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Characterization of the PGE_2 receptor subtype in bovine chondrocytes in culture

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1 Prostaglandin E_2 (PGE_2) is an autacoid that decreases proteoglycan synthesis, increases metalloprotease production by cultured chondrocytes, and can modulate some of the actions of interleukin-1 on cartilage. The objective of the present study was to characterize the subtype of prostaglandin E_2 receptor present in bovine chondrocytes in culture.

2 Primary cultures of articular chondrocytes were prepared from slices of bovine carpal cartilage by sequential digestion with type III hyaluronidase, trypsin, type II collagenase, followed by overnight incubation in Dulbecco's Modified Eagle's Medium (DMEM) with type II collagenase, washing, and seeding at a density of 2×10^5 cells cm^{-2} in DMEM with 10% foetal bovine serum.

3 PGE_2 and carbaprostacyclin induced dose-dependent increases in intracellular cyclic AMP in bovine chondrocytes in culture. The potencies of these compounds were different, and maximal doses of PGE_2 and carbaprostacyclin had an additive effect. PGD_2 induced a small increase in intracellular cyclic AMP only at a high concentration (10^{-5} M).

4 PGE_2 was more potent that the EP_2 agonist 11-deoxy-PGE_1 at inducing increases in intracellular cyclic AMP. The EP_2 agonist butaprost, however, induced only a small increase at a concentration of 10^{-5} M. 17-Phenyl-PGE_2 (EP_1 agonist), sulprostone and MB 28767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid) (EP_3 agonists) did not induce an increase in intracellular cyclic AMP at concentrations up to 10^{-5} M.

5 The EP_4 antagonist AH 23848B ([1α(Z),2β,5α]-($\pm$)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-5-heptenoic acid) antagonized PGE_2 but not carbaprostacyclin effects on intracellular cyclic AMP. The Schild plot slope was different from 1 but this could be due to an interaction of PGE_2 with IP receptors in high doses. The exact nature of the antagonism by compound AH 23848B could not be definitely established in these experimental conditions.

6 Neither PGE_2 nor any of its analogues inhibited the increase in intracellular cyclic AMP induced by forskolin, and pertussis toxin did not alter the response to PGE_2, suggesting that no Gi-coupled PGE_2 receptors are present in these cells. Stimulation with PGE_2 did not induce significant increases in intracellular inositol-trisphosphate levels nor increases in intracellular free calcium as determined by confocal microscopy, suggesting the absence of phospholipase-C-coupled or of calcium channel-coupled PGE_2 receptors in bovine chondrocytes in these experimental conditions.

7 These results show for the first time that bovine chondrocytes in culture present a functional PGE_2 receptor that has some pharmacological characteristics of an EP_4 subtype, as well as an IP receptor.

Keywords: Prostaglandin receptors; prostaglandin E_2; prostacyclin; chondrocyte; cartilage; osteoarthritis; arthritis

Introduction

Chondrocytes are the cells responsible for the maintenance of cartilage integrity and turnover, possessing the abilities both to destroy and to synthesize cartilage matrix. The catabolic aspect of these cells is the production of metalloproteases, and the anabolic counterpart the synthesis of proteoglycans, collagens, and tissue inhibitor of metalloproteases (TIMP) (Pelletier & Martel-Pelletier, 1993). The maintenance of the cartilage integrity depends on the balance of chondrocyte anabolic and catabolic functions, and there are many indications that prostanoids act as important modulators of these functions. Chondrocytes possess the enzyme phospholipase A_2 (PLA_2), and the activity of this enzyme is higher in osteoarthritic than in normal human cartilage showing a positive and linear relation to the proteoglycanase activity (Vignon *et al.*, 1989) of the cartilage. Chondrocytes not only produce prostaglandins but are also exposed to high concentrations of these substances produced by other cells during inflammatory processes such as rheumatoid arthritis (Dayer *et al.*, 1977). High levels of prostaglandin E_2 (PGE_2) have also been described in synovial fluid from patients with osteoarthritis (Atik, 1990). Prostaglandins may increase proteoglycan degradation and latent metalloprotease release in cultured chondrocytes (Malemud & Sokoloff, 1977). Prostanoids may be important modulators of the actions of interleukin-1 (IL-1) on cartilage, as suggested by several studies showing the inhibition of the effects of that particular cytokine on chondrocytes and cartilage by non-steroidal anti-inflammatory agents (Pelletier & Martel-Pelletier, 1989).

Prostanoid actions are mediated by specific receptors that have been classified on the basis of their sensitivity to the five naturally occurring eicosanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TxA_2) and named DP, EP, FP, IP and TP respectively (Kennedy *et al.*, 1982). With the use of synthetic prostaglandin analogues three subtypes of PGE_2 receptors have been characterized and called EP_1, EP_2 (Coleman *et al.*, 1984) and EP_3 (Coleman *et al.*, 1987). More recently a new subtype, the EP_4 receptor, has been described (Coleman *et al.*, 1994a). EP_1 receptors are present in smooth muscles, mediate a contractile response (Lawrence *et al.*, 1992; Sheldrick *et al.*, 1988), and have been shown to be coupled to phosphoinositide hydrolysis through regulatory G proteins (Creese & Denborough, 1981). There are selective agonists for the EP_1 receptor but none is highly selective, while several specific antagonists have been developed (Coleman *et al.*,

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1990). EP_2 receptors are widely distributed in smooth muscle and mediate relaxation (Coleman *et al.*, 1984) through interaction with a Gs protein and stimulation of AMP-cyclase (Jumblatt & Paterson, 1991). Specific EP_2 agonists but no specific antagonists are available. EP_3 receptors also have a wide distribution and can mediate contraction of smooth muscle (Coleman *et al.*, 1990), and inhibit adipocyte lipolysis (Strong *et al.*, 1992), autonomic neurotransmitter release (Ohia & Jumblatt, 1990), and gastric acid secretion (Coleman *et al.*, 1988). EP_3 receptors were characterized by the use of specific agonists, specific antagonists being not currently available. EP_4 receptors cause relaxation of the piglet saphenous vein and can be characterized by the use of antagonists that also block TP receptors (Coleman *et al.*, 1994a). Mouse (Watanabe *et al.*, 1993; Honda *et al.*, 1993; Namba *et al.*, 1993) and human (An *et al.*, 1993; Funk *et al.*, 1993; Adam *et al.*, 1994; Bastien *et al.*, 1994) EP_1, EP_2 and EP_3 receptors have been cloned. They are all rhodopsin-type receptors with 7 transmembrane domains. Several isoforms of the EP_3 receptor can be produced by alternative splicing of the C-terminus (Negishi *et al.*, 1993; Irie *et al.*, 1993; Breyer *et al.*, 1994; Adam *et al.*, 1994). These isoforms have different G-protein specificities and can, thus, activate different second messenger systems (Namba *et al.*, 1993) with minimal changes in the affinity for agonists. Recently a new clone of the human EP_2 receptor was described (Regan *et al.*, 1994). Its sequence and pharmacological characteristics differ from the previously described EP_2 clone (Bastein *et al.*, 1994) but are much closer to the pharmacologically defined EP_2 (Coleman *et al.*, 1994a). It is possible that one of these clones is, in fact, the EP_4 receptor (Pierce *et al.*, 1995). This has been shown to be the case for the cloned mouse receptors. In this species the cDNA clone for a PGE_2 receptor positively coupled to adenylate cyclase and initially described as an EP_2 receptor (Honda *et al.*, 1993) was later shown to have pharmacological characteristics of an EP_4 receptor (Nishigaki *et al.*, 1995). This receptor was not sensitive to butaprost, an EP_2 agonist, but could be activated by 11-deoxy-PGE_1, also known as an EP_2 agonist. Compound AH 23848B not only displaced [^{3}H]-PGE_2 binding to the cloned receptor but was a competitive antagonist of PGE_2-stimulated adenosine 3',5'-cyclic monophosphate (cyclic AMP) formation.

Knowledge of the distribution of receptor subtypes may allow pharmacological intervention aimed at restricted targets, thus decreasing undesired effects on other systems. Although PGE_2 is an important autacoid for chondrocytes the characterization of receptor subtypes on these cells has not yet been performed. The aim of the present study was to characterize the subtypes of PGE_2 receptors present on bovine chondrocytes in primary culture. Our first attempts were to perform binding assays using tritiated PGE_2 as the radioactive ligand. The specific binding obtained, however, was too low to allow an accurate evaluation of the system. An alternative approach was then envisaged, that of characterizing the receptor subtype through the actions of prostanoids on the intracellular levels of the second messengers that could be implicated in message transduction and on the biological actions of these autacoids. Using these methods we obtained results showing that the EP receptor present in bovine chondrocytes in culture has some characteristics of the EP_4 subtype.

Methods

Cell culture

Articular chondrocytes were prepared from bovine carpal cartilage. The articulations were obtained from a local slaughter house. After thorough washing in phosphate buffered saline (PBS) the articular capsules were opened under sterile conditions, slices of cartilage were cut out and reduced to small fragments measuring approximately $2 \times 2 \times 0.5$ mm. These fragments were sequentially treated at room temperature with 0.5 mg ml^{-1} type III hyaluronidase from sheep testes in PBS for 10 min, followed by 2.5 mg ml^{-1} trypsin in PBS for 15 min and by 2 mg ml^{-1} type II collagenase for 30 min in PBS to remove cells other than chondrocytes eventually attached to the fragments. They were then incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS), 50 u ml^{-1} penicillin, 150 μg ml^{-1} streptomycin, and 1.5 mg ml^{-1} type II collagenase at 37°C in a 5% CO_2 atmosphere. After the overnight incubation the detached cells were washed in PBS, resuspended in the same culture medium without collagenase and seeded at a density of 2×10^5 cells cm^{-2}. The medium was replaced at 3 to 4 day intervals. Confluent cells were incubated for 24 h in DMEM with 2% FBS and then used in the experiments.

Determination of the levels of intracellular cyclic AMP

Confluent primary cultures of bovine chondrocytes in 24 well plates were incubated in DMEM with 2% FBS for 24 h. The medium was changed and the cells were incubated for 15 min at 37°C in serum-free DMEM containing 0.1 mM 3-isobutyl-1-methyl-xanthine, then stimulated for 15 min with PGE_2 or its analogues. To test for the presence of AMP-cyclase-inhibiting receptors, the cells were stimulated with 10 μM forskolin immediately before application of the stimulus. All stimulations were stopped by aspiration of the medium and lysis of the cells with 0.1 M HCl at room temperature for 3 h. Aliquots of the supernatants were stored at −80°C. Intracellular cyclic AMP level was determined, after acetylation, using a commercially available ELISA kit (Cayman, Ann Arbor, MI, U.S.A.). The results are expressed as a function of the concentration of protein in the cells as determined by the Bradford (1976) method with BSA as standard. For assays with pertussis toxin (PTx) the cells were incubated with 10 ng ml^{-1} PTx for 12 h at 37°C before application of the stimuli.

Determination of the levels of intracellular inositol trisphosphate

Confluent primary cultures of chondrocytes in 24 well plates were used in these studies. Labelling and stimulation were done according to a previously described method (Boulay *et al.*, 1990). Briefly, the medium was changed for inositol-free DMEM without FBS containing 10^6 c.p.m. ml^{-1} myo-[2-^{3}H]-inositol (10–20 Ci mmol^{-1}) for 12 h. The labelled cells in 24 well plates were washed with 1 ml of PBS containing 5.5 mM dextrose and incubated with 225 μl of medium M199 (Sigma Chem. Co. St. Louis, MI) containing 25 mM HEPES, 1 mg ml^{-1} BSA, pH 7.4, for 10 min at 37°C. The cells were then stimulated with PGE_2 or analogues for 5 min. The reactions were terminated with the addition of 100 μl of a solution of 17.5% (v/v) ice-cold perchloric acid and 50 μl of 10 mg ml^{-1} BSA, followed by incubation for 30 min at 0°C. The cells were scraped with the aid of a rubber policeman, transferred to Eppendorf tubes and centrifuged 5 min at $13\,000 \times g$ at 4°C. The pellet-associated radioactivity was determined in a scintillation counter. Perchloric acid was extracted from the supernatants with a 1 : 1 mixture of freon and tri-n-octyl-amine. After neutralisation, samples were applied to a column of Dowex AG1-X8 formate form. The radioactive inositol phosphates were separated by elution of the column with 9 ml of 0.1 M formic acid containing 1.0 M ammonium formate, and the amount of radioactive inositol in the eluate evaluated in a scintillation counter. Results were calculated as a percentage of the amount of radioactivity in control, non-stimulated cells.

Determination of intracellular calcium

Laser confocal scanning microscopy was used to determine Ca^{2+} concentration and distribution within individual cells. Freshly obtained bovine chondrocytes were seeded at a density of 40 000 cells cm^{-2} onto 25 mm round glass coverslips coated with 2.5 μg ml^{-1} poly-L-lysine as previously described (Bkaily,

1994). The plates wee incubated for 72 h in the usual culture medium, washed twice, and incubated for 1 h in Tyrode buffer containing 0.1% BSA and 13 μM Fluo3-AM, then 15 min in the same buffer without BSA. Individual coverslips were placed in an observation chamber with Tyrode buffer containing 1.8 mM $CaCl_2$. Cells were examined with a Molecular Dynamics Multiprobe 2001 confocal argon-laser scanning system equipped with a Nikon Diaphot inverted epi-fluorescence microscope and a 60 x 1.4 N.A. Nikon Oil Plan achromat objective. The 488 mm argon laser line (7.5 mV) was directed to the sample via a primary dichronic filter and attenuated with 1–3% neutral density filters to reduce photobleaching. PGE_2 10^{-6} M, carbaprostacyclin 10^{-6} M, or bradykinin 10^{-6} M was added to the chamber and records done every 30 s for 6 min. Serial confocal images were collected at 0.18 μm step-size increments. Between 35 and 65 optical sections were obtained depending on the flatness of the adherent cell, with an image resolution of 512 x 512 pixels per scan. Images were collected by the Image Space software provided by Molecular Dynamics and stored in a Silicon Graphics Indy R3000 workstation. Image analysis and 3-D reconstructions were done in a Silicon Graphics Indy R4000 workstation by the Volume Work tools of the Image Space software, and images printed on a Mitsubishi CP-210 colour processor. Intracellular free calcium intensity levels are represented as pseudo-coloured images with an intensity scale of 0 to 255, with lowest intensity in black and highest in white.

Compounds

PGE_2, 11-deoxy-PGE_1, 17-phenyl-ω-trinor-PGE_2, PGD_2, $PGF_{2\alpha}$ and carbaprostacyclin were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). The following compounds were gifts which we gratefully acknowledge: sulprostone from Prof H. Vorbrüggen, Schering AG, Berlin, Germany; MB 28767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid) from Rhône-Poulenc Rorer Ltd, Eastbourne, U.K.; butaprost from Dr Harold Kluender, Bayer Corporation, West Haven, CT, U.S.A.; AH 23848B ([1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-5-heptenoic acid) from Dr R.A. Coleman, Glaxo Res. Dev. Ltd, Herefordshire, U.K.; SMS 201-995 (D-Ph-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)) from Dr J. Morisset, Université de Sherbrooke. Forskolin was purchased from ICN Biochemicals (Cleveland, OH, U.S.A.) and fluo3-AM from Molecular Probes Inc. (Eugene, OR, U.S.A.). All other substances were bought from Sigma Chem. Co. (St. Louis, MI, U.S.A.).

Analysis of data

EC_{50}s of dose-response curves were calculated by linear regression of the points situated between 20% and 80% of the maximum response plotted against the log of the concentration. Differences between points in different curves were compared by analysis of variance, and Student's t tested used for the other results. All data are presented as means ± s.e.mean of n different observations. P values of <0.05 were considered to be statistically significant. Schild plots were done by plotting log (DR – 1) against log of the concentration of antagonist as described elsewhere (Kenakin, 1987).

Results

The effect of prostanoids on intracellular cyclic AMP levels

Both PGE_2, the stable and specific PGI_2 agonist carbaprostacyclin (Whittle *et al.*, 1980; Aiken & Shebuski, 1980) and PGD_2 induced significant increases in intracellular cyclic AMP levels in bovine chondrocytes in culture (Figure 1a) when compared to non-stimulated cells; this effect was concentration-dependent. Carbaprostacyclin was the most potent agonist tested, presenting an $EC_{50} = 1.49 \pm 0.8$ nM ($n = 3$), followed by PGE_2 with an $EC_{50} = 55.11 \pm 8.91$ nM ($n = 6$), and by PGD_2 ($EC_{50} = 1.65 \pm 1.12$ μM, $n = 4$). The differences between these EC_{50}s were statistically significant for the three compounds tested. Among the PGE_2 analogues tested sulprostone, 17-phenyl-PGE_2 and MB 28767 were ineffective in inducing increases in intracellular cyclic AMP. 11-Deoxy-PGE_1 was effective at concentrations greater than 10^{-7} M, and butaprost at 10^{-5} M. Since no plateau was reached in concentrations up to 10^{-5} M the EC_{50}s for these two compounds were not calculated

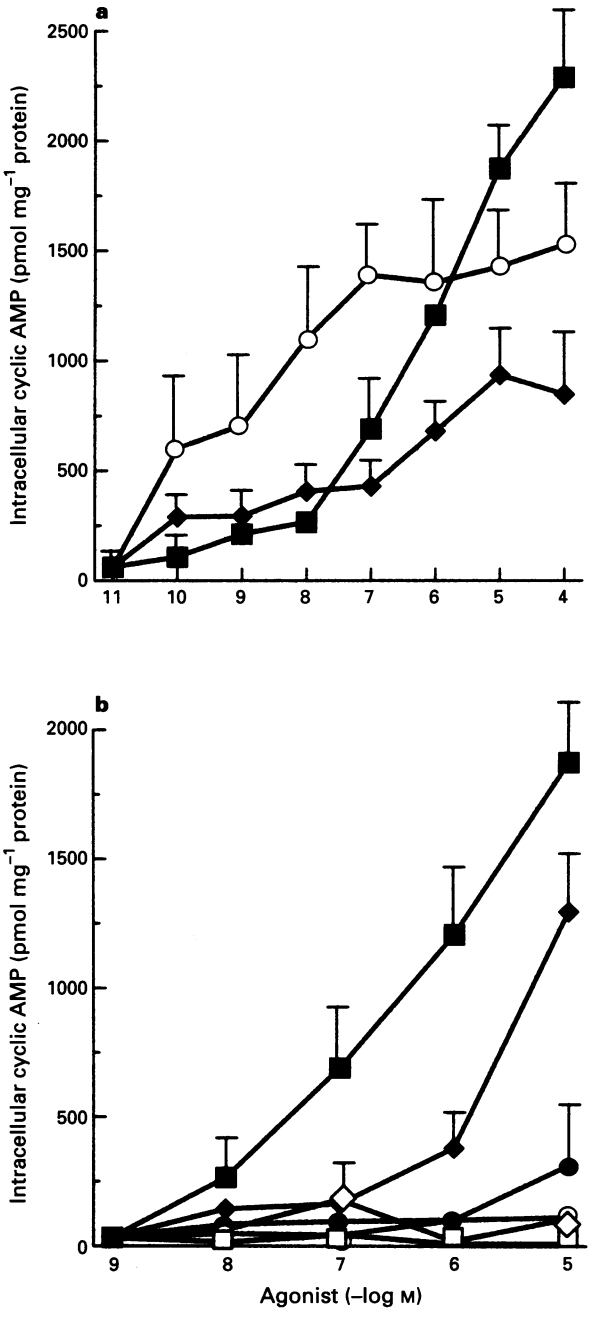

Figure 1 Effects of prostaglandin E_2 (PGE_2), PGE_2 agonists, PGD_2 and carbaprostacyclin on intracellular cyclic AMP in bovine chondrocytes in culture determined by ELISA. For comparison, the same curve for PGE_2 is shown in (a) and (b). (a) Carbaprostacyclin (○, $n = 3$); PGE_2 (■, $n = 8$); PGD_2 (◆, $n = 4$). (b) PGE_2 (■, $n = 8$); sulprostone (□, $n = 3$); 17-phenyl-ω-trinor PGE_2 (◇, $n = 3$); MB 28767 (○, $n = 3$); 11-deoxy-PGE_1 (◆, $n = 3$); butaprost (●, $n = 3$). Data represent the means and vertical lines show s.e.mean.

(Figure 1b). AH 23848B shifted the PGE_2 concentration-response curve to the right, and this shift depended on the concentration of AH 23848B (Figure 2a). A small depression of the maximum response was seen in the presence of the antagonist and the Schild regression yielded a slope of 0.43 ± 0.07 ($n=3$). The pK_B of the competitor was not calculated because the slope of the Schild regression was significantly different from one. AH 23848B did not displace the dose-response curve to carbaprostacyclin (Figure 2b). When tested together 10^{-5} M PGE_2 and 10^{-5} M carbaprostacyclin showed an additive effect on the increase in intracellular cyclic AMP (Figure 3).

Evaluation of G_i-coupled PGE_2 receptors

To evaluate the possibility of the existence of G_i-coupled PGE_2 receptors the cells were stimulated with 10 μM forskolin and treated with two concentrations of PGE_2 (10^{-10} and 10^{-5} M). As can be seen in Figure 4a PGE_2 did not significantly reduce the response to forskolin. The predominant intracellular cyclic AMP-increasing effect of PGE_2 could, however, conceal the existence of a G_i-coupled receptor. Since pertussis toxin is known to inhibit signal transduction through G_i proteins (Katada & Ui, 1979; 1981), we assessed if the increase in intracellular cyclic AMP in cells treated with both PTx and PGE_2 was higher than in the cells treated with PGE_2 alone, which would indicate the presence of PGE_2 receptors coupled to a G_i protein. SMS 201-995, a long-acting octapeptide analogue of somatostatin (Pless *et al.*, 1986), a hormone acting through receptors coupled to G_i protein, was used was a control for PTx. As can be seen in Figure 4b PTx did not alter the response to PGE_2. Pertussis toxin alone had a small but significant effect on the basal levels of intracellular cyclic AMP. SMS 201-995 alone decreased both the basal level of intracellular cyclic AMP and the response to PGE_2, and PTx reversed the inhibitory effect of SMS 201-995 on the response to PGE_2.

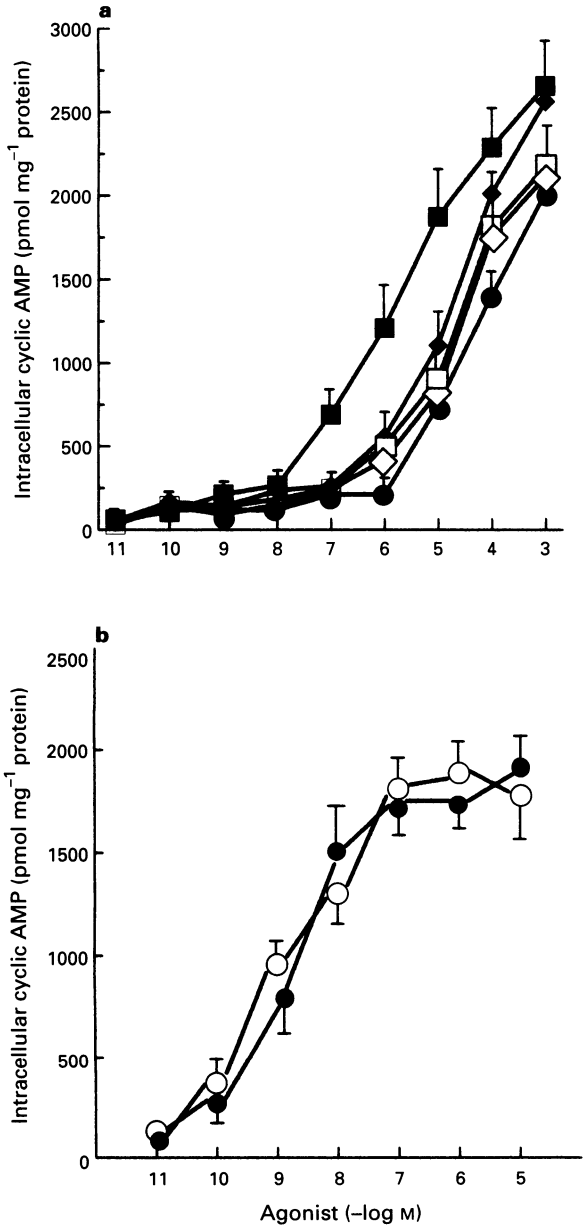

Figure 2 Effect of AH 23848B on the intracellular cyclic AMP levels induced by prostaglandin E_2 (PGE_2) and carbaprostacyclin. (a) PGE_2 (■, $n=8$); PGE_2 + AH 23848B 1 μM (◆, $n=3$); PGE_2 + AH 23848B 3 μM (□, $n=3$); PGE_2 + AH 23848B 10 μM (◇, $n=3$); PGE_2 + AH 23848B 30 μM (●, $n=6$). (b) Carbaprostacyclin (○, $n=3$); carbaprostacyclin + AH 23848B 30 μM (●, $n=3$). Data represent means and vertical lines show s.e.mean.

The effect of prostanoids on inositol phosphates turnover

Activation of EP_1 and some isotypes of EP_3 receptors may lead to increases in intracellular calcium through the activation of phospholipase C and inositol phosphates turnover (Coleman *et al.*, 1994b). Bovine chondrocytes in culture were stimulated with 10^{-5} M PGE_2 and the concentrations of intracellular IP_3 determined after 0, 1, 5 and 10 min of incubation. PGE_2 failed to induce significant increases of intracellular IP_3 levels at any of the times tested, although bradykinin, used as a positive control, doubled the intracellular concentrations of total inositol phosphates from 1 to 10 min (Figure 5).

The effect of prostanoids on intracellular calcium

Increases in intracellular calcium upon stimulation with prostaglandins may also occur through not well defined inositol phosphate-independent mechanisms (Coleman *et al.*, 1994b). To verify if prostaglandin receptors, by use of these mechanisms, were present in bovine chondrocytes in culture we stimulated the cells and determined intracellular calcium levels by confocal microscopy. As can be seen in Figure 6, PGE_2 10^{-5} M

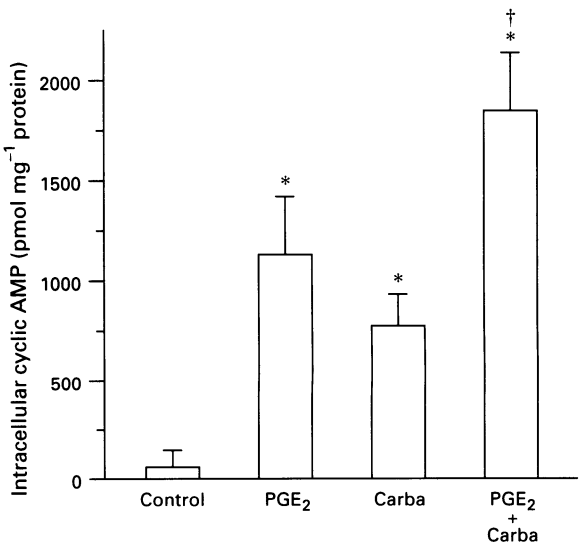

Figure 3 Additive effect of maximal doses of prostaglandin E_2 (PGE_2) and carbaprostacyclin on intracellular cyclic AMP levels in bovine chondrocytes in culture. PGE_2 10^{-5} M ($n=3$); carbaprostacyclin 10^{-5} M ($n=6$); PGE_2 10^{-5} M + carbaprostacyclin 10^{-5} M ($n=3$). Data represent means and vertical lines show s.e.mean. $^*P<0.05$ compared to control, Student's t test. $^\dagger P<0.05$ compared to PGE_2 alone, Student's t test. Carba = carbaprostacyclin.

failed to induce increases in intracellular calcium, although bradykinin, which was used as a positive control, was effective in this regard.

Discussion

Prostaglandins are probably important autacoids for the regulation of cartilage metabolism, as indicated by their biological effects on chondrocytes (Malemud & Sokoloff, 1977), their local synthesis by these cells (Dayer *et al.*, 1977), and by their presence in synovial fluids from inflammatory (Dayer *et al.*, 1977) and degenerative (Atik, 1990) joint diseases. Characterization of the types and subtypes of prostanoid receptors in chondrocytes and the availability of prostanoid analogues may allow a better understanding of the role of these substances in the control of cartilage metabolism and lead to better pharmacological approaches of these diseases. The objetive of the present study was, thus, to characterize the EP receptor subtype present in bovine chondrocytes in culture. Since, however, binding studies yielded a very low specific binding of [^{3}H]-PGE_2, we used the alternative approach of determining the effects of prostaglandins and analogues on the intracellular second messengers coupled to the specific receptors.

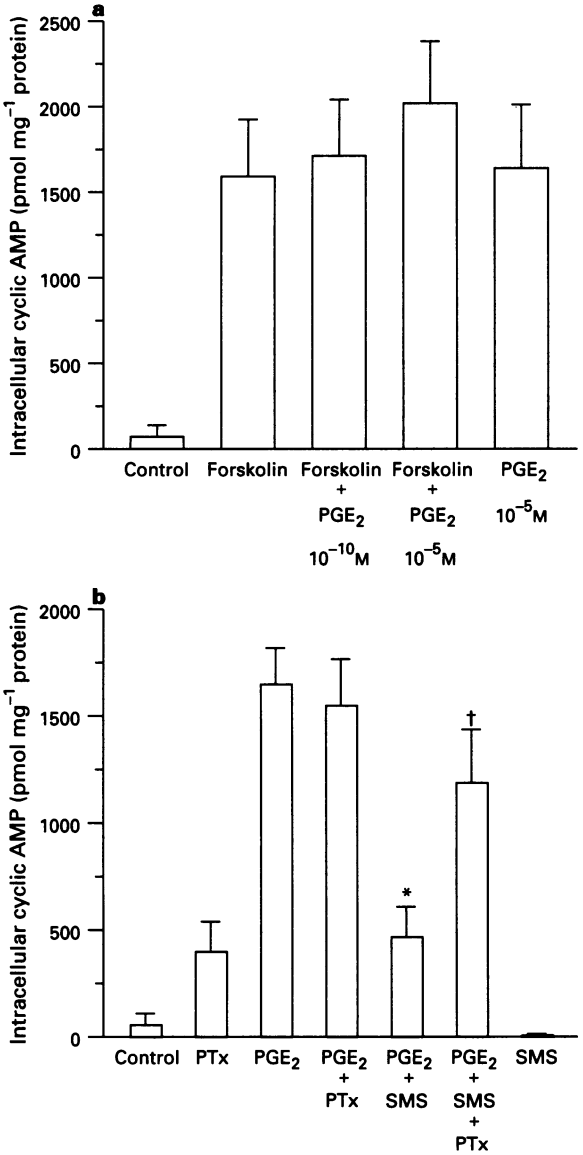

Figure 4 Effect of prostaglandin E_2 (PGE_2) on the intracellular levels of cyclic AMP in the presence of forskolin, pertussis toxin, and SMS 201-995, a somatostatin analogue. (a) Forskolin 10 μM ($n=3$); forskolin 10 μM + PGE_2 10^{-10} M ($n=3$); forskolin 10 μM + PGE_2 10^{-5} M ($n=3$). (b) Control, non-stimulated cells ($n=3$); pertussis toxin (PTx) ($n=3$); PGE_2 10^{-5} M ($n=3$); PGE_2 10^{-5} M + PTx 10 ng ml^{-1} ($n=3$); PGE_2 10^{-5} M + SMS 201-995 10^{-7} M ($n=3$); PGE_2 10^{-5} M + SMS 201-995 10^{-7} M + PTx 10 ng ml^{-1} M ($n=3$); SMS 201-995 10^{-7} M ($n=3$). Data represent means and vertical lines show s.e.mean. $^*P<0.05$, Student's t test, in comparison to PGE_2 alone. $^\dagger P<0.05$, Student's t test, in comparison to PGE_2 plus SMS 201-995. SMS = SMS 201-995.

Characterization of the types of prostanoid receptors in chondrocytes

Both PGE_2, carbaprostacyclin and PGD_2 significantly increased intracellular levels of cyclic AMP in bovine chondrocytes in culture in the following order of potency: carbaprostacyclin > PGE_2 > > PGD_2. Carbaprostacyclin showed an EC_{50} significantly lower than PGE_2, and this could suggest that both agonists interact with an IP receptor. However, maximal doses of PGE_2 and carbaprostacyclin had an additive effect on intracellular cyclic AMP levels, suggesting that these substances are acting through different receptors. This hypothesis is reinforced by the results within the EP_4 antagonist AH 23848B, which displaced the concentration-response curve of PGE_2 but not that of carbaprostacyclin. These results indicate the existence of two groups of receptors, namely IP and EP. DP receptors are coupled to adenylate cyclase in platelets (Gorman *et al.*, 1977) and in CCL 44 cells (Ito *et al.*, 1990). On bovine chondrocytes, however, PGD_2 was effective only in very high concentrations (10^{-6} M and higher), suggesting that this effect could be the result of a non specific interaction with IP or EP receptors.

Characterization of the subtype of EP receptor

Two subtypes of EP receptors are linked to and stimulate adenylate cyclase, EP_2 and EP_4 (Coleman *et al.*, 1994b). An isoform of EP_3 receptors ($EP_{3\gamma}$) can also couple to G_s protein and stimulate adenylate cyclase (Irie *et al.*, 1993). To determine the isotype implicated in adenylate cyclase stimulation in bovine chondrocytes we compared the pharmacological profiles, in terms of adenylate cyclase activation, of PGE_2 and several

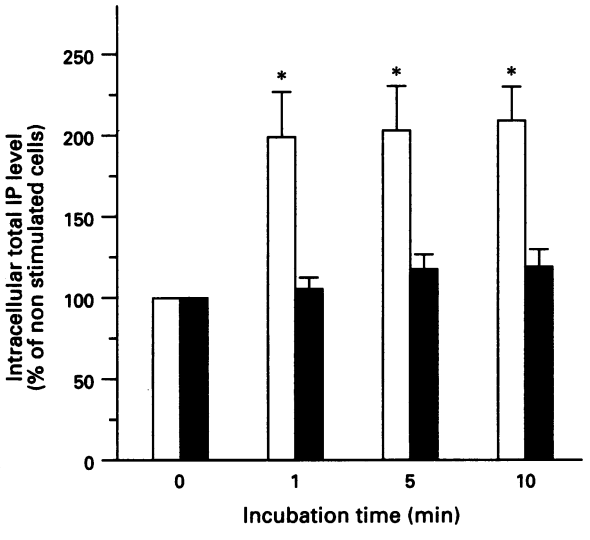

Figure 5 Effect of prostaglandin E_2 (PGE_2) on the intracellular levels of total inositol-phosphates (IP) determined by chromatography in bovine chondrocytes in culture. Bradykinin 10^{-6} M (open columns, $n=3$); PGE_2 10^{-5} M (solid columns, $n=3$). Data represent means and vertical lines show s.e.mean. Standard error bars were too small to be depicted in time zero. $^*P<0.05$ compared to time zero.

subtype-specific PGE_2 agonists. The EP_1 agonist 17-phenyl PGE_2, sulprostone (EP_1 and EP_3 agonist), and MB28767 (EP_3 agonist) (Coleman *et al.*, 1994b) were unable to induce any significant increase in intracellular cyclic AMP levels. These results indicate the absence of EP_1 and EP_3 receptor subtypes coupled to adenylate cyclase in the system studied. The EP_2 agonist 11-deoxy PGE_1 significantly increased intracellular cyclic AMP levels in concentrations of 10^{-6} M and 10^{-5} M, suggesting an interaction with an EP_2 receptor. Butaprost, however, which is also an EP_2 agonist, had a small effect only at a very high concentration (10^{-5} M). This relative lack of efficiency of butaprost is surprising for an EP_2 receptor. Since, to our knowledge, 11-deoxy PGE_1 activity on EP_4 receptors has not been tested we speculated that the observed effect of this compound could be due to an interaction with an EP_4 receptor. To test this hypothesis the cells were incubated with PGE_2 in the presence of AH 23848B, an EP_4 antagonist. AH 23848B shifted the PGE_2 concentration-response curve to the right and this shift depended on the concentration of the compound. This effect was specific to PGE_2 since it could not be observed when carbaprostacyclin was used in the presence of AH 23848B. A small depression of the maximum response to PGE_2 was observed and the Schild regression yielded a slope significantly different from one. These results suggest a non-competitive antagonism, although other reasons leading to similar results should be considered, such as drug-disposition mechanisms or non-equilbrium steady-state, the existence of a heterogeneous receptor population subserving the same response, or multiple drug properties in the concentrations used in the experiment (Kenakin, 1987). Of special concern is the possible interaction of PGE_2 in high concentrations with the IP receptor, which could lead to distorted Schild plots and stimulate a non-competitive antagonism. It would be interesting to perform these assays in the presence of a specific IP antagonist to determine if these results are due to an interaction of PGE_2 with an IP receptor. Unfortunately, such an antagonist is not available to allow the testing of this hypothesis. Although the present results with AH 23848B suggest a non-competitive antagonism it is not possible, in our model, to draw definite conclusions regarding the nature of this antagonism.

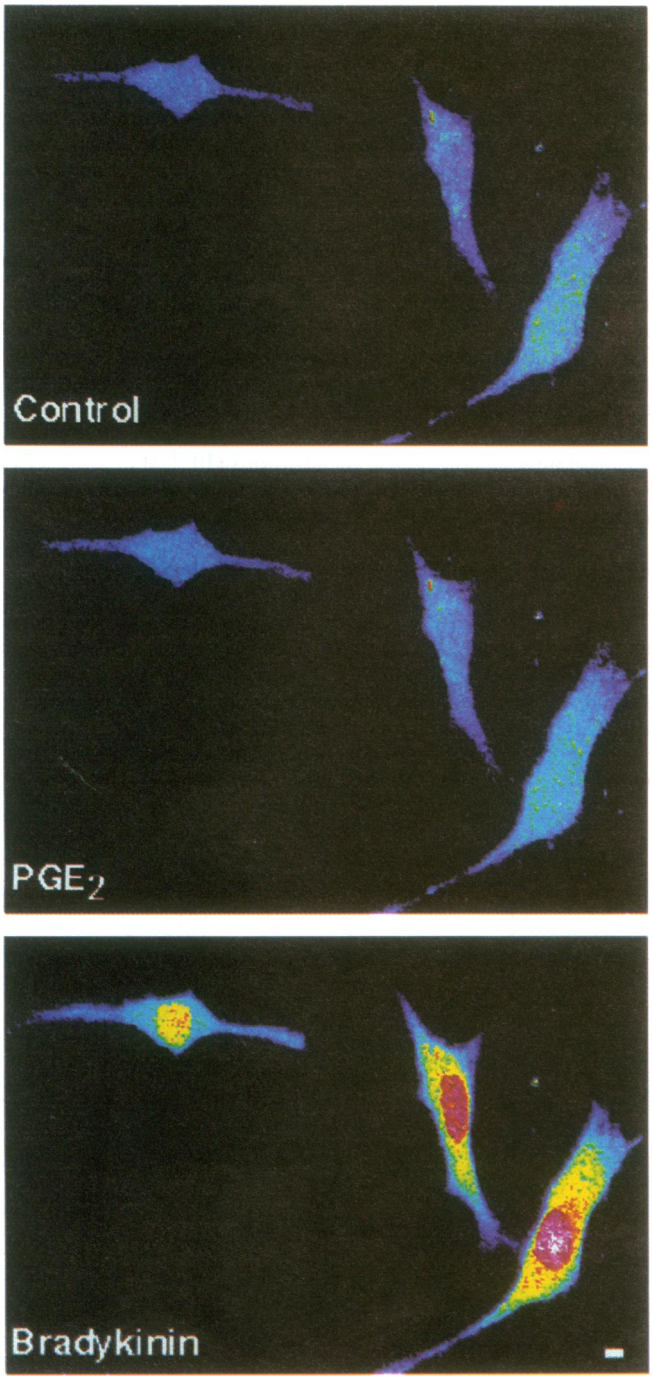

Figure 6 Effects of prostaglandin E_2 (PGE_2) and bradykinin on the intracellular Ca^{2+} determined by confocal microscopy in bovine chondrocytes in culture. Pseudocolor imaging of intracellular calcium concentration, white > red > yellow > blue > black. Control cells (upper panel); PGE_2 10^{-5} M (middle panel); bradykinin 10^{-6} M (lower panel). These are the results of a single experiment representative of three. Black corresponds to no calcium and white maximum amount of calcium. Bar = 5 μM.

A recently published article, in which the T-cell line Jurkat was used (De Vries *et al.*, 1995) described an EP receptor positively coupled to adenylate cyclase that was stimulated by PGE_2 and 11-deoxy-PGE_1, but not by butaprost. Unlike the originally described EP_4 receptor, EP receptors in Jurkat cells responded weakly to 16,16-dimethyl PGE_2 and not at all to 1-OH PGE_1. The effect of PGE_2 was antagonised by AH 23848 but again, unlike the EP_4 receptor, the antagonism was non-competitive. Based on these data the authors postulated the existence, in Jurkat cells, of an EP receptor with a novel pharmacological profile different from the EP_4 receptor. In the present study bovine chondrocytes present adenylate-cyclase-coupled EP receptors with a pharmacological profile similar to the EP receptor described by De Vries *et al.* (1995). Since, however, the nature of the antagonism by AH 23848B in bovine chondrocytes could not be precisely established, the characterization of this receptor as EP_4 or as a new variant or subtype of EP receptor, as suggested by De Vries *et al.* (1995), will need further study.

Are there other subtypes of EP receptors in bovine chondrocytes?

EP_3 receptors can couple to adenylate cyclase via a G_i protein (Coleman *et al.*, 1994b). To verify the existence of this receptor subtype the cells were treated with forskolin, a direct adenylate cyclase activator, and stimulated with PGE_2. No inhibitory effect of PGE_2 on intracellular cyclic AMP levels could be detected. However, since the predominant effect of PGE_2 is to increase intracellular cyclic AMP levels in bovine chondrocytes, there is the possbility that the existence of a G_i-coupled receptor of the EP_3 subtype could be masked by the predominant stimulant effect of PGE_2. If this were the case one would expect an inhibitor of G_i protein to increase the stimulant effect of PGE_2 on intracellular cyclic AMP levels. In the present study we used pertussis toxin, which is known to inhibit 'G_i-like' proteins, i.e., G_i1, 2, 3, G_o1, 2, and Td1, 2 (Katada & Ui, 1979; 1981), to test this hypothesis. Pertussis toxin catalyses the mono-ADP-rybosylation of G_i proteins which are maintained in their inactive $\alpha\beta\delta$ heterotrimeric

conformation; receptor activation of G_i is attenuated (Burns *et al.*, 1983) and the inhibitory effect of receptor activation on adenylate cyclase is decreased (Kurose *et al.*, 1986). In the present study pertussis toxin alone had a small but significant effect on the basal intracellular levels of cyclic AMP but did not change the response to PGE_2 (Figure 4b). As a control for the system we used SMS 201-995, a somatostatin analogue (Pless *et al.*, 1986). Somatostatin induces multiple biological actions by interacting with a family of specific receptors, referred to as SSTR1-SSTR5 (Law *et al.*, 1995), and all five receptors can be coupled to G_i protein and inhibit adenylate cyclase (Viollet *et al.*, 1995). In the present study SMS 201-995 reduced the basal level of cyclic AMP and the response to PGE_2, and this inhibitory effect was reduced by pre-incubation with pertussis toxin. These results show that stimulation of G_i-coupled receptors can reduce the increase in intracellular cyclic AMP induced by PGE_2 and that pertussis toxin can effectively block this effect. If G_i-coupled EP_3 receptors were present in significant amounts in the preparation, an increase in the response to PGE_2 in the presence of pertussis toxin should have been detected. Although it is difficult to conclude definitely that EP_3 receptors are absent, the present results suggest that G_i-coupled EP receptors, if they exist at all in this cell type, are not pharmacologically relevant under these experimental conditions.

EP_1 receptors induce increases in intracellular calcium concentrations, but the exact molecular mechanism implicated in this action has not been competely elucidated (Coleman *et al.*, 1994b). PGE_2-induced contraction of guinea-pig trachea is dependent on extracellular calcium, but phosphatidyl inositol turnover may also be implicated in this phenomenon (Watanabe *et al.*, 1993). In our system we tested the effect of PGE_2 on inositol phosphates turnover and on intracellular calcium levels, and neither parameter was increased upon stimulation with PGE_2. As a positive control we used bradykinin, which is known to increase inositol phosphates turnover and possibly intracellular calcium in chondrocytes (Baragi *et al.*, 1989). This lack of response to PGE_2 in both parameters tested strongly suggests that EP_1 receptors are not present or are inactive in bovine chondrocytes in culture.

The present results may have relevant pharmacological implications. To our knowledge bovine chondrocytes are the first isolated cell type to present just one functional EP receptor subtype that can be antagonized by AH 23848B. If the same receptor subtype is present in human chondrocytes these findings may have therapeutic implications. As has been described above, PGE_2 may participate in the pathophysiology of cartilage destruction in arthritis and osteoarthritis, and blockage of either the synthesis or of the effect of PGE_2 on cartilage may be important for the treatment of these diseases. When compared to cyclo-oxygenase inhibitors, the use of specific antagonists would have the advantage of not disturbing the physiological effects of PGE_2 on the stomach and the kidneys, where the main EP receptors are of the EP_3 subtype (Perkins *et al.*, 1991; Breyer *et al.*, 1993). We are presently working on the characterization of the EP receptor subtype in human chondrocytes.

In conclusion, the present results show that bovine chondrocytes in culture present a functional PGE_2 receptor that stimulates adenylate cyclase and is antagonized by AH 23848B. This receptor has some of the pharmacological characteristics of the EP_4 receptor subtype, and is probably the only EP receptor present or functional in this cell type in our experimental conditions. Our results suggest that an IP receptor is also present in the preparation.

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