Prostaglandin E_2 stimulates cyclic AMP-mediated hyaluronan synthesis in rabbit pericardial mesothelial cells

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We studied the effects of prostaglandin E_2 (PGE_2) on hyaluronan synthesis in rabbit pericardial mesothelial cells, and the following results were obtained. (1) PGE_2 (10–1000 ng/ml) stimulated hyaluronan synthesis and the level of hyaluronan synthase activity in a dose- and time-dependent manner, but $PGF_{2\alpha}$ did not. (2) Cyclic AMP (cAMP) levels in the cells peaked (about a 7-fold increase) at 5–10 min after adding PGE_2 (1000 ng/ml). (3) Increased hyaluronan synthesis induced by PGE_2 was significantly inhibited after pretreatment with either an adenylate cyclase inhibitor (2′,5′-dideoxyadenosine) or a cAMP-dependent protein kinase inhibitor (PKI 5-24), but there was no inhibition with the protein kinase C inhibitor H-7. (4) When the intracellular cAMP level was raised by manipulating the levels of dibutyryl cyclic AMP or forskolin, hyaluronan synthesis and the level of hyaluronan synthase activity were also stimulated. These results suggest that PGE_2 produced by cells stimulates hyaluronan synthesis in rabbit pericardial cells and that the stimulation mechanism involves the cAMP-mediated protein kinase signal transduction process.

INTRODUCTION

Although little attention has been paid to the physiological functions of the pericardium [1], we have shown previously that rabbit pericardium actively produces both prostaglandins (PGI_2 and PGE_2) [2] and a high-molecular-mass hyaluronan that increases the viscosity and affects the rheological properties of the pericardial fluid [3,4]. In order to clarify the biological functions of prostaglandins with special reference to hyaluronan in the pericardial fluid, we investigated the effects of prostaglandins on hyaluronan synthesis in rabbit pericardial cell cultures [5]. It is reported that hyaluronan synthesis is stimulated by many different compounds, e.g. fetal bovine serum [6], epidermal growth factor (EGF) [5,7,8], cyclic AMP (cAMP) [6], oestrogen [9], retinoic acid [10], interleukin 1 [11], vanadate [12], insulin-like growth factor-I (IGF-I) [13], platelet-derived growth factor (PDGF)-BB [14,15], transforming growth factor β (TGF-β) [16], phorbol 12-myristate 13-acetate (PMA) [15], and a 150 kDa glycoprotein in sera from bovine fetuses and from breast cancer patients [17] in various tissues or cell culture systems. However, the molecular mechanism of stimulation of hyaluronan synthesis is not yet clear. We reported that growth factors such as IGF-I and/or EGF stimulate hyaluronan synthesis by a transmembrane signalling process, involving receptor tyrosine kinases, that is responsive to IGF-I and EGF [5]. Recently, it was also reported by Heldin et al. [15] that tyrosine phosphorylation is involved in stimulation of hyaluronan synthase activity by PDGF-BB in human mesothelial cells. Our work reported here investigates whether there are other signal transduction pathways for stimulating hyaluronan synthesis in rabbit pericardial cells.

MATERIALS AND METHODS

Materials

The following commercial materials were used: minimum essential medium (MEM; Eagle's) from Gibco; fetal bovine serum (FBS) from Whittaker M.A. Bioproducts; streptomycin sulphate and crystalline penicillin G potassium from Meiji Seika, Tokyo, Japan; calcium- and magnesium-free phosphate-buffered saline [PBS(−)] from Nissui Seiyaku Co., Tokyo, Japan; trypsin from Difco Laboratories, Detroit, MI, U.S.A.; D-[6-^{3}H]glucosamine hydrochloride (40.4 Ci/mmol), uridine 5′-diphosphate-[^{14}C]glucuronic acid (UDP-[^{14}C]glucuronic acid; 328.2 mCi/mmol) from New England Nuclear Research Products, Boston, MA, U.S.A.; [5-^{3}H]uridine (29 Ci/mmol) and high-specific-radioactivity ^{3}H-labelled L-amino acid mixture (1 mCi/ml) from Amersham International; [*methyl*-^{3}H]thymidine (24 Ci/mmol) from ICN Biomedicals, Irvine, CA, U.S.A; UDP-*N*-acetylglucosamine, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and dibutyryl (AMP (Bt_2-cAMP) from Sigma Chemical Co., St. Louis, MO, U.S.A.; cholera toxin from Calbiochem Co., La Jolla, CA, U.S.A.; hyaluronidase (EC 4.2.2.1) from *Streptomyces hyalurolyticus*, whale cartilage chondroitin sulphate A-Na salt, the cAMP-dependent protein kinase inhibitor PKI 5-24) and the protein kinase C (PKC) inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) from Seikagaku Kogyo Co., Tokyo, Japan; actinase E (1×10^6 tyrosine units/g) from Kaken Chemicals Co., Tokyo, Japan; 3,5-diaminobenzoic acid dihydrochloride (DABA) from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; *N*-cetylpyridiniumchloride monohydrate from Merck, Darmstadt, Germany; Sepharose CL-6B and 2′,5′-

Abbreviations used: cAMP, cyclic AMP; Bt_2-cAMP, N^6,2′-*O*-dibutyryl cAMP; EGF, epidermal growth factor; DABA, diaminobenzoic acid; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; IGF-I, insulin-like growth factor-I; MEM, minimum essential medium; PSB(−), Ca^{2+}/Mg^{2+}-free PBS; PDGF, platelet-derived growth factor; PG, prostaglandin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor β.

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dideoxyadenosine from Pharmacia Fine Chemicals, Uppsala, Sweden; protein assay dye reagent kit from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Yamasa cAMP assay kit from Yamasa Shoyu, Co., Chiba, Japan.

Prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ were kindly provided by Ono Pharmaceutical Co., Osaka, Japan.

Cultures of pericardial cells from the rabbit pericardial cavity

Cultures of pericardial cells from the rabbit pericardial cavity were carried out as described previously [5]. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Once the cells had become confluent (about 2 weeks after seeding), they were subcultured in 75 cm^2 T-flasks by dispersing them with a 5 min incubation in 0.025% trypsin and 0.025% K_2EDTA in PBS(−), pH 7.3. The cells were split at a ratio of 1:3 and cultured in 10% FBS/MEM containing penicillin (100 i.u./ml) and streptomycin (100 μg/ml). The culture medium was changed every 3 days. Experiments on hyaluronan synthesis were performed on cells of less than five passages.

Assay for glycosaminoglycan synthesis

Pericardial cells were inoculated at about 2×10^5 cells/ml in plastic microwells and maintained in MEM supplemented with 10% FBS. After growth had ceased at confluency, the medium was replaced with fresh MEM. After preincubation for 16 h at 37 °C, PGE_2 and $PGF_{2\alpha}$ at various concentrations (10–1000 ng/ml), or ethanol as a vehicle, was added to the microwells, and then [6-^{3}H]glucosamine (10 μCi/ml) was added. After incubation for the indicated periods (6–48 h) at 37 °C, either without or with PGE_2 and $PGF_{2\alpha}$, the medium (medium fraction) was removed from each microwell and the cells (cell fraction) were rinsed with cold PBS(−). The cells, to which 0.5 M NaOH (1 ml) was added, were harvested with a rubber policeman.

Hyaluronan synthesis and hyaluronidase digestion of glycosaminoglycans

^{3}H-labelled glycosaminoglycans from the medium and cell fractions were prepared after actinase E (protease) digestion and cetylpyridinium chloride treatment [18]. Hyaluronan synthesis was determined by measuring the radioactivity of *Streptomyces* hyaluronidase-digested material in the [^{3}H]glycosaminoglycans. Digestion of the [^{3}H]glycosaminoglycans with *Streptomyces* hyaluronidase at 60 °C for 2 h has been described [19]. Sulphated glycosaminoglycan synthesis was determined by subtracting the radioactivity of [^{3}H]glycosaminoglycan in the sample.

Gel chromatography

Gel chromatography was carried out on a Sepharose CL-6B column (1.2 cm × 60 cm) equilibrated and eluted with 50 mM Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl, at a flow rate of 15 ml/h at 4 °C. Fractions (1 ml) were collected and assayed for radioactivity.

Assay of hyaluronan synthase activity

Pericardial cells were incubated at 2×10^5 cells/ml in 60 mm-diam. plastic culture dishes and cultured in 10% FBS/MEM until the stationary phase of growth. The medium was then replaced with serum-free MEM. After preincubation for 16 h at 37 °C, medium was replaced with MEM containing PGE_2 and $PGF_{2\alpha}$, or ethanol as a vehicle. After cells had been incubated for 6 h at 37 °C, they were harvested from the dishes with a rubber policeman. Hyaluronan synthase activity was measured as described previously [5]. Briefly, the harvested cells were sonicated twice for 5 s each at 50 W with an ultrasonic cell disruptor (Heat Systems-Ultrasonics, Farmingdale, NY, U.S.A.). The cell sonicate was centrifuged at 800 *g* for 5 min at 4 °C, and the supernatant was separated and recentrifuged at 20000 *g* for 10 min at 4 °C. More than 80% of the hyaluronan synthase activity was recovered in the 20000 *g* cell sonicate pellet. To this pellet was added 100 μl of reaction buffer (pH 7.0) containing 40 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.4 mM UDP-*N*-acetylglucosamine (Sigma), and 0.1 μCi of UDP-D-[^{14}C]-glucuronic acid (328.2 mCi/mmol). After incubation for 1 h at 37 °C, 100 μl of 2% SDS was added to the reaction mixture, which was then boiled for 2 min to stop the reaction. The sample was subjected to descending paper chromatography on Whatman no. 3MMChr filter paper in isobutyric acid/1 M NH_4OH (5:3, v/v). After development for 24 h, the spotted origins of the chromatogram were cut out, and radioactivity was measured in 10 ml of scintillation fluid [2 g of 2,5-diphenyloxazole (DPO), 0.15 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) and 500 ml of toluene] in a liquid scintillation counter. The specific activity of this enzyme was expressed as d.p.m. of UDP-D-[^{14}C]glucuronic acid incorporated into hyaluronan/6 h per mg of protein.

Assay for cellular cAMP

After confluent pericardial cells (in 35 mm-diam. plastic culture dishes) had been preincubated in MEM for 16 h at 37 °C, PGE_2 (1 μg/ml) or $PGF_{2\alpha}$ (1 μg/ml) plus 0.1 mM IBMX were added to the dishes. Cells were incubated for the periods shown at 37 °C in a CO_2 incubator, and then the medium was immediately discarded. The cells were washed with cold PBS(−), and cold 7% (w/v) trichloroacetic acid was added to the dishes. The cells were scraped off the dishes with a rubber policeman, and sonicated for 3 × 5 s at 50 W at 4 °C. The cell sonicate was centrifuged at 1300 *g* for 5 min at 4 °C, and then the supernantants were extracted twice with water-saturated diethyl ether in a shaker to remove trichloroacetic acid (Yayoi Co., model YS-8D). cAMP contents in the aqueous solution were radioimmunoassayed by the method of Honma et al. [20] with a Yamasa cAMP assay kit. ^{125}I radioactivity was determined using an auto-well γ-radiation counter (Aloka-ARC-2000).

Assay for DNA, RNA and protein synthesis

Assays for DNA, RNA and protein synthesis were described in our previous paper [13]. After the preincubation of confluent cells on microwells with MEM for 16 h at 37 °C, the medium was replaced with 1 ml of MEM containing [*methyl*-^{3}H]thymidine (1 μCi/ml), [5-^{3}H]uridine (1 μCi/ml) or [^{3}H]amino acid mixture (1 μCi/ml) without or with PGE_2 (1 μg/ml). After a 2 h incubation at 37 °C the medium was removed from the microwells. The cells were washed with PBS(−), and then 60% trichloroacetic acid was added to give a final concentration of 10%. Pellets were dissolved in 0.5 M NaOH, the alkali solution was neutralized with 0.5 M HCl and then the sample radioactivity was measured.

Assay of DNA content

The DNA content of cultured cells was determined by a fluorescence DNA assay [21]. Briefly, pericardial cells treated

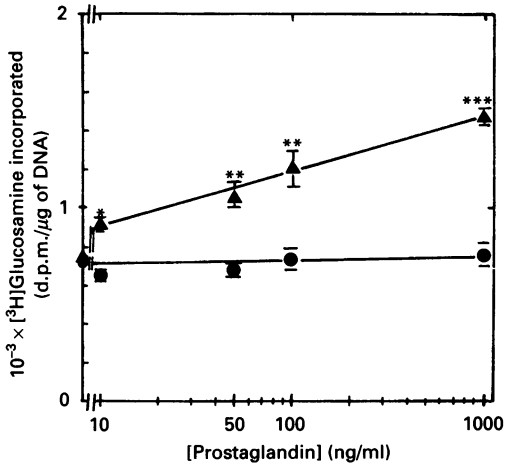

Figure 1 Dose-dependence of the effects of prostaglandins on glycosaminoglycan synthesis in rabbit pericardial cells

Confluent pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were cultured without or with PGE_2 (▲) or $PGF_{2\alpha}$ (●) (10–1000 ng/ml) for 24 h at 37 °C. Glycosaminoglycan-synthesizing activities from [6-^{3}H]glucosamine (10 μCi/ml) per μg of DNA were assayed as described in the Materials and methods section. Values represent means ± S.D. from three individual wells. Significant differences from untreated cells: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

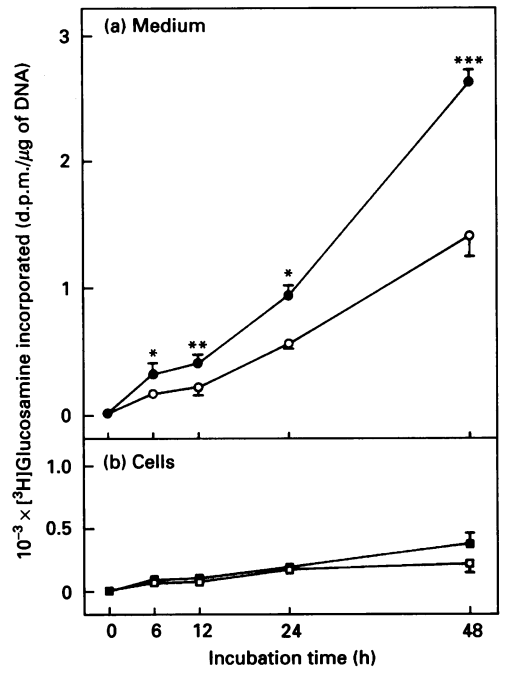

Figure 2 Time course of the effect of PGE_2 on glycosaminoglycan synthesis in rabbit pericardial cells

Confluent rabbit pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were incubated with [6-^{3}H]glucosamine (10 μCi/ml) and without (○, □) or with (●, ■) PGE_2 (1000 ng/ml) for the indicated times. [^{3}H]Glycosaminoglycans from medium (**a**) or cell (**b**) fractions were prepared as described in the Materials and methods section. Values represent means ± S.D. from three individual wells. Significant differences from untreated cells: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with or without prostaglandins were rinsed with PBS(−) and fixed directly in wells with absolute ethanol (1 ml) for 5 min on ice. The ethanol fixative solution was removed and the cell layers

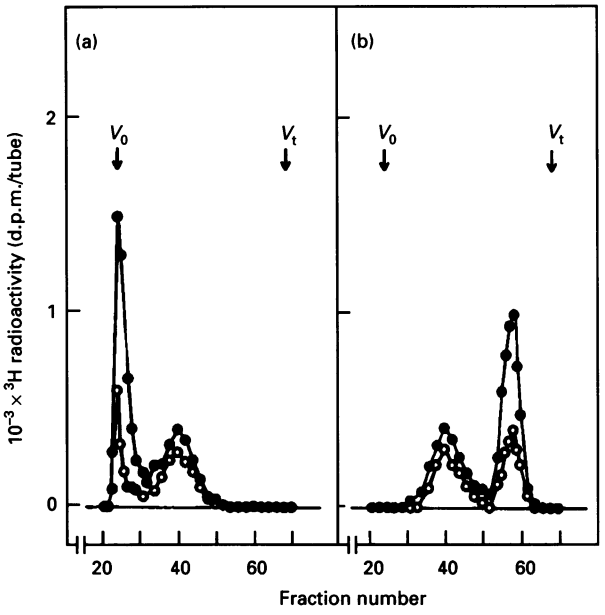

Figure 3 Elution profiles from Sepharose CL-6B of ^{3}H-labelled glycosaminoglycans synthesized in rabbit pericardial cells

Confluent rabbit pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were incubated with [6-^{3}H]glucosamine (10 μCi/ml) and without (○) or with (●) PGE_2 (1000 ng/ml) for 24 h. The [^{3}H]glycosaminoglycans isolated from the medium of rabbit pericardial cells were applied to a Sepharose CL-6B column (1.4 cm × 65 cm) and eluted with 50 mM Tris/HCl buffer containing 0.15 M NaCl (pH 7.4). Fractions (1 ml/tube) were collected and assayed for radioactivity. (**a**) and (**b**) show samples respectively before and after *Streptomyces* hyaluronidase digestion. The arrows indicate void volume (V_0) and total volume (V_t).

were air-dried in the wells for 1 day at room temperature. To each well was then added 200 μl of freshly prepared DABA (4 g/10 ml), and the assay plate was heated at 60 °C for 45 min. The DABA–DNA reaction was stabilized by adding 1.5 ml of 1 M HCl to each well. The complete reaction mixture (350 μl) was transferred into a well on a Nunc-immuno plate and then read in a Corona MTP-32 microplate reader (Corona Electric Co., Ibaraki, Japan) set at an excitation of 365 nm and an emission of 530 nm. DNA from salmon sperm was used as a standard.

Protein assay

Protein contents were determined by Bradford's method [22].

Measurement of radioactivity

An 8 ml portion of ACS-II (Amersham) and 0.5–1.0 ml of the sample solution to be assayed were mixed in a vial bottle, and then radioactivity was measured in a liquid scintillation counter (Aloka LSC-1000).

Statistics

A probability level of $P < 0.05$ was considered significant when results were subjected to Student's t test.

RESULTS

Stimulatory effect of PGE_2 on hyaluronan synthesis in rabbit pericardial cells

Figure 1 shows the dose-dependence of the effects of PGE_2 and $PGF_{2\alpha}$ (10–1000 ng/ml) on glycosaminoglycan synthesis from

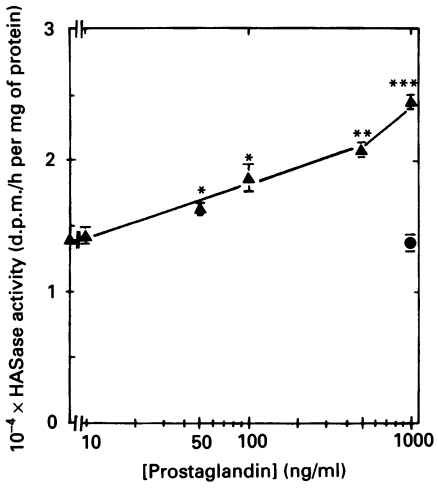

Figure 4 Dose-dependence of the effects of PGE_2 and $PGF_{2\alpha}$ on hyaluronan synthase activity in rabbit pericardial cells

Confluent pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were cultured with various concentrations of PGE_2 (▲) or $PGF_{2\alpha}$ (●; 1000 ng/ml) for 6 h at 37 °C. Hyaluronan synthase (HASase) activity in cells was determined as described in the Materials and methods section. Values represent means ± S.D. from three individual wells. Significant differences from untreated cells: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

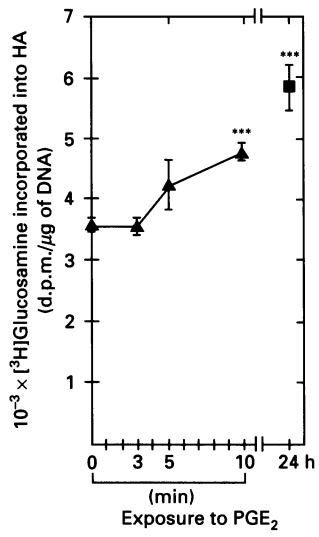

Figure 5 Increase in hyaluronan synthesis by pulse–chase experiments with PGE_2 in rabbit pericardial cells

Confluent pericardial cells were preincubated with serum-free MEM for 16 h at 37 °C. Cells were then exposed to PGE_2 (▲; 1000 ng/ml)-containing MEM for the short time indicated. After PGE_2 was removed from the culture medium, cells were incubated in PGE_2-free MEM with [6-^{3}H]glucosamine (10 μCi/ml) for 24 h. The symbol ■ indicates the data point represented by exposing cells to PGE_2 (1000 ng/ml) with [6-^{3}H]glucosamine (10 μCi/ml) for 24 h. Hyaluronan (HA) synthesis was assayed as described in the Materials and methods section. Values represent means ± S.D. from three individual wells. Significant difference from untreated cells: $^{***}P < 0.001$.

[6-^{3}H]glucosamine in confluent pericardial cells after a 24 h exposure to each prostaglandin. Significant stimulation of ^{3}H-labelled glycosaminoglycan synthesis by PGE_2 was seen at a concentration of 10 ng/ml (30 nM), and the synthesis increased linearly up to a concentration of 1000 ng/ml (3 μM). Synthesis stimulated by PGE_2 (1000 ng/ml) was twice that of the control. However, $PGF_{2\alpha}$ (10–1000 ng/ml) did not stimulate glycosaminoglycan synthesis.

Figure 2 shows the time course of glycosaminoglycan synthesis from [6-^{3}H]glucosamine in pericardial cells after addition of PGE_2 (1000 ng/ml) or vehicle to pericardial cells at confluence. A significant stimulatory effect of PGE_2 treatment on glycosaminoglycan synthesis was seen in the medium fraction after 6 h, and total activity stimulated by PGE_2 was about twice that of the control after a 48 h culture period.

To identify what kind of glycosaminoglycan was stimulated by PGE_2, ^{3}H-labelled glycosaminoglycan was subjected to Sepharose CL-6B column chromatography and *Streptomyces* hyaluronidase digestion. Two radioactive peaks (void volume and $K_{av.} = 0.36$) were observed (Figure 3). Material eluted in the void volume was identified as hyaluronan, as it was completely digested by *Streptomyces* hyaluronidase. Hyaluronan synthesis was stimulated by PGE_2 (1000 ng/ml). Materials retarded by Sepharose CL-6B gel chromatography were not digested by *Streptomyces* hyaluronidase, suggesting that they are sulphated glycosaminoglycans. The synthesis of sulphated glycosaminoglycans was not significantly stimulated by PGE_2. These results show that PGE_2 specifically stimulates hyaluronan synthesis in a dose- and time-dependent manner in pericardial cells.

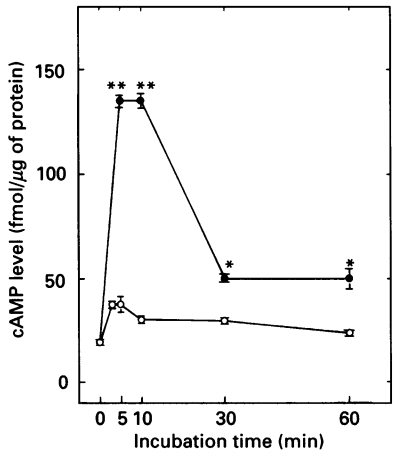

Figure 6 Changes in the accumulation of intracellular cAMP in rabbit pericardial cells stimulated by PGE_2

Confluent pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were cultured with IBMX (100 μM) (○) or PGE_2 (1000 ng/ml) plus 100 μM IBMX (●) for the indicated periods. After incubation, media were removed and 7% (w/v) ice-cold trichloroacetic acid was immediately added to cultured dishes to stop the reaction. Cellular cAMP contents extracted were measured using a radioimmunoassay kit (Yamasa). Values represent means ± S.D. from duplicate cultures. Significant differences from untreated cells: $^*P < 0.05$; $^{**}P < 0.01$.

Mechanism of stimulation of hyaluronan synthesis by PGE_2 in rabbit pericardial cells

To clarify the mechanism of PGE_2 stimulation of hyaluronan synthesis in pericardial cells, hyaluronan synthase activity was measured in pericardial cells treated or not with PGE_2 or $PGF_{2\alpha}$. Figure 4 shows that PGE_2 activated hyaluronan synthase activity in a dose-dependent manner, and the activity at a concentration of PGE_2 of 1000 ng/ml was about double that of controls. However, $PGF_{2\alpha}$ did not activate hyaluronan synthase activity even at a concentration of 1000 ng/ml.

We evaluated the exposure time needed to stimulate

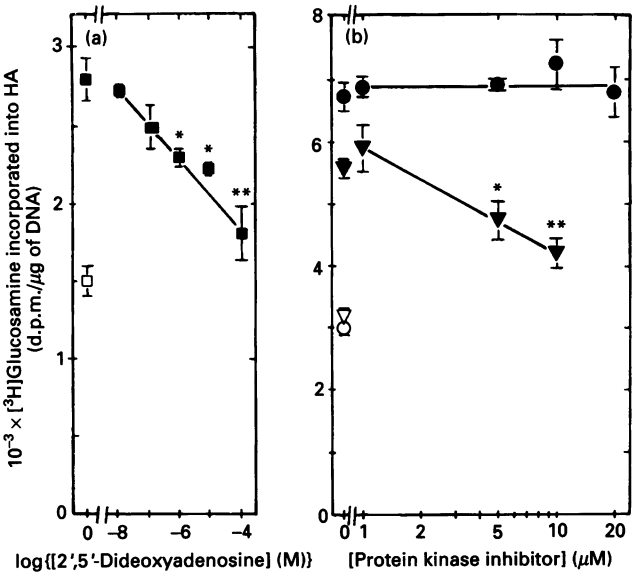

Figure 7 Effects of 2',5'-dideoxyadenosine (a) and protein kinase inhibitors (b) on hyaluronan (HA) synthesis enhanced by PGE_2 in rabbit pericardial cells

Confluent pericardial cells preincubated with serum-free MEM for 16 h at 37 °C were pretreated for 30 min at 37 °C with various concentrations of (**a**) 2',5'-dideoxyadenosine (■; 0.01–100 μM) and (**b**) the protein kinase A inhibitor PKI 5–24 (▼; 1–10 μM) and the PKC inhibitor H-7 (●; 1–20 μM), before a 24 h incubation at 37 °C without (□, ▽, ○) or with (■, ▼, ●) PGE_2 (1000 ng/ml). The inhibitors were left in the culture after the addition of PGE_2. Values represent means ± S.D. from three individual wells. Significant differences from PGE_2 (1000 ng/ml)-treated cells without inhibitors: $^*P < 0.05$, $^{**}P < 0.01$.

hyaluronan synthesis in pericardial cells by exposing cells to PGE_2 in pulse–chase experiments (Figure 5). Hyaluronan synthesis significantly increased only when pericardial cells were exposed to PGE_2 (1000 ng/ml) for 10 min or more, suggesting the involvement of some second messenger of PGE_2 in stimulating hyaluronan synthesis.

Figure 6 shows the changes in the accumulation of intracellular cAMP in pericardial cells stimulated by PGE_2 (1000 ng/ml) in the presence of IBMX (100 μM). cAMP levels in the cells peaked (about a 7-fold increase) 5–10 min after addition of PGE_2, and after that dropped to about 50 fmol/μg of protein by 30 min after addition of PGE_2, and maintained this level for 60 min.

We next studied the effect of an adenylate cyclase inhibitor, 2',5'-dideoxyadenosine, on hyaluronan synthesis enhanced by PGE_2. Figure 7(a) shows that pretreatment for 30 min with 2',5'-dideoxyadenosine at concentrations of 0.01–100 μM caused a significant inhibition of the enhancement of hyaluronan synthesis by PGE_2 (1000 ng/ml), with near-complete inhibition to the control level at a concentration of 100 μM. However, 2',5'-dideoxyadenosine itself did not cause a direct inhibitory effect on hyaluronan synthesis even at a concentration of 100 μM. These results suggest that the stimulation of hyaluronan synthesis by PGE_2 is due to cAMP produced by adenylate cyclase in pericardial cells.

We examined whether or not a potent synthetic peptide (PKI 5-24; 1–10 μM), which inhibits cAMP-dependent protein kinase, affects hyaluronan synthesis enhanced by PGE_2 in pericardial cells. Figure 7(b) shows that pretreatment for 30 min with PKI 5–24 (10 μM) caused a significant inhibition of the enhancement of hyaluronan synthesis by PGE_2 (1000 ng/ml). However, cells treated with PKI 5-24 alone (10 μM) maintained about 95% of the general protein synthesis levels of controls (100%), as determined by ^{3}H-labelled amino acid incorporation into cells. These results suggest that the potent inhibitor PKI 5-24 of the cAMP-dependent protein kinase causes inhibition of the enhancement of hyaluronan synthesis by PGE_2, probably due to inhibition of the kinase activity in pericardial cells.

In contrast, the PKC inhibitor H-7 (1–20 μM) did not inhibit the enhancement of the hyaluronan synthesis by PGE_2 (1000 ng/ml) (Figure 7b).

When the intracellular cAMP level was raised by manipulating the levels of either a membrane-permeable cAMP analogue (Bt_2-cAMP; 10 mM) or adenylate cyclase activators (forskolin, 10 μM; cholera toxin, 10 ng/ml), both hyaluronan synthesis and activity of hyaluronan synthase were also stimulated (Table 1).

Table 1 Relationship between the intracellular cAMP content, hyaluronan synthase activity and hyaluronan synthesis in rabbit pericardial cells

Confluent pericardial cells were preincubated with serum-free MEM for 16 h at 37 °C. Cells were then cultured with PGE_2 (1000 ng/ml), Bt_2-cAMP (10 mM), forskolin (10 μM) or cholera toxin (10 ng/ml) for 5 min for the assay of cAMP content at 37 °C, for 6 h for the assay of hyaluronan synthase (HASase) activity, and for 24 h for the assay of hyaluronan (HA) synthesis. Cellular cAMP contents were measured using a radioimmunoassay kit (Yamasa). Results are the means of duplicate experiments. All values are within ± 10% of the mean. Hyaluronan synthase activity was determined as described in the Materials and methods section. Hyaluronan synthesis was determined by measuring the radioactivity in *Streptomyces* hyaluronidase-digested materials of [^{3}H]glycosaminoglycans. Results represent the means ± S.D. from three individual dishes or wells. Significant differences from untreated control cells: $^{**}P < 0.01$; $^{***}P < 0.001$.

Stimulant	cAMP (fmol per μg of protein)	10^{-4} × HASase activity (d.p.m./h per mg of protein)	10^{-4} × HA synthesis (d.p.m./24 h per mg of DNA)
Control	37.5	1.3 ± 0.3	25.3 ± 2.1 (100%)
PGE_2 (1000 ng/ml)	135.1	2.5 ± 0.3***	46.3 ± 2.4** (183%)
Bt_2-cAMP (10 mM)	51351.4	2.6 ± 0.1***	99.5 ± 8.0*** (393%)
Forskolin (10 μM)	3919.0	2.3 ± 0.1***	78.8 ± 2.9*** (312%)
Cholera toxin (10 ng/ml)	105.4	2.0 ± 0.2**	42.1 ± 1.6** (166%)

DISCUSSION

This study demonstrates that PGE_2 stimulates hyaluronan synthesis in rabbit pericardial mesothelial cells and the mechanism of stimulation by PGE_2 involves cAMP-mediated protein kinase signal transduction.

The involvement of PGE_2 in an adenylate cyclase system stimulating hyaluronan synthesis in pericardial cells is confirmed by experiments with the following molecular pharmacological reagents: (1) the diterpene forskolin, known to be an adenylate cyclase activator by direct stimulation of the catalytic enzyme subunit [23], (2) a non-competitive adenylate cyclase inhibitor, 2',5'-dideoxyadenosine [24–26], that blocks prostaglandin-stimulated cAMP accumulation [27], (3) cholera toxin, which causes ADP-ribosylation of a G-protein, thus altering the G-protein to a state of permanent activation, leading to stimulation of the catalytic moiety of adenylate cyclase, and (4) the membrane-permeant cAMP analogue Bt_2-cAMP, which is used to elevate intracellular cAMP concentration directly. Involvement of a signal transduction pathway from cAMP to protein kinase A, not PKC, for hyaluronan synthesis in pericardial cells is shown by using PKI 5-24, a potent synthetic peptide inhibitor of cAMP-dependent protein kinase A [28–30]. Our results support the existence of an activation mechanism for hyaluronan synthesis involving protein kinase A-mediated phosphorylation.

i.e. PGE_2 acts on PGE receptors coupled to a G-protein [31] on the plasma membrane, leading to adenylate cyclase activation, thus increasing cellular cAMP levels and activating protein kinase A and in turn hyaluronan synthase activity in pericardial cells.

Several signal transduction pathways have been suggested for activating hyaluronan synthesis. We have shown that IGF-I [13], EGF [5] and vanadate [12] stimulate hyaluronan synthesis by inducing or activating hyaluronan synthase in pericardial cells. The stimulatory effects of IGF-I and EGF on hyaluronan synthesis were blocked by the tyrosine kinase inhibitor genistein [5]. These results suggest that enhanced hyaluronan synthesis induced by IGF-I and/or EGF could be mediated by a receptor tyrosine kinase-dependent transmembrane signalling process. Heldin et al. [15] also suggested that tyrosine phosphorylation is involved in the stimulation of hyaluronan synthase activity by PDGF-BB in human mesothelial cells. Combined exposure of pericardial cells to a growth factor, IGF-I (100 ng/ml) or EGF (100 ng/ml), plus PGE_2 (1000 ng/ml) showed additive effects on hyaluronan synthesis and on hyaluronan synthase activity (results not shown). These results suggest that two different extracellular signals operate co-operatively through different receptors and subsequent signal transduction pathways to activate hyaluronan synthesis in pericardial cells. PMA (100 nM) also stimulates hyaluronan synthesis in mesothelial cells [15], suggesting involvement of a PKC-mediated signal transduction pathway in hyaluronan synthesis. Uzuka et al. [9] reported another signal transduction pathway for stimulating hyaluronan synthesis using oestrogen, i.e. oestrogen passes through the plasma membrane of cells, then binds to a cytosolic receptor forming an oestrogen–receptor complex; this complex then moves to chromatin before inducing hyaluronan synthesis in oestrogen-treated mouse skin. Although various signal transduction pathways and the cross-talk for hyaluronan synthesis described above may be cell-type-specific, the mechanism of stimulation of endogenous hyaluronan synthesis by various extracellular stimuli will be a basis for developing a new type of drugs, e.g. lubricants.

When exogenous arachidonic acid (30 μM) was injected into a normal rabbit pericardial cavity followed by incubation for 5 min in *ex vivo* experiments, concentrations of PGE_2 reached from about 1 ng/ml to 50 ng/ml of pericardial fluid. Thus it is possible that, in some pathological situations, especially pericarditis, substantial amounts of PGE_2 could be formed by the pericardium itself or by inflammatory cells, and accumulate in the pericardial space [2]. Because PGE_2 is a potent stimulator of cAMP-mediated hyaluronan synthesis in pericardial cells and the hyaluronan molecular domain includes a large amount of water in solution, PGE_2 in the pericardial fluid could be very important in influencing further inflammation and fluid accumulation *via* hyaluronan in this vital physiological space.

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