleman *et al.* 1997). In the present experiments the secretory period was extended to 6 h because cells *in vitro* respond maximally to PKC over 3–6 h (Heldin *et al.* 1992). Previous control studies using metabolic blockers showed that the hyaluronan recovered in the final set of washouts represents newly synthesised material, not pre-existing hyaluronan that has diffused into the cavity (Coleman *et al.* 1997).

Sample preparation

Freeze-dried aspirates were reconstituted in 300 μl Ringer solution and treated with papain (5.6 units at 60–65 °C for 1 h; Sigma, UK) to prevent partial masking of the small hyaluronan band by the shoulder of a large albumin band; interstitial albumin diffuses into the joint from the surrounding tissues. The papain digestion step does not alter the molecular weight of the hyaluronan (Coleman *et al.* 1997). Samples were centrifuged at 7.2 *g* for 5 min prior to high pressure liquid chromatography (HPLC).

Hyaluronan analysis by HPLC

The recovered hyaluronan mass was quantified using size exclusion HPLC. The system comprised a Waters 2690 separation module (Waters Ltd, Watford, UK) with a TosoHaas TSK G6000 PW_{XL} 300 mm × 7.8 mm column and PW_{XL} 40 mm × 6 mm guard column (Anachem Ltd, Luton, UK). The Tosohaas TSK-Gel had a nominal resolution range of 40–8000 kDa (pore size > 100 nm, particle size 13 μm). Elution profiles were measured by a Waters 486 programmable ultraviolet absorbance detector set at 206 nm for hyaluronan analysis. Retention times and peak heights were analysed using Waters Millenium[32] software. Injection volume was 50–100 μl and a column flow 1 ml min^{-1}. The eluent was Ringer solution (composition as in 'Materials') at room temperature adjusted to pH 7.4 by 1 M sodium hydroxide solution. This was vacuum filtered through a 0.2 μm membrane (Gelman Bioscience, Northhampton, UK).

A calibration curve was constructed for each batch of samples using known concentrations of rooster comb sodium hyaluronate (Sigma, Poole, UK). The molecular mass of the standard, ~2000 kDa, is close to that of endogenous rabbit hyaluronan. The calibration curve was linear from 0.006 to 0.400 mg ml^{-1} (Coleman *et al.* 1997). A molecular size calibration curve was constructed by measuring the retention times of hyaluronan standards of mean molecular mass 210–5500 kDa donated by Dr O. Wik (New Pharmacia, Uppsala, Sweden). The standard molecular weights had been determined by laser light scattering. Retention times were essentially insensitive to pH over the range 7.0–7.4.

Protocols

Four sets of experiments were carried out. In the first the effect of PMA at concentrations ranging from 20 to 2000 ng ml^{-1} was explored. In the second, injections of 200 ng ml^{-1} PMA plus 3.3 μg ml^{-1} BIM were used to test whether the stimulatory effect of PMA is mediated specifically through kinase activation. In the third set, injections of 200 ng ml^{-1} PMA plus 100 μg ml^{-1} CX were used to test whether the stimulatory effect of PMA depends on protein synthesis *de novo* (cf. activation of pre-existing synthase). The fourth set examined the effects of BIM and CX on basal hyaluronan secretion rate, in order to test whether the basal secretion of hyaluronan into the joint depends on basal PKC activity and/or short-term protein synthesis.

Subsidiary experiments were carried out to test whether PMA causes significant synovial inflammation. Inflammation was assessed as increased fluid filtration into the joint and increased permeation of plasma albumin labelled with Evans blue into the joint cavity, as described by Poli *et al.* (2001, 2002). Other subsidiary experiments tested whether the organic solvents in the vehicle, namely DMSO and ethanol, influenced hyaluronan secretion.

Statistical analysis

Means ± S.E.M. are cited throughout. One-way ANOVA followed by the Newman-Keuls test was used for multiple comparisons. All distributions passed the Kolmogorov-Smirnov normality test at $P > 0.10$, including those in Fig. 5. The results in the middle panel of Fig. 5 appear to be skewed, however (KS statistic = 0.31). For this reason the nonparametric Mann-Whitney *U* test was used for unpaired groups. Significance was accepted at $P \leqslant 0.05$.

RESULTS

Basal hyaluronan secretion rate and endogenous hyaluronan turnover time

The hyaluronan concentration in each wash decreased exponentially with wash number and fell below the HPLC detection limit of 3 μg ml^{-1} by wash 12–14. The chromatogram retention time of 7.05 ± 0.01 min corresponded to an average molecular mass for endogenous hyaluronan of 2.2–2.3 × 10^6 Da (95 % confidence intervals). The total hyaluronan mass recovered per joint was 181 ± 8 μg (n = 90 knees) and net volume recovery was 94 ± 1 % of the injectate. The endogenous hyaluronan mass divided by the mean hyaluronan concentration in rabbit knee synovial fluid, 3.6 mg ml^{-1} (Price *et al.* 1996), indicated that the native synovial fluid volume was 50 μl.

The mean hyaluronan secretion rate into the control joints, i.e. those receiving drug-free injections, was 4.35 ± 0.37 μg h^{-1} (n = 26 joints), close to that in a smaller study of basal secretion by Coleman *et al.* (1997), namely 4.80 ± 0.77 μg h^{-1} (n = 5). The basal secretion rate divided by the mean endogenous mass gave the turnover rate constant k, 0.0244 h^{-1}. This is equivalent to a hyaluronan intra-articular half-life of 28 h (ln 2/k) and a turnover time for the endogenous hyaluronan mass of 41 h.

Effect of PMA on synovial hyaluronan secretion

Initially a set of eight paired experiments was carried out with PMA in one joint and vehicle in the contralateral joint

(Fig. 1, upper panel). The PMA concentration of 200 ng ml^{-1} (0.32 μM) was based on work on cultured B6 hybrid cells (Klewes & Prehm, 1994). In eight pairs of rabbit knees the PMA raised the hyaluronan secretion rate > 3-fold, from 3.4 ± 0.6 μg h^{-1} in the vehicle-treated control joints to 10.7 ± 1.6 μg h^{-1} in the PMA-treated joints ($P = 0.002$, paired t test). In a total of 14 joints treated with 200 ng ml^{-1} PMA the mean hyaluronan secretion rate, 10.1 ± 1.2 μg h^{-1}, was 2.3 times the mean basal rate, 4.35 ± 0.37 μg h^{-1} ($n = 26$; $P < 0.0001$, Mann-Whitney U test) (Fig. 1, lower panel).

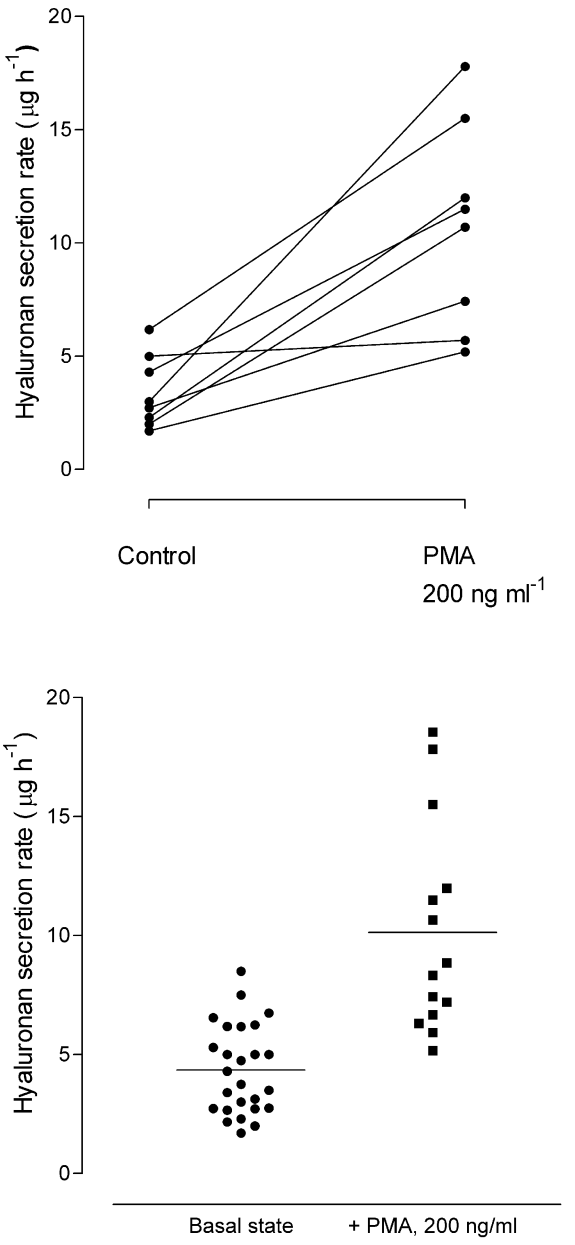

Figure 1. Stimulation of hyaluronan secretion into the cavity of the rabbit knee by phorbol-12-myristate-13-acetate (PMA)

Upper panel, paired experiments in which joint 1 received control injections of vehicle and joint 2 of the same animal received PMA at 200 ng ml^{-1} in vehicle ($P = 0.002$, paired t test). Lower panel, pooled results and mean (horizontal line) from 26 control joints and 14 PMA-treated joints. The secretion rate was significantly raised by PMA ($P < 0.0001$, Mann-Whitney U test).

The HPLC retention times for the PMA-stimulated hyaluronan, 7.24 ± 0.08 min, corresponded to 1.8–2.2 × 10^6 Da (95 % confidence intervals), showing that PMA-stimulated synovium continued to synthesise long HA chains. This was in keeping with the finding that, of the three mammalian isoforms HAS1, HAS2 and HAS3, rabbit synovium expresses predominantly HAS2 mRNA, which generates chains of > 2 × 10^6 Da (Spicer & Nguyen, 1999; Ohno *et al.* 2001; Momberger *et al.* 2003). HAS3, which is upregulated by pro-inflammatory cytokines, synthesises shorter chains (0.1–1.0 × 10^6 Da; Itano *et al.* 1999; Tanimoto *et al.* 2001).

Dose–response relation

To assess the dose–response relation, joints received intra-articular injections of 1 ml vehicle (control, $n = 26$) or PMA at 20 ($n = 5$), 100 ($n = 3$), 200 ($n = 14$), 500 ($n = 3$) and 2000 ng ml^{-1} ($n = 5$). The hyaluronan secretion rate increased as a function of PMA concentration ($P < 0.0001$, one-way ANOVA), though not in a simple log–sigmoidal manner (Fig. 2). At 20, 100 and 200 ng ml^{-1} PMA the hyaluronan secretion rate concentration increased to 7.6 ± 2.4, 9.8 ± 4.2 and 10.1 ± 1.2 μg h^{-1}, respectively. At 500 ng ml^{-1} PMA there was no further significant increase in the secretion rate (8.5 ± 0.8 μg h^{-1}) and the differences between this value and those at 20, 100 and 200 ng ml^{-1} PMA were not significant ($P > 0.05$, Newman-Keuls multiple comparison test). Because the shape of dose–response curve up to 500 ng ml^{-1} hinted at a possible decay above 200 ng ml^{-1}, the study was extended to 2000 ng ml^{-1} PMA (3.2 μM). This caused a further, very large increase in hyaluronan secretion rate to 21.7 ± 5.0 μg h^{-1}, which was more than double the stimulated rates evoked by 200–500 ng ml^{-1} PMA ($P < 0.01$, Newman-Keuls multiple comparison test) and five times greater than the basal rate. Given the shape of the dose–response relation in Fig. 2, it is not possible to define an EC_{50}. The hyaluronan chromatography retention times at the highest secretion rates (6.87 ± 0.01 min, $n = 49$ samples) were almost identical to those of the matched endogenous hyaluronan washouts (6.88 ± 0.01 min; $n = 58$; $P = 0.4$, Mann-Whitney test), showing that hyaluronan of high molecular mass was still being secreted.

Effect of BIM on PMA-stimulated hyaluronan secretion

The effect of 200 ng ml^{-1} PMA (0.32 μM) in the presence of the PKC inhibitor BIM (3.3 μg ml^{-1}, 8 μM) was studied in order to test whether PMA stimulated hyaluronan secretion through kinase activation. The selected BIM concentration was above that which causes 100 % inhibition of PMA-induced protein phosphorylation in cultured Swiss 3T3 fibroblasts (5 μM, Toullec *et al.* 1991). BIM completely abolished the stimulation of

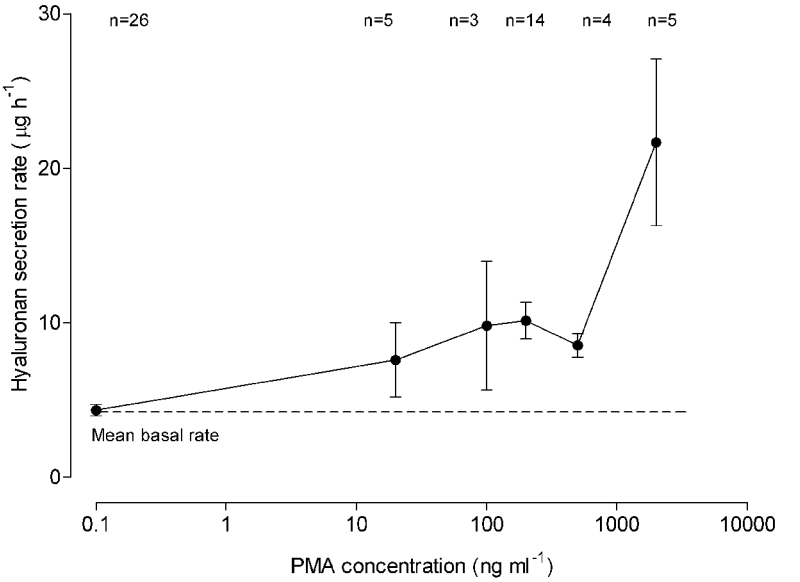

Figure 2. Dose–response relation for effect of PMA on hyaluronan secretion into joints (means ± S.E.M.)

Basal secretion rate of hyaluronan in the absence of PMA is plotted at 0.1 ng ml^{-1} on the logarithmic scale.

hyaluronan secretion by PMA (Fig. 3). In nine joints treated with both PMA and BIM the mean hyaluronan secretion rate, 3.16 ± 0.56 μg h^{-1}, was one-third of that evoked by the PMA alone ($P < 0.001$, Newman-Keuls multiple comparison test). The secretion rate in the presence of PMA and BIM was not significantly different from the basal secretion rate of 4.4 ± 0.4 μg h^{-1} ($P > 0.05$, Newman-Keuls multiple comparison test), which leads to the inference that most of the basal hyaluronan secretion is not dependent on a basal level of PKC activity. This issue was subsequently tested directly (see below).

The above PMA + BIM experiments were unpaired because early paired experiments, in which PMA + BIM were injected into one knee and PMA alone into the contralateral knee, indicated that sufficient BIM is absorbed into the circulation *in vivo* to inhibit the contralateral joint (Fig. 3, right column). In seven animals treated with 200 ng ml^{-1} PMA in one joint and PMA + BIM in the contralateral joint, the hyaluronan secretion rate in the PMA-alone joint was 3.9 ± 0.6 μg h^{-1} ($n = 7$ 'cross-circulation' joints), compared with 10.1 ± 1.2 μg h^{-1} ($n = 14$) in joints injected with 200 ng ml^{-1} PMA in the absence of circulating BIM ($P < 0.001$, Mann-Whitney U test). The IC_{50} of BIM is ~0.01 μM *in vitro* (Toullec *et al.* 1991), and a calculation based on the intra-articular half-life and replacement protocol indicates that whole-body extracellular fluid concentration in a 3 kg rabbit should reach 0.01 μM within the first hour, neglecting renal excretion.

Effect of CX on PMA-stimulated hyaluronan secretion

To test whether the stimulatory effect of PKC on hyaluronan secretion is mediated by the induction of new protein synthesis, the ribosome translation inhibitor CX (100 μg ml^{-1}, 0.36 mM) was infused with PMA (200 ng ml^{-1}) into eight joints. The concentration of CX exceeded that which completely inhibits protein synthesis in cultured chondrocytes (Bansal & Mason, 1986),

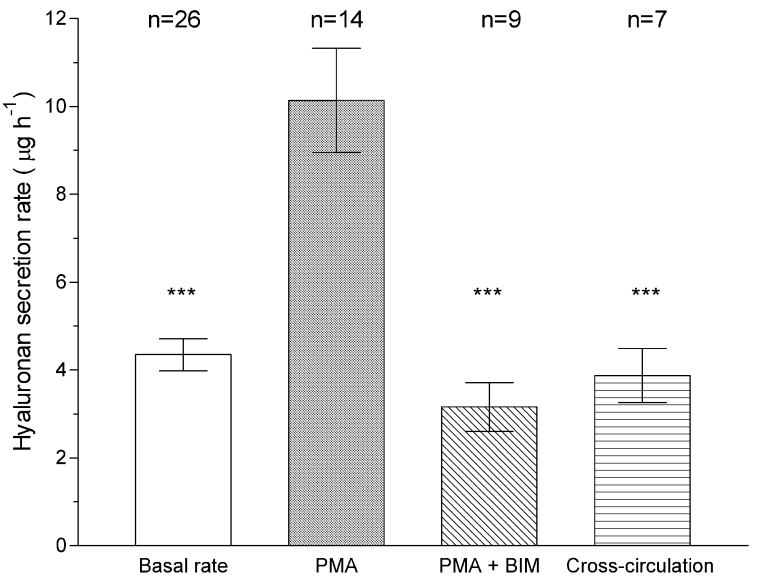

Figure 3. Inhibition of PMA-induced hyaluronan secretion by bisindolylmaleimide (BIM, 3.3 μg ml^{-1})

The infused PMA concentration was zero for the left column (basal rate) and 200 ng ml^{-1} for the other 3 columns. The column labelled 'cross-circulation' shows the secretion rate in joints that received PMA while the contralateral joint simultaneously received PMA + BIM; the low secretion rate in the PMA-stimulated joints is attributed to circulating BIM. Means ± S.E.M.; *** $P < 0.001$, Newman-Keuls test comparison with PMA; differences between columns 1, 3 and 4 were not significant.

fibroblasts (Smith *et al.* 1995) and smooth muscle (Bartlett *et al.* 1999) but is not cytotoxic (Mapleson & Buchwald, 1981). CX substantially reduced the stimulatory effect of PMA on hyaluronan secretion, though not to the same degree as BIM; the hyaluronan secretion rate remained above the basal level. In four paired experiments where one joint received PMA and the other PMA + CX, CX reduced the secretion rate in every case, from 13.0 ± 3.8 μg h^{-1} in the presence of PMA to 8.1 ± 3.3 μg h^{-1} in the presence of PMA + CX ($P = 0.04$, paired t test) (Fig. 4, upper panel). In a total of eight joints treated with PMA + CX the hyaluronan secretion rate, 5.8 ± 1.7 μg h^{-1}, was substantially reduced compared with that evoked by PMA alone, 10.1 ± 1.2 μg h^{-1} ($n = 14$, 200 ng ml^{-1} PMA; $P < 0.01$, Newman-Keuls test) (Fig. 4, lower panel). Although the residual secretion rate was higher than the basal secretion rate (4.4 ± 0.4 μg h^{-1}, $n = 26$) or the PMA + BIM secretion rate (3.2 ± 0.6 μg h^{-1}, $n = 9$), the difference did not reach statistical significance ($P > 0.05$, Newman-Keuls test).

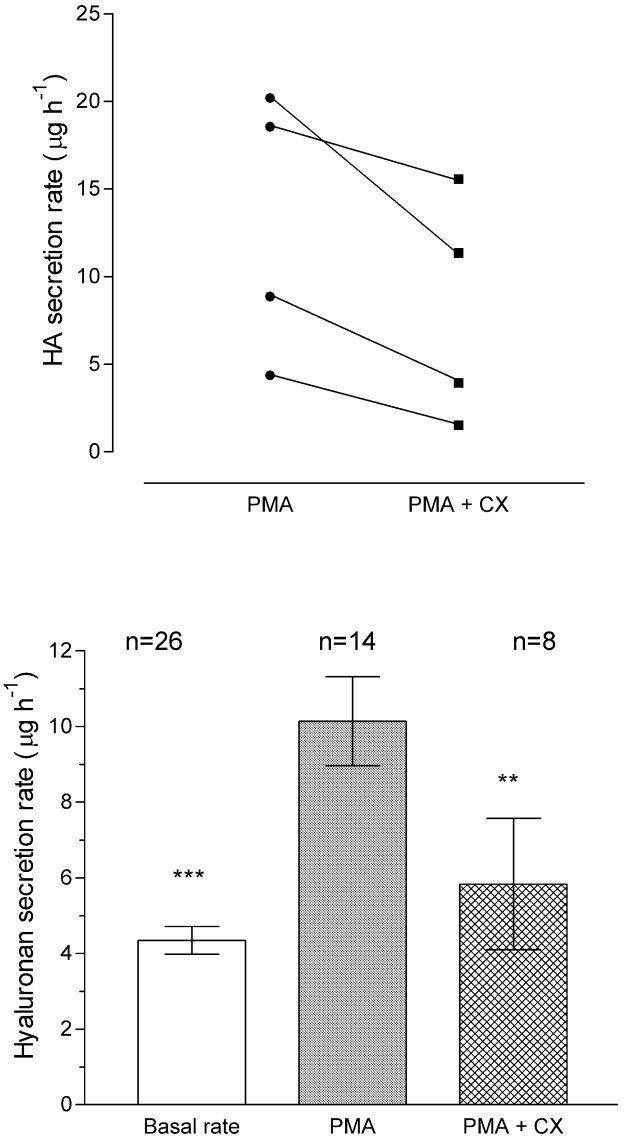

Figure 4. Partial inhibition of PMA-induced hyaluronan secretion by cycloheximide (CX, 100 μg ml^{-1})

Top panel, experiments on pairs of joints in same animal ($P = 0.04$, paired t test, $n = 4$ rabbits). Bottom panel, pooled results. The infused PMA concentration was 200 ng ml^{-1} except for the left column (basal rate). Means ± S.E.M.; *** $P < 0.001$, ** $P < 0.01$, Newman-Keuls test comparison with PMA; $P > 0.05$ for column 1 *vs.* column 3.

Effects of BIM and CX on basal secretion rate

To assess the contributions of basal PKC activity and protein turnover to basal hyaluronan secretion *in vivo*, 10 joints were treated with BIM alone (3.3 μg ml^{-1}) and eight joints with CX alone (100 μg ml^{-1}). No PMA was present in either joint in these experiments.

In non-stimulated joints treated with BIM a substantial hyaluronan secretion persisted, namely 3.07 ± 0.61 μg h^{-1} ($n = 10$) (Fig. 5). The secretion rate was 29 % lower, however, than the basal rate (4.35 ± 0.37 μg h^{-1}, $n = 26$) ($P = 0.03$, Mann-Whitney U test). Similarly, the secretion rate of 3.11 ± 0.40 μg h^{-1} for all the BIM-inhibited joints, pooling both the BIM-alone and PMA + BIM-treated joints ($n = 19$), was significantly lower than the basal secretion rate ($P = 0.02$, Mann-Whitney test).

CX treatment had no detectable effect on basal hyaluronan secretion rate over 6 h (Fig. 5). The mean secretion rate in CHX-treated joints, 4.20 ± 0.67 μg h^{-1} ($n = 8$), was only slightly less than the mean basal rate of 4.35 ± 0.37 μg h^{-1} ($P = 0.9$, Mann-Whitney test).

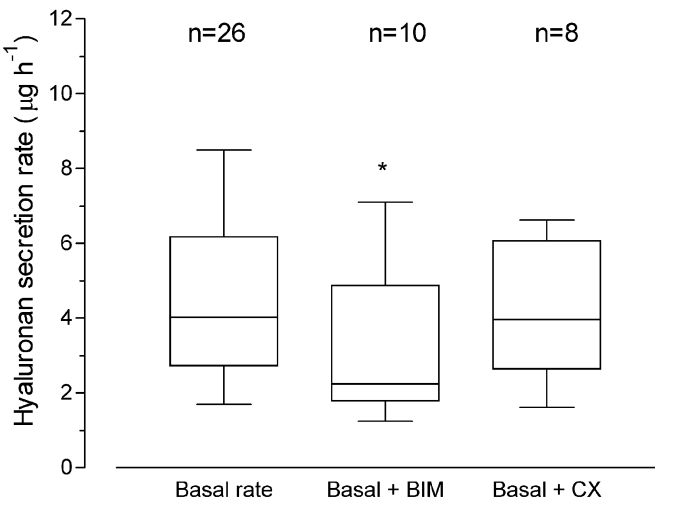

Figure 5. Effects of bisindolylmaleimide (BIM, 3.3 μg ml^{-1}) and CX (100 μg ml^{-1}) on basal, unstimulated rate of hyaluronan secretion into joint cavity

The boxes show quartiles and medians, and the bars show ranges. BIM reduced the basal secretion rate (* $P = 0.03$, Mann-Whitney U test). CX did not significantly alter basal secretion rate over 6 h.

Effect of PMA on indices of synovial inflammation

PMA might, in principle, indirectly stimulate hyaluronan secretion by triggering an inflammatory synovitis. We tested this in a joint using the inflammation-assay method of Poli *et al.* (2001, 2002). First, a 150 μl bolus of 200 ng ml^{-1} PMA was injected into the joint cavity and the pressure recorded through a cannula. The rate of pressure decay after a bolus injection is an index of net trans-synovial fluid escape. The observed decay over 30 min, −0.03 cmH_2O min^{-1}, was in the normal range for Ringer solution (mean −0.05 ± 0.01 cmH_2O min^{-1}, Poli *et al.* 2001). By contrast intra-articular pressure increases with time during an inflammatory response, due to increased synovial capillary filtration. Next, a bolus of Evans blue-labelled albumin (EVA) was given intravenously and the joint cavity infused continuously with the PMA solution at a controlled pressure, which was raised at 15 min intervals to a final intra-articular volume of ~2 ml (20 cmH_2O). Trans-synovial flow at each pressure was measured as described by Poli *et al.* (2002). EVA clearance from plasma into the joint cavity was measured by cavity washout every 15 min for 2 h. The net EVA clearance rate, 11.1 μl h^{-1} over 2 h, was at the upper limit of the range for Ringer-infused joints (mean 6.2 ± 1.4, range 1.5–11.1 μl h^{-1}). This contrasts with EVA clearances of >100 μl h^{-1} after endothelial barrier breakdown (Poli *et al.* 2002). The hydraulic conductance of the PMA-treated synovium, calculated as trans-synovial flow per unit increase in joint pressure, was 0.65 ± 0.09 μl min^{-1} cmH_2O^{-1}, similar to the mean for Ringer solution (0.87 ± 0.22 μl min^{-1} cmH_2O^{-1}) cf. 1.86 ± 0.11 μl min^{-1} cmH_2O^{-1} after barrier breakdown (Poli *et al.* 2002). There was thus no evidence of an acute inflammatory response to intra-articular PMA.

Effect of vehicle DMSO and ethanol on hyaluronan secretion

We tested whether 0.01–0.10 % DMSO–ethanol solution, the solvent concentrations in 200–2000 ng ml^{-1} PMA solutions, stimulated hyaluronan secretion. The mean hyaluronan secretion rate in joints receiving 0.01 % DMSO–ethanol in Ringer solution, 3.4 ± 0.6 μg h^{-1} (n = 8), was not significantly different from that in 14 joints receiving plain Ringer solution, namely 4.7 ± 0.7 μg h^{-1} (n = 14; P = 0.20, Mann-Whitney test). In a single joint treated with 0.1 % DMSO–ethanol in Ringer solution the hyaluronan secretion rate, 2.2 μg h^{-1}, was within the Ringer control range (1.6–8.5 μg h^{-1}). The results showed that the stimulation of hyaluronan synthesis by PMA-containing solutions is not caused by the traces of organic solvent in the vehicle. Similarly, Suzuki *et al.* (1995) found that DMSO at 0.02–0.20 % has little effect on hyaluronan secretion by cultured human fibroblasts; 0.50 % DMSO has no detectable effect on synovial permeability (Poli *et al.* 2002); and DMSO at 0.50 % is not toxic to hippocampal cells (Doerner *et al.* 1990).

DISCUSSION

The principle new findings were that PMA stimulates the secretion of hyaluronan into joint fluid up to 5-fold *in vivo*: BIM completely blocks the response: CX partially blocks it: and control hyaluronan secretion rate is reduced by BIM but not CX over 6 h.

Methodological issues

The administration of a 1 ml intra-articular loading dose, to distribute the drug throughout the joint cavity, distends the cavity initially to ~6 cmH_2O. This may in principle cause a moderate stimulation of the control hyaluronan secretion rate above true basal level. The basal rate in non-distended joints was 4.8 + 0.8 μg h^{-1} in a previous study, and the rate in volume-expanded joints (2 ml) was 5.8 ± 0.8 μg h^{-1} (Coleman *et al.* 1997). A comparison of these results with the present control rate, 4.4 ± 0.4 μg h^{-1}, provides no evidence for an increase above basal rate in the controls. Comparisons within the present study were always between joints receiving identical volumes of injectate.

Effect of PMA on hyaluronan secretion

The demonstration of PMA-induced hyaluronan secretion into joints extends previous work *in vitro* to an intact organ system *in vivo*. PMA stimulates hyaluronan secretion between 2- and 10-fold in cultured B6, testicular peritubular, glioma and mesothelial cell lines over 6 h (Heldin *et al.* 1992; Klewes & Prehm, 1994; Asplund *et al.* 1998; Thiébot *et al.* 1999). Since hyaluronan is not stored in vesicles and the total quantity of pre-existing hyaluronan in rabbit synovium is only 6–11 μg (Price *et al.* 1996; Levick *et al.* 1999), the PMA-stimulated secretion rates must reflect increased net hyaluronan synthase (HAS) activity.

PMA activates both the conventional and novel isoforms of PKC, including the α, δ and ϵ isoforms recently demonstrated in rabbit synoviocytes (Momberger *et al.* 2003). BIM blocks the catalytic domain of PKC α, β, δ and ϵ. The complete inhibition of PMA-induced hyaluronan secretion by BIM supports the view that activated PKC mediates the response. BIM is highly selective for PKC, with a 200-fold greater affinity for PKC over PKA (Toullec *et al.* 1991). The latter group showed that 5 μM BIM (their highest concentration) had no effect on protein kinase A-dependent phosphorylation in cultured fibroblasts.

CX-suppressible component of stimulated response

Since CX inhibited 75 % of the response to PMA, it appears that PKC acts in part by inducing the synthesis of new protein(s) – either HAS itself or a regulatory protein. By contrast, Suzuki *et al.* (1995) observed little effect of CX on PMA-stimulated hyaluronan secretion in fibroblasts *in vitro*. Other groups, however, report that CX almost totally abrogates the HAS activity increase in cultured mesothelial

cells, B6 cells and orbital fibroblasts stimulated respectively by platelet-derived growth factor, fetal calf serum and leukoregulin (Heldin *et al.* 1992; Klewes & Prehm, 1994; Smith *et al.* 1995), implicating gene transcription and protein synthesis *de novo*.

PKC can induce gene transcription through activation of the ERK-kinase cascade, which activates the gene regulatory protein Elk-1; and through phosphorylation of Iκ-B leading to the release of the gene regulatory protein NF-κB. PKC may upregulate HAS2, since HAS2 transcription is upregulated by growth factors that activate PKC (Feusi *et al.* 1999; Pienimäki *et al.* 2001). Alternatively, regulatory proteins or precursor enzymes such as uridine diphosphoglucose dehydrogenase (UDP) could be upregulated, as happens during joint cavitation (Ward *et al.* 1999). It seems unlikely, however, that precursor supply is rate-limiting, because supplementary UDP-glucose and glucosamine do not enhance hyaluronan synthesis *in vitro* (Sisson *et al.* 1980). A further possibility is that HAS mRNA translation rather than transcription might be enhanced, since the translation initiation factor e1F-4E is activated by PMA-induced phosphorylation in cardiac myocytes (Wada *et al.* 1996).

CX-resistant component of stimulation

About 25 % of the PMA-stimulated hyaluronan secretion was not inhibited by CX at a concentration that completely blocks protein synthesis (Bansal & Mason, 1986; Smith, Wang & Evans, 1995; Bartlett, Sawdy & Mann, 1999). This finding, like the work of Klewes & Prehm (1994) and Suzuki *et al.* (1995) *in vitro*, indicates that part of the stimulatory effect of PKC is unrelated to new protein synthesis. The residual stimulation has been attributed to serine phosphorylation of pre-existing HAS or associated 34 kDa, 52 kDa and larger protein co-factors. In support, alkaline phosphatase treatment of plasma membranes from stimulated cell lines reduces the synthase activity by 50 % (Klewes & Prehm, 1994). The intracellular domain of HAS2 possesses consensus sequences for phosphorylation by PKC, protein kinase A (PKA) and tyrosine kinase, but a specific regulatory domain has not been identified (Ohno *et al.* 2001). Our results in joints are compatible with both increased gene transcription/translation and the phosphorylation of pre-existing protein as mechanisms of increased hyaluronan synthase activity *in vivo*.

If the HAS half-life were short, CX-resistant stimulation could be brought about, in principle, by reduced HAS degradation. This cannot apply, however, if HAS activity terminates upon synthesis of a single hyaluronan chain (Kitchen & Cysyk, 1995). Nor can it apply if the half-life of synovial HAS *in vivo* is >6 h, as seems to be the case (see below). For the same reason HAS mRNA stabilisation is unlikely to be the regulatory mechanism.

Interactions amongst kinases

Activated PKCα commonly translocates to a particulate fraction to trigger the extracellular signal-regulated kinase (ERK) cascade (Kanashiro & Khalil, 1998), which in turn activates nuclear transcription factors (Schönwasser *et al.* 1998). In keeping with this, PKCα translocation and ERK1/2 phosphorylation have been observed in stretched, hyaluronan-secreting rabbit synoviocytes (Momberger *et al.* 2003). Likewise, phospho-ERK1 and hyaluronan appear in the joint line at the time of joint cavitation during embryogenesis (Ward *et al.* 1999).

It is possible that PKC might act partly through PKA activation. The action of PKC on G_i protein can enhance adenylate cyclase production of cAMP and hence PKA activity (Kanashiro & Khalil, 1998). cAMP-PKA activation stimulates hyaluronan synthesis *in vitro* (Sisson *et al.* 1980; Klewes & Prehm, 1994; Suzuki *et al.* 1995). Although PKC rather than PKA is the main regulator of HAS activity in fibroblasts *in vitro* (Suzuki *et al.* 1995), this aspect has not yet been investigated for synovium *in vivo*.

Stability of hyaluronan synthase *in vivo* cf. *in vitro*

Basal hyaluronan secretion into joints was unaffected by CX treatment over 6 h (Fig. 5), even though this concentration of CX was demonstrably active (Fig. 4). Basal hyaluronan secretion is not dependent, therefore, on the synthesis of HAS *de novo* over 6 h. It follows that the half-life of HAS is more than 6 h in synovium *in vivo*. This contrasts with the short half-life of HAS in chondrocytes and fibroblasts *in vitro*, namely 100–180 min (Bansal & Mason, 1986; Kitchen & Cysyk, 1995), and with the finding that CX reduces basal HAS activity by 50–65 % over 2–3 h in cultured mesothelial cells and 3T3 fibroblasts (Heldin *et al.* 1992; Kitchen & Cysyk, 1995). In subcultured skin and synovial fibroblasts the effect of CX on basal hyaluronan synthesis ranges from total suppression over 24 h through partial suppression to no effect at all (Smith & Hamerman, 1968; Sisson *et al.* 1980; Mapleson & Buchwald, 1981). The present results indicate that HAS stability is much greater in synoviocytes *in situ* than in most cultured cell lines. This in turn raises the issue of whether phenotypic, metabolic changes may occur in mesenchymal, integrin-bound cells removed from the influence of their normal, highly complex interstitial environment.

Does basal synovial hyaluronan secretion depend on basal PKC activity?

On this question too, the synovial results *in vivo* differ from those on cells *in vitro*. PKC inhibition greatly reduces basal hyaluronan synthesis by quiescent cultured fibroblasts (Suzuki *et al.* 1995) whereas it reduces basal hyaluronan synthesis by synovium *in vivo* by only 29 %. We conclude that ~29 % of the basal hyaluronan secretion into joints is driven by a basal level of PKC activity and the majority of the basal secretion, ~71 %, is not dependent on classical/novel PKC isoform activity.

Physiological and clinical significance of results

Hyaluronan must be secreted continuously into the joint fluid in order to maintain an effective concentration in the face of fluid turnover. Fluid turnover is brought about by synovial capillary filtration into the cavity and lymphatic clearance of fluid escaping from it. A high hyaluronan concentration is needed to prevent the synovial fluid from seeping too rapidly out of the joint cavity. A high hyaluronan concentration is also necessary for hydrodynamic lubrication during high frequency, low-load movements, because this form of lubrication depends on synovial fluid viscosity, which is hugely increased by hyaluronan. If hyaluronan secretion rate were fixed and unregulated, any increase in capillary filtration into the joint cavity would threaten viscous lubrication by diluting the hyaluronan. Distension of the cavity, however, by increased intra-articular fluid volume, stretches the synoviocytes (McDonald & Levick, 1988) and stimulates hyaluronan secretion (Coleman *et al.* 1997). This constitutes a homeostatic mechanism tending to maintain the intra-articular hyaluronan concentration. The demonstration that PKC activation powerfully stimulates intra-articular hyaluronan secretion provides a potential insight into the transduction pathway *in vivo* and is a *sine qua non* for our working hypothesis, namely that synovial PKC activation is part of a mechanotransduction pathway that links joint stretch to hyaluronan secretion.

Recent work on stretched synoviocytes *in vitro* supports the above hypothesis by showing that stretch activates PKCα and increases hyaluronan secretion (Momberger *et al.* 2003). Mechanical deformation of a cell is known to stress the integrin–matrix linkages at focal adhesion sites, where PKCα is concentrated (Kanashiro & Khalil, 1998; Mellor & Parker, 1998). This activates the ERK cascade, probably through the focal adhesion kinase–phospholipase C-γ1–DAG–PKC pathway (Schmidt *et al.*1998; MacKenna *et al.* 1998; Karim, 1998; Zhang *et al.* 1999).

Synovial PKC activation is also likely to be involved in the stimulation of hyaluronan secretion into joints with inflammatory arthritis. In these conditions the joint cavity is distended by a cytokine-rich effusion and contains a greatly increased mass of hyaluronan. Pro-inflammatory mediators in the effusion, such as interleukin β_1 and tumour growth factor β, stimulate hyaluronan synthesis through the activation of PKC (Heldin *et al.* 1992; Ito *et al.* 1993; Haubeck *et al.* 1995; Suzuki *et al.* 1995; Kolomytkin *et al.* 1999; Tanimoto *et al.* 2001). PKC activation is also reported to inhibit the progression of osteoarthritis in rabbit knees (Hamanishi *et al.* 1996).

In conclusion, the study showed that PKC is part of a transduction cascade that regulates hyaluronan secretion into intact synovial joints *in vivo*. The study also revealed substantial differences between organ-specific hyaluronan secretion *in vivo* and hyaluronan secretion by cell lines *in vitro*, which may reflect the physiological importance of cellular environment and matrix binding in determining metabolic function. Further work is needed to determine which proteins are upregulated by PKC and how the activity of pre-existing HAS is regulated.

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