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Phosphodiesterase isoenzyme families in human osteoarthritis chondrocytes – functional importance of phosphodiesterase 4

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1 We studied whether selective inhibitors of cyclic nucleotide hydrolysing phosphodiesterase (PDE) isoenzymes influence IL-1β-induced nitric oxide (NO) release from human articular chondrocytes. In addition, the pattern of PDE isoenzymes contributing to cyclic nucleotide hydrolysis in human chondrocytes was characterized.

2 Chondrocytes were isolated from human osteoarthritic cartilage and cultured in alginate beads. IL-1β-induced chondrocyte products (nitric oxide and prostaglandin E_2) were measured in culture supernatants after 48 h incubation time. PDE activities were assessed in chondrocyte lysates. Inducible nitric oxide synthase (iNOS) and PDE4A-D proteins were detected by immunoblotting.

3 The selective PDE4 inhibitors Piclamilast and Roflumilast partially attenuated IL-1β-induced NO production whereas selective inhibitors of PDE2 (EHNA), PDE3 (Motapizone) or PDE5 (Sildenafil) were inactive. Indomethacin reversed the reduction of IL-1β-induced NO by PDE4 inhibitors. It was shown that autocrine prostaglandin E_2 (PGE_2) enabled PDE4 inhibitors to reduce IL-1β-induced NO in this experimental setting.

4 Major PDE4 and PDE1 activities were identified in chondrocyte lysates whereas only minor activities of PDE2, 3 and 5 were found. IL-1β and cyclic AMP-mimetics upregulated PDE4 activity and this was associated with an augmentation of PDE4B2 protein.

5 Based on the view that nitric oxide contributes to cartilage degradation in osteoarthritis our study suggests that PDE4 inhibitors may have chondroprotective effects.
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Keywords: Chondrocytes; phosphodiesterase; cyclic AMP; nitric oxide; interleukin-1β

Abbreviations: BCA, bicinchoninic acid; COX, cyclooxygenase; IBMX, isobutylmethylxanthine; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LDH, lactat dehydrogenase; NO, nitric oxide; PDE, phosphodiesterase

Introduction

Over the past decade numerous studies established the view that selective inhibitors of cyclic AMP-hydrolysing PDE4 may represent a new class of anti-inflammatory drugs. In fact, at least two selective PDE4 inhibitors – SB207499 (Ariflo) (Barnette *et al*., 1998; Torphy *et al*., 1999) and Roflumilast (Hatzelmann & Schudt, 2001; Bundschuh *et al*., 2001) – are in advanced clinical development for inflammatory airway diseases. Numerous splicing variants of four PDE4 subtypes (PDE4A-D) are expressed in humans. Activities of the PDE4 variants are regulated by transcriptional and post-translational mechanisms (Houslay *et al*., 1998).

Osteoarthritis (OA) is characterized by progressive degradation of articular cartilage driven by abnormal function of the articular chondrocyte. Consequently, pharmacological restoration of normal chondrocyte function represents a strategy for OA treatment. One of the factors supporting cartilage degradation is IL-1β. *In vitro*, human chondrocytes exposed to IL-1β produce a panel of mediators promoting cartilage degradation such as nitric oxide (NO) (Palmer *et al*., 1993), prostaglandin E_2 (PGE_2) (Geng *et al*., 1995) or matrix metalloprotease-13 (MMP-13, collagenase-3) (Reboul *et al*., 1996). NO induces chondrocyte apoptosis (Blanco *et al*., 1995) and inhibits proteoglycan (Häuselmann *et al*., 1994b) and collagen II synthesis (Cao *et al*., 1997). Conversely, selective inhibition of inducible NO synthase (iNOS) alleviated OA in an animal model (Pelletier *et al*., 2000).

Previous work suggests that cyclic AMP-mimetics and PDE4 inhibitors may interfere with human chondrocyte functions (Martel-Pelletier *et al*., 1999; DiBattista *et al*., 1996; Geng *et al*., 1998). The purpose of this study was to investigate effects of selective and non-selective PDE inhibitors on NO release from human chondrocytes isolated from OA cartilage. This work was complemented by an analysis of the phosphodiesterases contributing to cyclic nucleotide hydrolysis in chondrocytes.

Methods

Reagents

Pronase and collagenase P were from Calbiochem (Bad Soden, Germany) and Roche Molecular Biochemicals (Mannheim, Germany) respectively. Alginate (Keltone LV)

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was obtained from Kelco (Chicago, IL, U.S.A.). DMEM/F12 1:1 medium, FBS, glutamine and gentamicin were from Life Technologies (Karlsruhe, Germany). IL-1β was purchased from Peprotech (London, U.K.). The PGE_2 kit was from R&D systems (Wiesbaden, Germany). The LDH assay kit was purchased from Promega (Mannheim, Germany). Tritiated cyclic nucleotides and QAE Sephadex A25 were obtained from AP Biotech (Freiburg, Germany). Rothi®-Load electrophoresis loading buffer and acrylamide/bisacrylamide (Rotiphorese 30) were from Carl Roth GmbH (Karlsruhe, Germany). A rabbit polyclonal antibody raised against a peptide corresponding to the aminoterminal end of iNOS of human origin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Goat anti-rabbit IgG coupled to horse radish peroxidase was from Jackson ImmunoResearch (Richmond, NJ, U.S.A.). The BCA protein assay was from Pierce (Rockford, IL, U.S.A.) and Lumi-Light^{PLUS} Western Blotting Substrate was purchased from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals were of reagent grade and purchased from several different companies. The selective PDE3 inhibitor Motapizone was a gift from RPR (Cologne, Germany), the selective PDE inhibitors Piclamilast (PDE4) (Karlsson *et al*., 1995), Roflumilast (PDE4) (Hatzelmann & Schudt, 2001; Bundschuh *et al*., 2001) and Sildenafil (PDE5) were prepared at the chemical facilities of Byk Gulden. The protein kinase A activator Sp-5.6-cBIMPS (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole 3′,5′ cyclic mono-phosphorothioate, Sp-isomere) was from Biolog (Bremen, Germany).

Cell cultures

Cartilage was obtained from patients undergoing total knee or hip replacement surgery due to osteoarthritis in local orthopaedic hospital units and with institutional approval. OA cartilage was aseptically dissected from underlying bone. Fibrocartilaginous areas were discarded and the gross morphology of the cartilage specimen was classified as moderate to severe OA. Cartilage digestion and alginate culture of chondrocytes was performed as previously described (Häuselmann *et al*., 1994a;b; 1998) with minor modifications. Briefly, chondrocytes were isolated by sequential digestion of cartilage specimen with pronase (4 mg ml^{-1}) over 90 min and collagenase P (0.5 mg ml^{-1}) overnight in DMEM/F12 supplemented with 5% FBS, 2 mM L-glutamine and 50 μg ml^{-1} gentamicin. Chondrocytes were encapsulated in alginate beads. To this end cells (4×10^6 cells ml^{-1}) were suspended in 1.2% alginate in 150 mM sodium chloride. The suspension was added drop-wise to a 102 mM $CaCl_2$ solution with constant stirring. Alginate beads were extensively washed with saline and medium. Beads were cultured in DMEM/F12 with 20% FBS, 2 mM L-glutamine, 1 mM L-cysteine, 25 μg ml^{-1} L-ascorbate and 50 μg ml^{-1} gentamicin over 5–10 days. Where required, chondrocytes were recovered from alginate beads by exposure to 150 mM sodium chloride, 55 mM sodium citrate, 1 mg ml^{-1} collagenase P for 15 min at 37°C.

Chondrocyte NO and PGE_2 generation

To assess IL-1β-induced generation of chondrocyte-derived NO and PGE_2 alginate beads (2 beads per well) were placed in 24-well dishes and cultured in DMEM/F12 with 10% FBS, 1 mM L-cysteine, 1.1 mM L-arginine, 2 mM L-glutamine, 25 μg ml^{-1} ascorbate and 50 μg ml^{-1} gentamicin. Cells were preincubated with cyclic AMP modifiers for 30 min and stimulated with IL-1β (0.002–2 ng ml^{-1}) over 0.5–48 h in a volume of 300 μl. The final DMSO concentration was 0.3% in all experiments. NO and PGE_2 were measured in culture supernatants.

NO was measured as accumulated nitrite by using the Griess assay. Diluted culture supernatant (180 μl) was mixed with 20 μl Griess reagent (1% sulfanilamide in 1 M HCl and 0.1% N-naphtylethylenediamine dihydrochloride). Color development was measured spectrophotometrically (OD_{544}). Chondrocyte-derived NO was converted to nitrite and nitrate. The proportion of nitrite to total nitrite/nitrate release from chondrocytes was $66\pm5.1\%$ (mean$\pm$s.e.m., $n=14$) as detected in experiments including enzymatic nitrate reduction. To exclude interference of substances used in the experiments with the Griess assay effects of compounds on the absorption (OD_{544}) of 30 μM nitrate was measured. Nitrate was converted to nitrite by adding an excess of nitrate reductase. None of the substances changed the absorption induced by 30 μM nitrate in these control experiments. In other experiments it was shown that substances used in this study affected chondrocyte nitrite and nitrite/nitrate release (measured in presence of nitrate reductase) in an identical manner. Therefore, the compounds did not change the ratio of NO metabolization towards nitrite and nitrate. As a consequence the majority of the experiments relied on nitrite measurements as a surrogate parameter of NO formation. PGE_2 in culture supernatants was assessed by ELISA according to the instructions of the manufacturer. In experiments directed to investigate the time-dependency of IL-1β-stimulated chondrocyte nitrite and PGE_2 generation medium was changed several times before the start of the incubation corresponding to the longest stimulation period (i.e. 48 h) and IL-1β was added at the appropriate times during the total incubation period (i.e. 48 h). The amount of chondrocyte-derived nitrite or PGE_2 was related to total cell LDH activity. A linear relationship between total cell LDH activity and chondrocyte cell numbers was shown in separate experiments. To measure LDH activity in chondrocyte lysates cells were recovered from alginate beads and chondrocytes were lysed with Triton X-100 (final concentration 0.2% v v^{-1}). Lysates were diluted in PBS and LDH was measured with 'Cyto Tox 96 Nonradioactive Cytotoxicity Assay'. The test is based on the colorimetric determination of formazan formed in a reaction mixture containing NAD^+, lactate, the tetrazolium salt INT, diaphorase and cell lysates. Alginate beads without cells served as controls. Additional measurements of LDH in culture supernatants were used to estimate cytotoxicity. At the concentrations used in the experiments neither the different compounds nor IL-1β increased LDH release into the culture supernatants compared to controls.

Measurements of phosphodiesterase isoenzyme activities and preparation of cellular extracts

Chondrocytes (1×10^6) were washed twice in phosphate buffered saline (4°C) and resuspended in 1 ml homogenization buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4,

1.5 mM KH_2PO_4, 10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 5 μM pepstatin A, 10 μM leupeptin, 50 μM phenylmethylsulfonyl fluoride, 10 μM soybean trypsin inhibitor, 2 mM benzamidine, pH 8.2). Cells were disrupted by sonication (Branson sonifier, 3×15 s) and lysates were immediately used for phosphodiesterase (PDE) activity measurements.

PDE activities were assessed in cellular lysates as described (Thompson & Appleman, 1979) with some modifications (Bauer & Schwabe, 1980). The assay mixture (final volume 200 μl) contained (mM): Tris HCl 30; pH 7.4, $MgCl_2$ 5, 0.5 μM either cyclic AMP or cyclic GMP as substrate including [^{3}H]cAMP or [^{3}H]cGMP (about 30 000 c.p.m. per well), 100 μM EGTA, PDE isoenzyme-specific activators and inhibitors as described below and chondrocyte lysate. Incubations were performed for 60 min at 37°C and reactions were terminated by adding 50 μl 0.2 M HCl per well. Assays were left on ice for 10 min and then 25 μg 5′- nucleotidase (Crotalus atrox) was added. Following an incubation for 10 min at 37°C assay mixtures were loaded onto QAE-Sephadex A25 columns (1 ml bed volume). Columns were eluted with 2 ml 30 mM ammonium formiate (pH 6.0) and radioactivity in the eluate was counted. Results were corrected for blank values (measured in the presence of denatured protein) that were below 2% of total radioactivity. cyclic AMP degradation did not exceed 25% of the amount of substrate added. The final DMSO concentration was 0.3% (v v^{-1}) in all assays. Selective inhibitors and activators of PDE isoenzymes were used to determine activities of PDE families as described previously (Rabe *et al*., 1993) with modifications. Briefly, PDE4 was calculated as the difference of PDE activities at 0.5 μM cyclic AMP in the presence and absence of 1 μM Piclamilast. The difference between Piclamilast-inhibited cyclic AMP hydrolysis in the presence and absence of 10 μM Motapizone was defined as PDE3. The fraction of cyclic GMP (0.5 μM) hydrolysis in the presence of 10 μM Motapizone that was inhibited by 100 nM Sildenafil reflected PDE5. At the concentrations used in the assay Piclamilast (1 μM), Motapizone (10 μM) and Sildenafil (100 nM) completely blocked PDE4, PDE3 and PDE5 activities without interfering with activities from other PDE families. PDE1 was defined as the increment of cyclic AMP hydrolysis (in the presence of 1 μM Piclamilast and 10 μM Motapizone) or cyclic GMP hydrolysis induced by 1 mM Ca^{2+} and 100 nM calmodulin. The increase of cyclic AMP (0.5 μM) degrading activity in the presence of 1 μM Piclamilast and 10 μM Motapizone induced by 5 μM cyclic GMP represented PDE2. The PDE2 inhibitor EHNA (100 μM) completely inhibited this cyclic GMP-induced activity increment further verifying this activity as PDE2.

Detection of PDE4 and iNOS protein expression in chondrocytes stimulated with IL-1β

Chondrocyte beads were preincubated with cyclic AMP modifiers or Indomethacin and stimulated with IL-1β (200 pg ml^{-1} or 2 ng ml^{-1}) over 12 h. At the end of the incubation period chondrocytes were recovered from beads, washed twice in ice-cold PBS and resuspended in lysis buffer (150 mM sodium chloride, 5 mM EDTA, 50 mM TrisHCl, 0.1% sodium azide, 0.5% Triton X-100, 5 μM pepstatin A, 10 μM leupeptin, 50 μM phenylmethylsulfonyl fluoride, 10 μM soybean trypsin inhibitor and 2 mM benzamidine) at 1×10^6 cells per 50 μl buffer. The suspension was incubated for 30 min at 4°C and then centrifuged at $1000 \times g$ for 15 min at 4°C. Supernatants were removed and an aliquot was taken for protein measurements. The remaining supernatant was mixed with one third of its volume of a modified Laemmli buffer (Roti®-Load1), boiled for 5 min and frozen at −80°C for later immunoblotting. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (10% acrylamide/0.34% bisacrylamide) under reducing conditions. After transfer to PVDF membranes proteins were immunostained with polyclonal rabbit antibodies to human PDE4A-D or iNOS. Bound antibodies were detected by goat-anti rabbit IgG coupled to horsh radish peroxidase and visualized using the LumiLight^{PLUS} Western Blotting Substrate by Fuji LAS-1000 CCD camera and AIDA Version 2.0 software. Polyclonal antibodies against human PDE4A-D were obtained from a commercial source and raised in rabbits according to standard procedures. Antibodies are directed against the following PDE4-subtype specific peptide se-

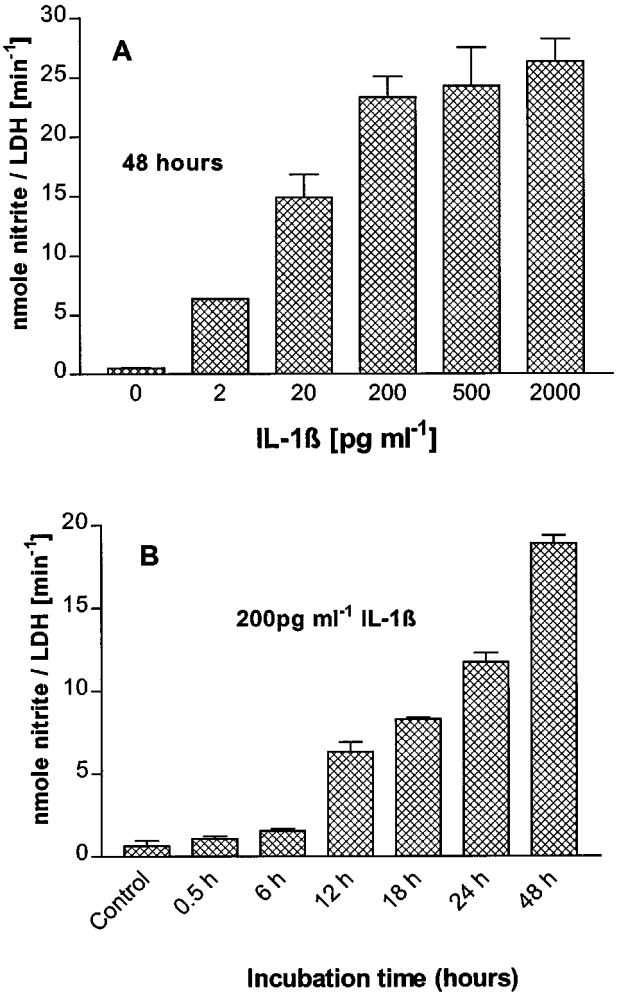

Figure 1 Time- and concentration-dependent induction of chondrocyte NO production by IL-1β. Chondrocytes cultured in alginate over 5–7 days were stimulated with IL-1β (0–2 ng ml^{-1}) for up to 48 h. Nitrite accumulation in culture supernatants was related to LDH in lysates. Results are given as the means±s.e.m. from 3–5 independent experiments.

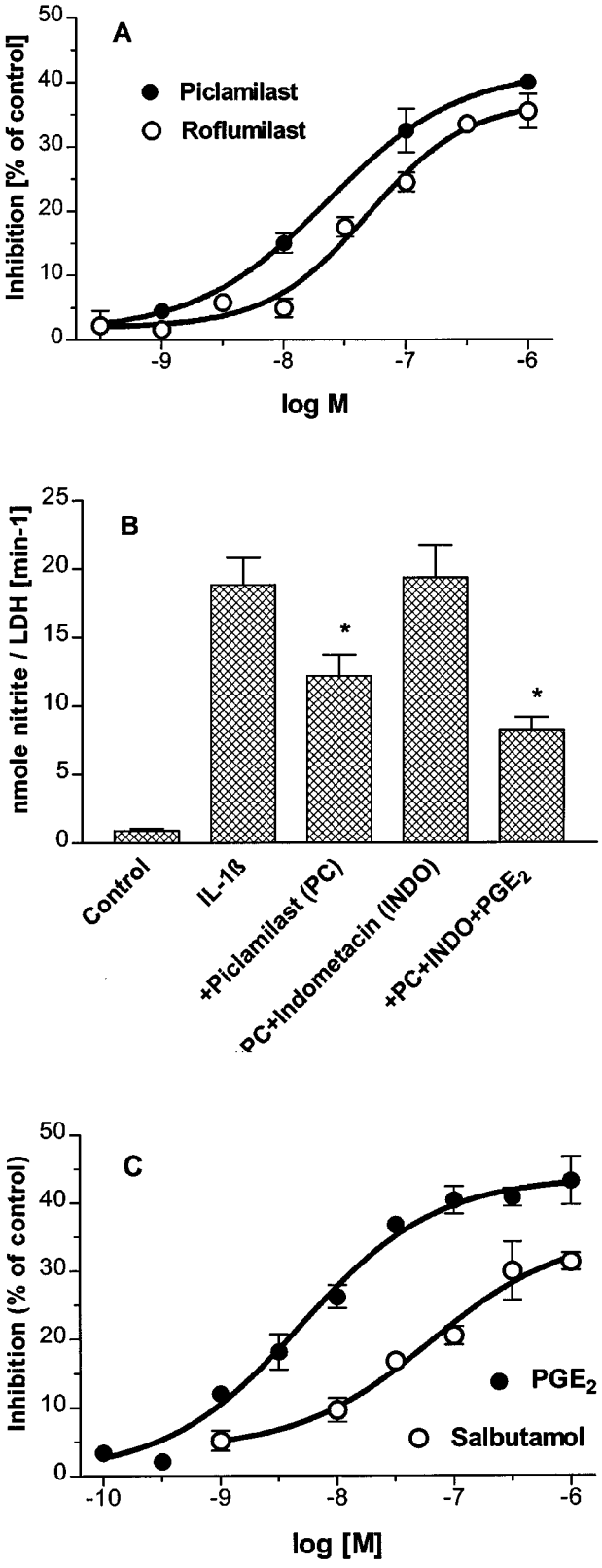

Figure 2 Concentration-dependent inhibition of IL-1β-induced chondrocyte nitrite accumulation by selective PDE4 inhibitors; reversal by Indomethacin. Chondrocytes were preincubated with the selective PDE4 inhibitors Piclamilast or Roflumilast (0.3–1000 nM), 10 μM Indomethacin, PGE_2 (0.1–1000 nM) or Salbutamol (1–1000 nM) before IL-1β (200 pg ml^{-1}) was added. Nitrite accumulation was measured after 48 h. (A) Concentration-dependent effects of the selective PDE4 inhibitors Piclamilast and Roflumilast (in the absence of Indomethacin) on nitrite accumulation. (B) Indomethacin reverses inhibition of nitrite accumulation by Piclamilast (1 μM). Addition of PGE_2 (100 nM) restored the inhibition of nitrite accumulation. Statistical significance: $^*P<0.05$ *versus* IL-1β and IL-1β in the presence of Piclamilast and Indomethacin (C) PGE_2 or Salbutamol inhibited nitrite accumulation in the presence of Indomethacin and 1 μM Piclamilast in a concentration-dependent manner. Results are given as the means ± s.e.m. from three (A,C) and six (B) experiments.

quences which were coupled to ovalbumin. PDE4A, STAAEVEAQREHQAAK; PDE4B, CVIDPENRDSLGETDI; PDE4C, CGPDPGDLPLDNQRT; PDE4D, EESQPEASVIDDRSPDT. The antibodies showed immunoreactivity with the corresponding subtype but no crossreactivity with any other PDE4 subtype (data not shown). Because the polyclonal antibodies were raised against peptides selected from the C-terminal ends of the PDE4A-D proteins they exhibited immunoreactivity against all of the splicing variants of a subtype as shown with recombinantly expressed proteins of human PDE4 variants in our experiments (data not shown). In contrast, corresponding preimmune serum did not interfere with any of the recombinant PDE4 variants. The expression of a certain splicing variant of a subtype was detected based on molecular weight and on comparison to the electrophoretic mobility of the recombinantly expressed PDE4 variants.

Recombinant human type 4 PDE proteins were expressed in the Sf9 baculovirus system according to standard methods (Richardson, 1995). The $1000 \times g$ supernatants of cellular lysates were used in the experiments.

Statistical analysis

Statistical analysis was based on Student's *t*-test (GraphPad Software, San Diego, CA, U.S.A.). Values are given as mean ± s.e.m.

Results

IL-1β-induced NO generation is partly reduced by PDE4 inhibitors; reversal by Indomethacin

OA chondrocytes cultured in alginate generate large amounts of NO after stimulation with IL-1β in a time-and concentration-dependent fashion (Figure 1). To investigate whether the selective PDE4 inhibitors Piclamilast and Roflumilast modulate IL-1β-induced NO release chondrocytes were stimulated with 200 pg ml^{-1} IL-1β and nitrite accumulation was measured after 48 h. Piclamilast and Roflumilast reduced chondrocyte IL-1β-induced nitrite accumulation in a concentration-dependent fashion (Figure 2A). Half-maximum inhibition for Piclamilast and Roflumilast was at 20 and 50 nM, respectively. At 1 μM (a concentration that completely blocks PDE4 but does not interfere with other isotypes investigated so far) the PDE4 inhibitors showed maximum inhibition of nitrite formation which was about 40% for both Piclamilast and Roflumilast. In our experimental setting, selective inhibitors of PDE3 (10 μM Motapizone), PDE5 (100 nM Sildenafil) and PDE2

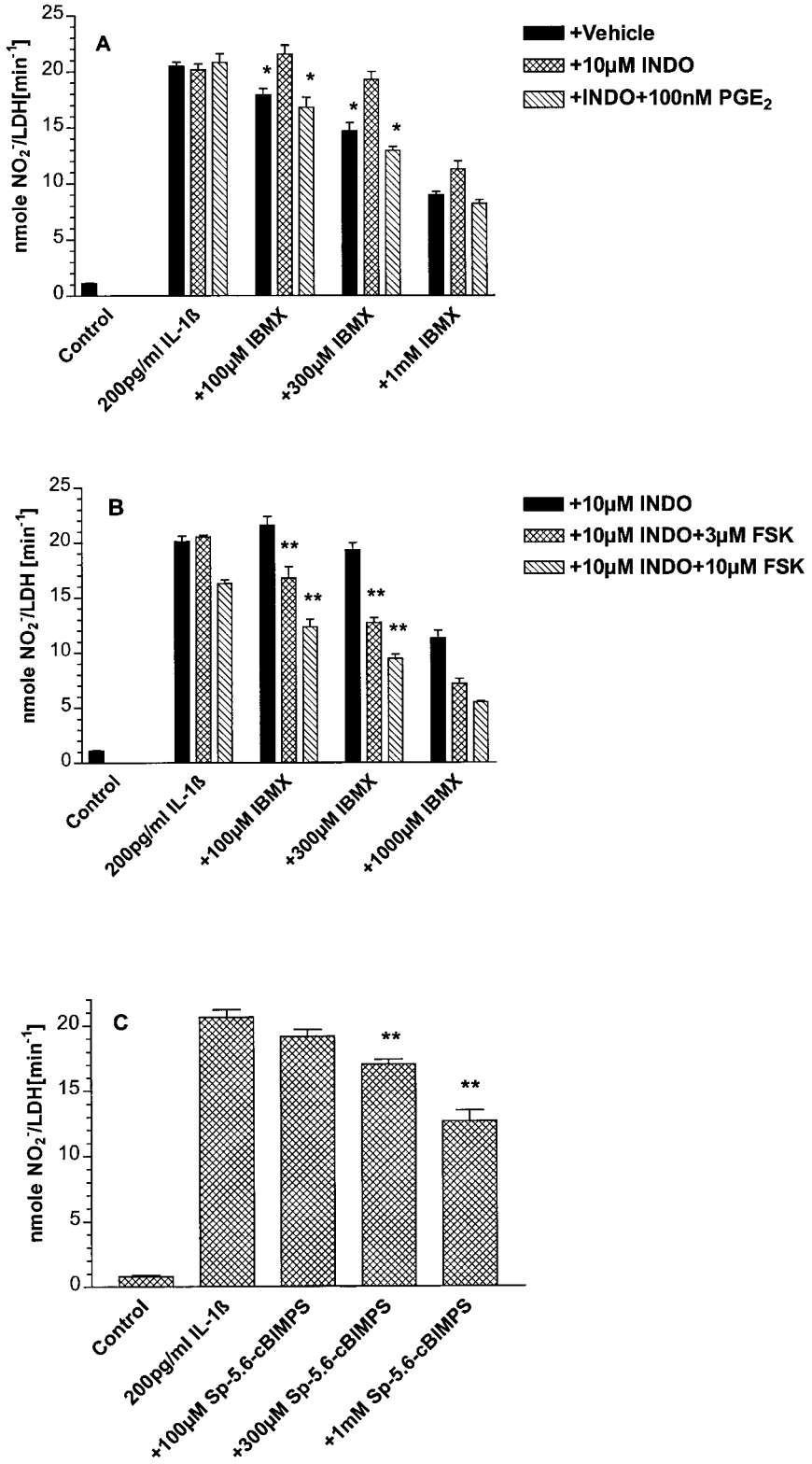

Figure 3 IBMX, forskolin and the protein kinase A activator Sp-5.6-cBIMPS inhibit IL 1β-induced chondrocyte nitrite accumulation. Chondrocytes were preincubated with IBMX (100–1000 μM), Forskolin (3, 10 μM), Indomethacin (10 μM), PGE_2 (100 nM) or Sp-5.6-cBIMPS (100–1000 μM) and stimulated with IL-1β (200 pg ml^{-1}) for 48 h. Nitrite accumulation was measured in culture supernatants. (A) Indomethacin (INDO) reverses the inhibition of nitrite accumulation by 100 and 300 μM IBMX. PGE_2, in the presence of Indomethacin, restores the inhibition of nitrite accumulation. Statistical significance: $^*P<0.05$ *versus* IL-1β with IBMX and in the additional presence of Indomethacin. (B) Forskolin (FSK) is synergistic with IBMX (100 or 300 μM) to inhibit nitrite accumulation if Indomethacin is present. Statistical significance: $^{**}P<0.01$ *versus* IBMX (100 or 300 μM) and Forskolin (3 and 10 μM) alone. (C) Concentration-dependent inhibition of nitrite accumulation by Sp-5.6-cBIMPS. Statistical significance: $^{**}P<0.01$ *versus* IL-1β alone. Results are shown as the means of three experiments $\pm$ s.e.m.

(10 μM EHNA) did not affect the extent of nitrite production stimulated by IL-1β when administered alone or even in combination (data not shown).

The reduction of IL-1β-induced NO release by Piclamilast (1 μM) was completely reversed by the cyclooxygenase inhibitor Indomethacin (10 μM) (Figure 2B). It is well known that chondrocytes produce PGE_2 as the major cyclooxygenase product following stimulation with IL-1β. In our experiments, 200 pg ml^{-1} IL-1β increased PGE_2 concentrations in culture supernatants of alginate beads from $\sim$5 nM at baseline to $\sim$110 nM at 6 h stimulation time (mean of two experiments). Indeed, the effect of Indomethacin to reverse Piclamilast-induced reduction of NO release was overcome by the addition of 100 nM PGE_2 (Figure 2B). In the presence of 1 μM Piclamilast and 10 μM Indomethacin the prostanoid inhibited IL-1β-stimulated chondrocyte nitrite formation in a concentration-dependent fashion (half-maximum inhibition at 4.9 nM) (Figure 2C). In parallel, Salbutamol (1 – 1000 nM) suppressed nitrite accumulation in the presence of 10 μM Indometacin and 1 μM Piclamilast (Figure 2C) but not in the absence of the PDE4 inhibitor. Neither Indomethacin (10 μM) nor PGE_2 (100 nM, 1 μM) nor Salbutamol (1 μM) on their own affected the extent of IL-1β-induced nitrite formation (data not shown).

IL-1β-induced NO formation is suppressed by cyclic AMP agonists

The non-specific PDE inhibitor IBMX triggered a concentration-dependent inhibition of IL-1β-induced nitrite accumulation from human chondrocytes. Indomethacin completely reversed inhibition of NO formation by 100 μM IBMX or 300 μM IBMX whilst having little effect on the inhibition induced by 1 mM IBMX. In agreement to the findings with Piclamilast PGE_2 restored inhibition of nitrite accumulation (Figure 3A). In the presence of Indomethacin Forskolin (3 or 10 μM) synergistically augmented the inhibition of IL-1β-induced NO generation by IBMX (100 or 300 μM) (Figure 3B). On the other hand, in the absence of Indomethacin Forskolin was only additive with IBMX to attenuate IL-1β-induced NO release (data not shown). Finally, inhibition of nitrite accumulation was also achieved with the protein kinase A activator Sp-5.6-cBIMPS (Figure 3C).

IL-1β-induced expression of iNOS protein is inhibited by cyclic AMP mimetics

Incubation of alginate-encapsulated chondrocytes with IL-1β (200 pg ml^{-1}) for 12 h resulted in the expression of iNOS protein ($\sim$130 kDa) that was detected by a polyclonal anti-iNOS antibody in immunoblotting experiments. iNOS protein was absent in nonstimulated cells. One mM IBMX attenuated the iNOS protein and this effect was further accentuated by adding 10 μM Forskolin (Figure 4A). In parallel, the PDE4 selective inhibitor Piclamilast (1 μM) reduced iNOS protein expression induced by IL-1β (Figure 4B).

Cyclic AMP inhibits IL-1β-induced PGE_2 synthesis from OA chondrocytes

Alginate-cultured human OA chondrocytes released PGE_2 measured over a 48 h incubation period under baseline

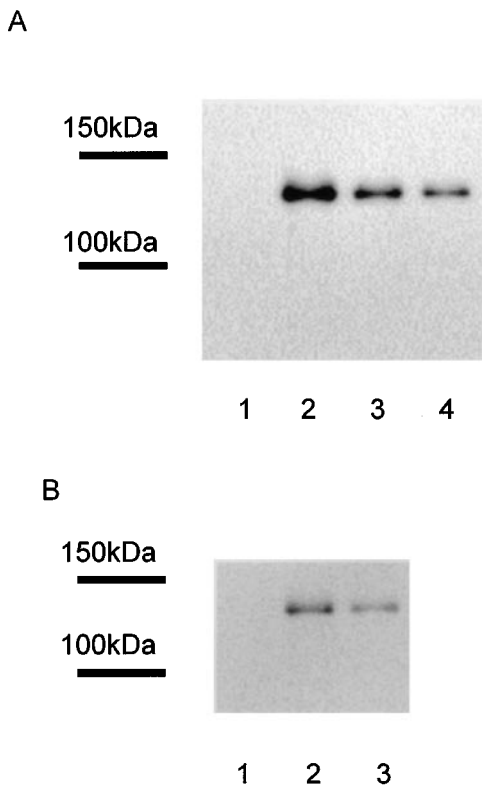

Figure 4 IBMX, Forskolin and IBMX, or Piclamilast inhibit chondrocyte iNOS expression induced by IL-1β. Chondrocytes were exposed to vehicle, 200 pg ml^{-1} IL-1β or 200 pg ml^{-1} IL-1β in the presence of 1 mM IBMX, 1 mM IBMX and 10 μM Forskolin or 1 μM Piclamilast over 12 h. Cellular extracts were prepared and immunoblotting was performed with a rabbit polyclonal iNOS antibody as described in the Methods section. Each lane was loaded with 20 μg protein. (A) Lane 1 - vehicle control, lane 2 - IL-1β, lane 3 - IL-1β with IBMX, lane 4 - IL-1β with Forskolin and IBMX. (B) Lane 1 - vehicle control, lane 2 - IL-1β, lane 3 - IL-1β with 1 μM Piclamilast. Blots represent typical examples of four (A) and three (B) experiments.

conditions, however, with considerable variability between the donors (mean of 121 nM PGE_2 in 13 donors, ranging from 4 nM to 532 nM). Stimulation with 200 pg ml^{-1} IL-1β over 48 h resulted in an increase in PGE_2 released into the culture supernatant to 1230$\pm$125 nM (mean$\pm$s.e.m. from 13 donors). IBMX at 100, 300 and 1000 μM reduced IL-1β (200 pg ml^{-1})-induced PGE_2 accumulation over 48 h by 21.7$\pm$6.2%, 47.3$\pm$6.1% and 81.2$\pm$3.3%, respectively (mean$\pm$s.e.m. from four experiments). PGE_2 release was also inhibited by dibutyryl cyclic AMP (79.8$\pm$2.7% at 1 mM) and Forskolin (32.1$\pm$3.6% at 10 μM, mean$\pm$s.e.m. from six experiments). In contrast, Piclamilast did not achieve significant inhibition of PGE_2 (11.7$\pm$8.9% at 1 μM, mean$\pm$s.e.m. from six experiments) and selective inhibitors of PDE2, 3 and 5 were inactive.

Activities of PDE isoenzymes in human OA chondrocytes

In alginate-cultured human OA chondrocytes total cyclic AMP and cyclic GMP hydrolysing PDE activities were 2554$\pm$221 pmol$\times$min$^{-1}\times10^8$ cells^{-1} and 808$\pm$62 pmol$\times$min$^{-1}\times10^8$ cells^{-1} (n=6), respectively, at 0.5 μM substrate concentrations. Based on the use of specific activators and

inhibitors of PDE isoenzymes, PDE1 and PDE4 were the predominant activities detected (Figure 5A). When PDE3, 4 and 2 were blocked the residual cyclic AMP hydrolysis was 252 ± 29 pmol $\times$ min$^{-1} \times 10^8$ cells^{-1}. When PDE2, 5 and 3 were blocked the residual cyclic GMP activity was 401 ± 54 pmol $\times$ min$^{-1} \times 10^8$ cells^{-1}.

Up-regulation of PDE4 activity and PDE4B protein by IL-1β and cyclic AMP in alginate-cultured human OA chondrocytes

Incubation of alginate-cultured human OA chondrocytes with 200 pg ml^{-1} IL-1β over 12 h resulted in an increase in total cyclic AMP hydrolysis by 48% (Figure 5B). The augmented cyclic AMP hydrolysis was entirely owed to a significant up-regulation of PDE4 activity by 75%. Activities of other PDE isoenzymes remained at control levels. Addition of 1 mM IBMX and 100 nM PGE_2 to alginate cultures incubated with 200 pg ml^{-1} IL-1β further accentuated the increase in PDE4 (to 134% of control) and total cyclic AMP-PDE (to 86% of control) activity.

Immunoblotting using a polyclonal antibody to PDE4A revealed a protein that migrated at slightly higher molecular weight than human recombinant PDE4A4 or PDE4A10 ($\sim$125 kDa). This variance in electrophoretic mobility may be caused by differences in postranslational modification. The PDE4A4 or PDE4A10 band remained unchanged with IL-1β in the presence or absence of 1 mM IBMX and 10 μM Forskolin (Figure 6A). PDE4A4 and the recently described PDE4A10 (Rena *et al*., 2001) could not be separated by means of a subtype selective PDE4A antibody as the molecular weight of these splicing variants was reported to be identical. Immunoreactivity comigrating with human recombinant PDE4A1 ($\sim$83 kDa) was not detected. A polyclonal antibody to human PDE4B detected immunoreactivity at $\sim$75 kDa in chondrocyte lysates that comigrated with the recombinant protein for human PDE4B2 (short form) but not PDE4B1 or PDE4B3 (long forms of $\sim$105 kDa) (Figure 6B). In parallel to PDE4 activity chondrocyte PDE4B2 protein is augmented following incubation of alginate cultures with IL-1β (200 pg ml^{-1}) over 12 h. Indomethacin (10 μM) partly reversed this effect. On the other hand, PDE4B2 protein was increased by a 12-h exposure to 1 μM PGE_2 (Figure 6B). The combination of 1 mM IBMX and 10 μM Forskolin or 1 μM Piclamilast further enhanced the IL-1β-induced upregulation of PDE4B2 expression (Figure 6B,C). Using a PDE4D antibody a doublet-band comigrating with human recombinant PDE4D3 was detected at $\sim$95 kDa. Incubation with IL-1β, IBMX and Forskolin induced an upward shift in the electrophoretic mobility with only the higher molecular weight band being found (Figure 6D). Immunoreactivity to an antibody against PDE4C was not detected in chondrocyte lysates.

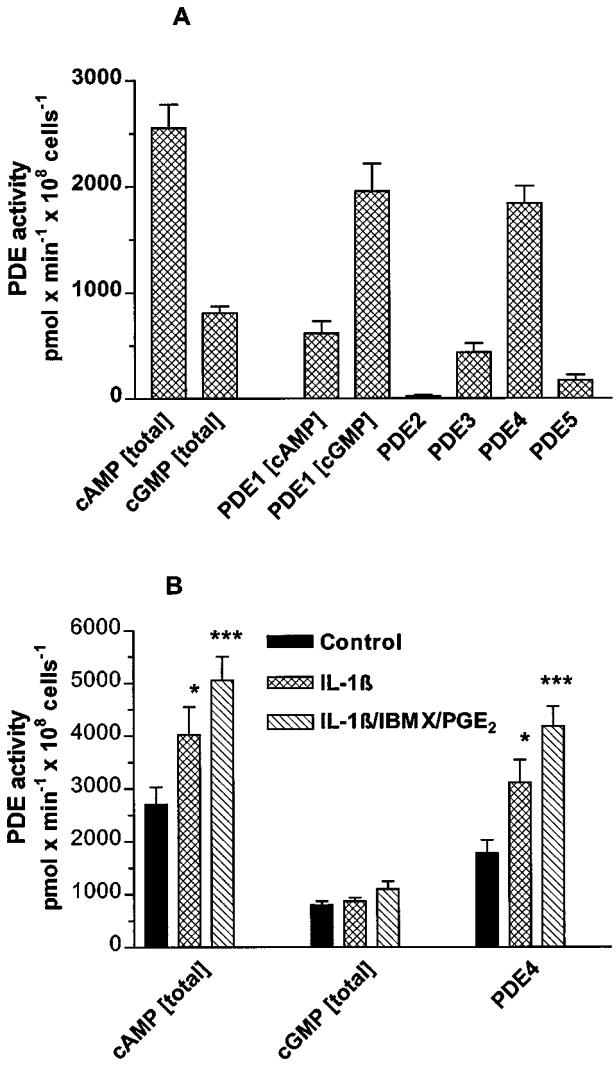

Figure 5 PDE1–5 activities in human OA chondrocytes. Upregulation of PDE4 by IL-1β and cyclic AMP agonists. (A) Chondrocytes were recovered from alginate beads and PDE activities were assessed in lysates at 0.5 μM cyclic AMP or cyclic GMP substrate concentrations. Results from six chondrocyte cultures derived from different donors are shown as the means $\pm$ s.e.m. (B) Chondrocytes were preincubated with vehicle or 1 mM IBMX and 100 nM PGE_2 and stimulated with 200 pg ml^{-1} IL-1β over 12 h. Chondrocytes were recovered from alginate beads and PDE activities were measured in lysates at 0.5 μM cyclic AMP or cyclic GMP substrate concentration. PDE4 was calculated as the fraction of total cAMP-PDE activity that was inhibited by 1 μM Piclamilast. Total cAMP-PDE and PDE4 were both increased by IL-1β and IL-1β and IBMX and PGE_2. Statistical significance: $^*P<0.05$; $^{***}P<0.001$ *versus* control activities. Changes in total cAMP-PDE and PDE4 activities with IBMX and PGE_2 added to IL-1β compared to IL-1β alone were not significant. Results are shown as the means of six experiments $\pm$ s.e.m.

Discussion

The major findings of the current study are that human OA chondrocytes express PDE4 and that its selective inhibition results in a partial reduction ($\sim$40%) of IL-1β-induced NO generation from alginate-cultured chondrocytes. The additional presence of PDE1 and of residual PDE activities probably corresponding to PDE7–11 may explain why selective PDE4 inhibition does not completely abolish chondrocyte nitrite production. A functional role of PDE4 in human OA chondrocytes was recently suggested based on the downregulation of IGFBP-3 transcripts by the selective PDE4 inhibitor Ro20-1724 (DiBattista *et al*., 1996), however, the occurence of PDE4 activity and the expression profile of PDE4 variants in human OA chondrocytes have not previously been demonstrated. It was shown that Indomethacin reversed the reduction

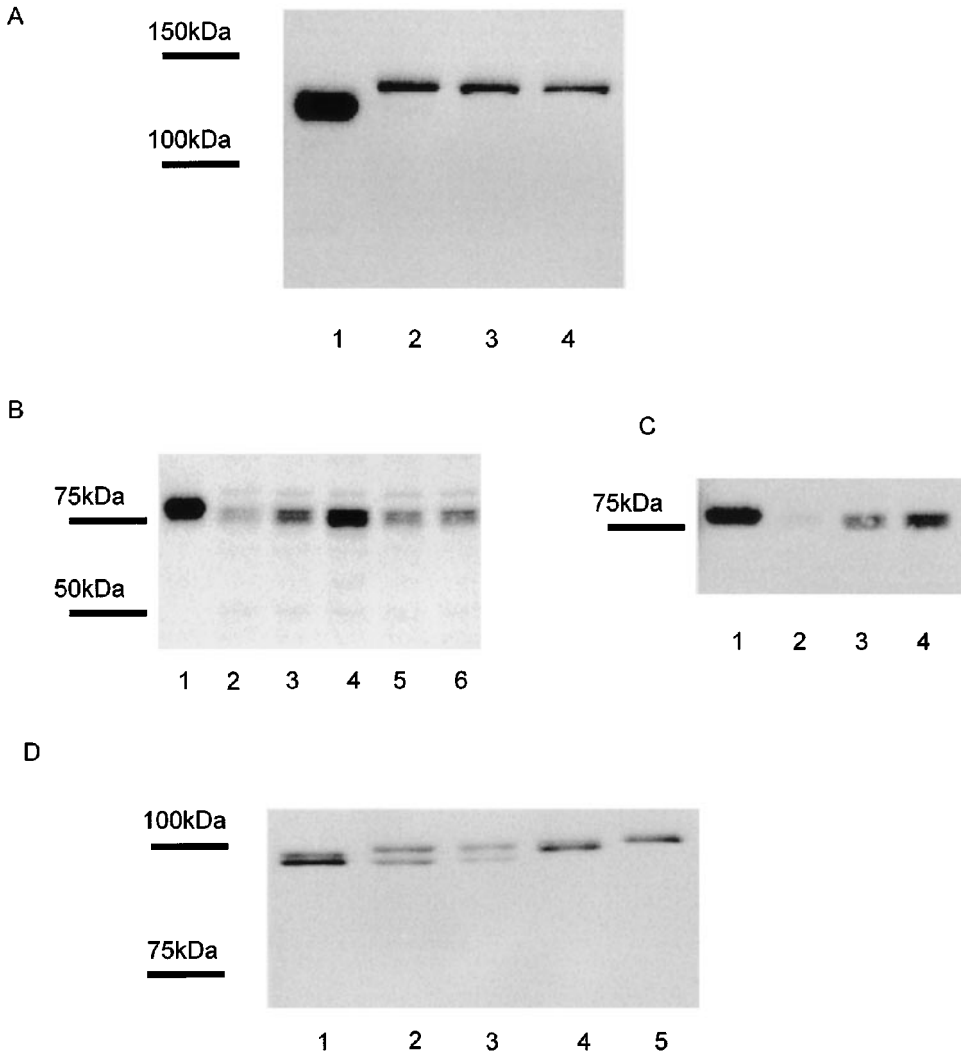

Figure 6 IL-1β and cyclic AMP-agonists increase PDE4B2 protein in human OA chondrocytes. Chondrocytes cultured in alginate beads were exposed to vehicle, 200 pg ml^{-1} IL-1β in the presence or absence of 10 μM Indomethacin or 10 μM Forskolin and 1 mM IBMX, or 1 μM PGE_2, or 1 μM Piclamilast for 12 h. Cellular extracts were prepared and immunoblotting was performed with rabbit polyclonal PDE4A, B or D antibodies as described in the Methods section. Each lane was loaded with 20 μg (for PDE4B and D) or 50 μg (for PDE4A) protein. (A) Antibody to PDE4A, lane 1 - recombinant human (rh) PDE4A4, lane 2 - control, lane 3 - IL-1β, lane 4 - IL-1β + Forskolin + IBMX. (B) Antibody to PDE4B, lane 1 - rh PDE4B2, lane 2 - control, lane 3 - IL-1β, lane 4 - IL-1β + Forskolin + IBMX, lane 5 - IL-1β + Indomethacin, lane 6 - PGE_2. (C) Antibody to PDE4B, lane 1 - rhPDE4B2, lane 2 - control, lane 3 - IL-1β, lane 4 - IL-1β + 1 μM Piclamilast. (D) Antibody to PDE4D, lane 1 - rh PDE4D3, lane 2 - control, lane 3 - IL-1β, lane 4 - IL-1β + IBMX + Forskolin, lane 5 - rhPDE4D5. The depicted blot is representative of 3–6 experiments.

of IL-1β-induced nitrite accumulation by Piclamilast. On the other hand, PGE_2 (100 nM) completely restored NO inhibition by 1 μM Piclamilast in the presence of Indomethacin. When cyclooxygenase (COX) and PDE4 were blocked, the half-maximum inhibition for suppression of IL-1β-stimulated nitrite accumulation by PGE_2 was 4.9 nM which corresponds to the affinity of the prostanoid to recombinant EP2 and EP4 receptors (Narumiya *et al*., 1999). PGE_2 constitutes the major COX product in IL-1β-stimulated chondrocytes (Geng *et al*., 1995). IL-1β over 6 h induced PGE_2 concentrations of about 100 nM matching levels of the prostanoid that achieve maximum inhibition of IL-1β-induced nitrite release in the presence of Piclamilast and Indomethacin. Collectively, we hypothesize that chondrocyte-derived PGE_2 acts as an autocrine adenylyl cyclase activator enabling PDE4 inhibitors to reduce IL-1β-stimulated chondrocyte NO. In our system PGE_2 or Salbutamol synergized with the PDE4 inhibitor Piclamilast to suppress IL-1β-induced nitrite release. After blocking endogenous PGE_2 production with Indomethacin, coincubation with PGE_2 (100 nM) or Salbutamol (1 μM) and Piclamilast (1 μM) resulted in $\sim$40% or $\sim$30% nitrite inhibition whilst the compounds were ineffective on their own. An enhanced expression of PDE4B2 induced by PGE_2 as shown in this study may provide one explanation for the inability of the prostanoid on its own to reduce chondrocyte NO formation and for the observed synergism to the selective PDE4 inhibitor. In parallel to the findings with Piclamilast the inhibition of IL-1β-induced nitrite accumulation by the non-specific PDE inhibitor IBMX at 100 μM and 300 μM was reversed by Indomethacin. In addition in the presence of Indomethacin both PGE_2 (100 nM) and Forskolin (3 and 10 μM) were synergistic to IBMX (100 or 300 μM) to reduce nitrite accumulation. Furthermore, the protein kinase A

activator Sp-5.6-cBIMPS attenuated IL-1β-induced NO formation. In summary cyclic AMP by activating protein kinase A is suggested as the common denominator which transduces the suppression of IL-1β-induced NO formation from human chondrocytes by PDE inhibitors in conjunction with adenylyl cyclase-stimulants. This suppression of NO formation may occur in consequence to an inhibition of iNOS expression because IBMX, IBMX and Forskolin, or Piclamilast attenuated IL-1β-induced iNOS protein as shown by immunoblotting.

Inhibition of IL-1β-triggered human chondrocyte iNOS mRNA and protein expression and NO production by IBMX has recently been described (Geng *et al*., 1998). However, the authors found that the selective PDE4 inhibitor Rolipram did not reduce IL-1β-induced nitrite release from normal human chondrocytes cultured as monolayers. The different culture conditions or donor populations used in this previous study compared to those in our experiments may account for this discrepancy.

Inhibition of IL-1β-induced NO production by 1 mM IBMX was only slightly reversed by Indomethacin and neither PGE_2 nor Forskolin were synergistic to 1 mM IBMX in the presence of the cyclooxygenase inhibitor. Previous descriptions of molecular targets other than PDE inhibition for the methylxanthine e.g. inhibition of G_i proteins and consecutive stimulation of cyclic AMP-synthesis (Parsons *et al*., 1988) or direct activation of protein kinase A (Tomes *et al*., 1993) may provide one explanation. In addition the strong suppression of chondrocyte-derived PGE_2 by 1 mM IBMX may also have contributed to the failure of Indomethacin to reverse the inhibition of IL-1β-triggered NO formation by 1 mM IBMX.

Total cyclic AMP phosphodiesterase activity in lysates of alginate-cultured human OA chondrocytes was $\sim$3 fold higher than cyclic GMP hydrolysis at 0.5 μM substrate concentration. PDE4 accounted for $\sim$70% of cyclic AMP degrading activity under our conditions. Only $\sim$15% of total cyclic AMP hydrolysis was PDE3 whereas PDE2 was undetectable. Immunoblotting indicated the presence of PDE4A4 or PDE4A10, PDE4B2 and PDE4D3 proteins. IL-1β enhanced PDE4 activity and in parallel increased PDE4B2 protein which was further augmented by the additional presence of IBMX and Forskolin, or Piclamilast. Intact OA cartilage autocrinuously produces IL-1β to functionally effective levels (Attur *et al*., 1998). Therefore, the role of PDE4 in degradation of chondrocyte cyclic AMP may be accentuated in intact OA compared to normal cartilage. An increase of chondrocyte PDE4B2 protein was also observed with 1 μM PGE_2 On the other hand, 10 μM Indomethacin reduced IL-1β-induced PDE4B2 expression. Collectively, these data suggest that IL-1β-induced PGE_2 partly mediates enhanced PDE4B2 expression by IL-1β. Cyclic AMP-triggered PDE4B2 upregulation has recently been shown in human myometrial cells (Méhats *et al*., 1999) and human monocytes (Manning *et al*., 1996). The upward-shift of electrophoretic mobility of PDE4D3 with IBMX and Forskolin may indicate protein kinase A-dependent phosphorylation (Maurice & Liu, 1999), however, other mechanisms cannot be excluded.

Ca^{2+}-calmodulin augmented cyclic GMP and cyclic AMP hydrolysis in human OA chondrocyte lysates is consistent with the occurence of PDE1. The presence of PDE1 was recently shown in murine chondroprogenitor ATDC5 cells (Fujishige *et al*., 1999). Owing to the absence of selective PDE1 inhibitors the functional role of PDE1 in chondrocytes could not be investigated.

In lysates of alginate-cultured human OA chondrocytes $\sim$20% of the hydrolysis of 0.5 μM cyclic GMP was attributed to PDE5. This PDE5 activity is consistent with the recent finding of a PDE5-related PCR product in human chondrocytes (Geng *et al*., 1998). The fraction of PDE5 in relation to total cyclic GMP hydrolysis may increase at higher cyclic GMP concentrations owing to the K_m for PDE5 of 5–6 μM. However, we found no change in the ratio of cyclic AMP to cyclic GMP hydrolysis at 20 μM substrate concentration compared to 0.5 μM (data not shown). Collectively, one might argue that baseline PDE5 activity *in situ* represents a minor component compared to PDE4 or PDE1. Consistent with this view Sildenafil which selectively blocks PDE5 did not influence IL-1β-induced NO formation. A recent report concluded a major role of cyclic GMP-PDE and PDE5 in human chondrocytes which appears to contradict our findings (Geng *et al*., 1998). A main difference between the studies was the selection of different substrate concentrations for PDE activity measurements. In the previous study measurements were at 0.05 μM cyclic AMP and 40 μM cyclic GMP. This may explain the prominent cGMP-PDE activity compared to cyclic AMP hydrolysis in that study. However, a final appreciation of PDE5 in human chondrocytes will await further investigations.

In summary, alginate-cultured human OA chondrocytes are harboring PDE4 activity and in parallel selective PDE4 inhibitors partly attenuate IL-1β-induced chondrocyte NO release. Indomethacin reversed the NO suppression by PDE4 inhibitors. Because this effect was overcome by PGE_2 and chondrocytes synthesized functionally relevant amounts of this prostanoid we postulate that autocrine PGE_2 enabled PDE4 inhibitors to reduce IL-1β-induced NO in this system. IL-1β and cyclic AMP upregulated PDE4 activity which was accompanied by an augmentation of PDE4B2 protein. Considering the importance of NO as a trigger of cartilage destruction we suggest that inhibition of PDE4 may have some chondroprotective effects. These chondroprotective effects may be reduced by the simultaneous presence of COX inhibitors which are frequently administered in osteoarthritis. However, other endogenously produced receptor agonists inducing cyclic AMP synthesis e.g. β-adrenoceptor agonists may then compensate for prostanoids. In fact, the PDE4 inhibitor Piclamilast suppressed IL-1β-induced NO formation in the presence of Indomethacin if Salbutamol was added. Further investigations particularly with animal models of osteoarthritis may reveal whether the *in vitro* findings with PDE4 inhibitors translate into chondroprotective effects *in vivo*.

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