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Dexamethasone down-regulates cAMP-phosphodiesterase in human osteosarcoma cells

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Abstract

Cyclic adenosine monophosphate (cAMP) is an important second messenger in the hormonal regulation of bone metabolism. cAMP is inactivated by the cyclic nucleotide phosphodiesterases (PDEs), a superfamily of enzymes divided into 11 known families, designated PDE1-11. Interference with the cAMP signaling pathway has been suggested as one mechanism causing glucocorticoid induced osteoporosis. We speculated that glucocorticoids could affect the cAMP pathway by a down-regulation of PDE-mediated cAMP hydrolysis. The main cAMP hydrolysing enzyme families of human MG-63 and SaOS-2 osteosarcoma cells were identified as PDE1 and PDE4 by assaying the PDE activity of Q-sepharose fractions and cell homogenates with selective inhibitors. Treatment with the glucocorticoid dexamethasone (Dex) decreased cAMP-PDE activity by up to 50%, without affecting cGMP-PDE activity. Dex treatment reduced the sensitivity of the total cAMP-PDE activity towards the PDE4 selective PDE inhibitor rolipram. Forskolin stimulated cAMP accumulation was increased 30–60-fold in the presence of rolipram. Treatment with Dex did not affect the basal or forskolin stimulated cAMP accumulation, but treatment resulted in a reduced effect of rolipram on cAMP accumulation. Expression of the following cAMP-PDE subtypes were detected by reverse transcriptase PCR (RT-PCR): PDE1A, PDE1C, PDE2A, PDE3A, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B, PDE8A, PDE10A and PDE11A. Using semi-quantitative RT-PCR, we detected a 50–70% decrease in the mRNA of PDE4A and PDE4B subtypes following Dex treatment. Further analysis revealed that Dex reduced the PDE4A4 and PDE4B1 isoforms. PDE4A1 PDE4A, PDE4A7, PDE4A10, PDE4B2 were also expressed, but Dex did not affect the transcription of these isoforms. We conclude that Dex treatment could affect the cAMP signaling pathway of human osteosarcoma cells by reducing type 4 cAMP-phosphodiesterase (PDE4).

Keywords: Osteoblast; Osteosarcoma; Dexamethasone; Glucocorticoids; Phosphodiesterase; cAMP

1. Introduction

Glucocorticoids play an important role in modifying the metabolic activity and proliferation of bone cells. Used in long-term therapy as immunosuppressive and anti-inflammatory drugs, glucocorticoid treatment leads to a reduction in bone formation, which enables the development of osteoporosis [1–4]. On a cellular level, glucocorticoids inhibit bone formation mainly by reducing the osteoblast life span, and by inhibiting the generation of new osteoblasts [5].

cAMP, a second messenger that has a role in a variety of cellular responses to extracellular agents such as hormones, neurotransmitters and cytokines, is involved in several bone formation-associated functions of the osteoblasts. PTH has been shown to decrease bone loss by reducing osteoblastic apoptosis by a cAMP-dependent mechanism [6]. Local anabolic actions involving increased osteoblastic secretion of insulin-like growth factor I are

Abbreviations: cAMP, cyclic adenosine 3′,5′monophosphate; Dex, dexamethasone; PDE, cyclic nucleotide phosphodiesterase; PTH, parathyroid hormone; BSA, bovine serum albumin; cGMP, cyclic guanosine 3′,5′monophosphate

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also mediated by cAMP [7–9]. Furthermore, cAMP is linked to the regulation of osteocalcin secretion by both human and rat osteoblast-like cells [10–12].

The intracellular concentration of cAMP is determined mainly by adenylate cyclase dependent formation and by the degradation rate due to the action of PDEs. The amplitudes and duration of cAMP-mediated hormonal responses are therefore largely determined by the PDEs [13,14]. The PDEs constitute a large superfamily of 11 distinct gene families which often show specific tissue, cellular and subcellular distribution. Each of these families contains one to four genes, and these genes often express several splice-products [14,15]. The PDEs are an important part of the signal transduction of the cell, but very little is known about them in bone cells. The aim of the present study was to investigate the effects of Dex, a synthetic glucocorticoid, on cAMP-phosphodiesterase expression in human osteoblast-like cells, at both enzyme and mRNA level.

2. Materials and methods

2.1. Cell culture

Two human osteosarcoma cell lines, both widely used as models for osteoblast studies were used in the present study. The MG-63 cells, which are considered to represent a mature osteoblastic phenotype was the primarily used cell line, whereas SaOS-2 cells were mainly used to confirm results obtain with MG-63 cells. Both cell lines were purchased from the American Type Culture Collection. MG-63 cells were cultured in α-MEM supplemented with 10% foetal calf serum and SaOS-2 cells in McCoy's 5A, supplemented with 12.5% foetal calf serum, with antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Radiochemicals

[^{125}I] cAMP (adenosine 3′,5′-cyclic phosphoric acid 2′-0-succinyl 3-[^{125}I] iodotyrosine methyl ester, 2000 Ci/mmol) for cAMP radioimmunoassays and [^{3}H]cAMP [8-[^{3}H]] adenosine 3′,5′-cyclic phosphate, 24 Ci/mmol)-for PDE activity assays were from Amersham international.

2.3. Treatments

Cells were grown to confluence and then maintained in serum-free media with 0.1% BSA (MG-63 cells) or in low-serum (2–5%) media (SaOS-2 cells) for 72 h before the beginning of the treatments. Dex was dissolved in ethanol. The concentration of ethanol vehicle in the media did not exceed 0.001%. Cells were treated with vehicle alone, or with various concentrations of Dex for indicated times. In these conditions, the Dex treatment did not affect cell number or viability. Each experiment was repeated at least three times unless differently specified.

2.4. Q-sepharose chromatography

Five confluent cultures of MG-63 cells, grown on 100 mm plastic dishes were washed twice with PBS buffer, harvested with a cell scraper into 6 ml ice-cold homogenization buffer (buffer A), containing 20 mM bis-Tris, pH 6.5, 5 mM mercaptoethanol, 0.1% Sigma protease inhibitor cocktail (PIC). The harvested cells were then homogenized by 10 passages on ice with a Teflon/glass homogenizer. The homogenization was repeated three times. The homogenate was then centrifuged for 20 min at 20,000 × *g*. Five millilitres of the supernatant was diluted with 5 mL buffer B, containing 20 mM bis-Tris, pH 6.5, 0.1 M sodium acetate, 0.02% PIC (v/v), 0.1 mM EDTA, 1 mM benzamidine and 1 mM mercaptoethanol, filtered through a 0.22 mM syringe filter, and applied to a column (5 mL bed volume) of Q-sepharose (HighTrap Q, Amersham Pharmacia Biotech) previously equilibrated with buffer B. After washing the column with 10 bed volumes, the PDE activities were eluted with a 0.05–1.2 M linear sodium acetate gradient in buffer B, at a flow-rate of 2.5 ml/min. Fractions of 2 ml were collected into tubes and assayed for cAMP-PDE activity as described below.

2.5. Assay of PDE activity

The PDE activity was assayed according to the method described by Thompson and Appleman [16]. Samples from either Q sepharose elutions or whole cell homogenates were added to incubation buffer (40 mM Tris–HCl, pH 8.0, 0.1% PIC, 0.05% BSA, 5 mM MgCl2, 1 mM EGTA, 0.5 μM cAMP and ^{3}H–cAMP (100.000 cpm/tube) to give a final reaction volume of 200 μl. For cGMP-PDE assay cAMP was replaced with 0.25 μM cGMP and ^{3}H–cGMP. The samples were incubated at 34 °C for 30–60 min, followed by boiling for 1 min. After cooling on ice, 50 μl *Crotalus atrox* snake venom nucleotidase (1 mg/ml, Sigma) was added and incubated at 34 °C for 10 min. Then 500 μl 1:3 slurry of AGI-X8 resin (BioRad) was added, and the tubes were mixed and centrifuged 5 min at 8000 rpm. The radioactivity of the supernatant was counted with a β-counter. Selective inhibitors for different PDE types were as followed: PDE1, 8-MMX (8-methoxymethyl-3-isobutyl-1-methylxanthine); PDE2, EHNA (erythro-9-[2-hydroxy-3-nonyl] adenine); PDE3, cilostamide (*N*-Cyclohexyl-*N*-methyl-4-[1,2-dihydro-2-oxo-6-quinolyloxy] butyramide) and PDE4, Rolipram (4-[3-{cyclopentyloxy}-4-methoxyphenyl]-2-pyrrolidinone). For non-specific PDE inhibition 3-isobutyl-1-methylxanthine (IBMX) was used.

Table 1
Primer pairs used to detect PDE subtypes

Subtype	Sense sequence	Anti-sense sequence	Product size	Reference
PDE1A	AAA ATG GGG ATG ACA AAA AAG AAA*	GAT ATT CAT TTC TTC TTC TTG CAT*	612	[19]
PDE1B	ATG CAG GAT GAT GAG ATG AA*	CGG AAG AAT TCC TCC AT*	272	[19]
PDE1C	AGA CAG GGT GAC AGA GAA GC*	TGG CCT TCT CCT CTT TGG GT*	397	[19]
PDE2A	ACC GGT CTG TCA GTG AGA TGGC	GAA GAC AGC GTC TAA TGC TGG TGT	577	[20]
PDE3A	TCA CCT CTC CAA GGG ACT CCT	CAG CAT GTA AAA CAT CAG TGG C	708	[21]
PDE3B	AGA GCT GGT GGT TTG TCC AG	AAT GTT TGA AGA CAG GCA GC	903	–
PDE4A	CTC GCA CAA GTT CAA AAG GAT G	GCC TCC AGC GTA ATC CGA CA	391	[22]
PDE4B	AGC TCA TGA CCC AGA TAA GTG	ATA ACC ATC TTC CTG AGT GTC	625	[23]
PDE4C	ACA CTG AAC TCC TGT CCC CTG AAG	GAT GTG ACT CAA GAG TGA CCA CTG G	410	[24]
PDE4D	CCC TGG ACT GTT ATC ATG CA	TGA TTG GAC ACA CCA GGA TG	262	[25]
PDE7A	GGA CGT GGG AAT TAA GCA AGC	TCC TCA CTG CTC GAC TGT TCT	285	[26]
PDE7B	TGC ACA GGA CAG GCA CTT TA	CTT CTG TGC TGC CTG GGC AA	347	–
PDE8A	CAG CCA GAG ACG ACA CTC TTC CAT	ATG ATC TTA GCG TTG ACT CGG AGC	502	[20]
PDE10A	GTG TGG TGC AGA TGG TCA AC	GGG TGA ATT GCA TGA GAC CT	234	–
PDE11A	ATC TGC CTC AGT ATC CCC CT	CCA CCA GGA AAA GAG AGC AG	199	–
GAPDH	AAGGCTGGG GCT CAT TTG	GTG TGG TGG GGG ACT GAG	784	[27]

All primer sequences used were designed as described previously, except primer pairs for 3B, 10A and 11A which were designed for the present study with Primer3 software. The primers were chosen that amplify common regions of all known splice variants (isoforms) of each subtype. Predicted product sizes are given as base pairs. The primers marked with * were originally designed according to bovine (PDE1A), mouse (PDE1B), and rat (PDE1C) species according to Sakakibara et al. [19], and were modified according to the corresponding human sequences.

2.6. Measurements of cAMP

Measurement of cAMP accumulation was determined using the method of Frandsen and Krishna [17] as described earlier [18]. Briefly, the cells were grown in 24-well plates, and treated with either vehicle (ethanol) or Dex (1–100 nM) for 48 h. The cells were then induced with forskolin for 7 min at 37 °C and rinsed twice with ice-cold PBS. For determination of basal cAMP levels, cells were incubated at 37 °C for 30 min before washing with ice-cold PBS. cAMP was extracted from the cells with 96% ethanol at −18 °C for 3 h. The extract was evaporated with N_2-gas and dissolved in assay buffer (0.05 M sodium acetate, pH 6.2). If necessary, the sample was further diluted with assay buffer, and the cAMP concentration was determined by radioimmunoassay with iodinated cAMP as tracer. Cell number of parallel cultures were counted with a haemocytometer.

2.7. RNA isolation and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells using the RNeasy Protect Mini Kit (Qiagen) according to the instructions of the manufacturer. cDNA was prepared from 1 μg total RNA by reverse transcription with following buffer and conditions: RT buffer (Promega) containing 50 mM Tris–HCl (pH 8.3) 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT, 13 U RNAguard, Rnase inhibitor (Amersham Pharmacia Biotech), 0.9 mM each of dATP, dCTP, dGTP and dTTP, 15 pmol Oligo(dT) 15 primer (Promega) and 100 U M-MLV-reverse transcriptase primer (Promega) in a final volume of 25 μl, with incubation for 1 h at 37 °C.

PCR amplifications were conducted in 30 μl containing 4 μl of cDNA, 1× buffer (Finnzymes), containing 10 mM Tris–HCl, pH 8.8 at 25 °C, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.35 μM of each primers and 1 U of DyNAzyme™ DNA polymerase (Finnzymes). The primers used are shown in Tables 1 and 2. The amplification protocol was run on a PTC-100 thermocycler (MJ Research). After denaturation at 94 °C for 4 min, the reactions underwent cycles of: 1 min at 95 °C, annealing for 90 s at 58–63 °C, and extension for 2 min at 72 °C. This was followed by a single step at 72 °C for 10 min. To ensure that the PCR was in the exponential phase, the conditions of the semi-quantitative RT-PCR

Table 2
Primer pairs used to detect PDE4A and 4B isoforms

Subtype	Sense sequence	Anti-sense sequence	Product size	Reference
PDE4A1	GCT GTT CAG AGG CAG GAA AC	CGT GTC ACC ATC ACA AGA CC	232	–
PDE4A4	ACC AAT GTG CCC GTT CCC AGT	GCC TCC AGC GTA ATC CGA CA	570	[28]
PDE4A7	CCA GTC GTG GTT CCT AT	CAG TAC GTG GGT GGA CTG C	589	–
PDE4A10	TCC GGG CAG ATC TGT CAG CTT	ACT GGG AAC GGG CAC ATT GGT	313	[29]
PDE4B1	AGC GGT GGT AGC GGT GAC TC	GCT GCG TGC AGG CTG TTG TG	680	[25]
PDE4B2	AGC GGT GGT AGC GGT GAC TC	ACC TTT CTT GGG CAC AGC CAG	680	[25]
PDE4B3	CTC CAC GCA GTT CAC CAA GGA AC	TGT GTC AGC TCC CGG TTC AGC	598	[30]

All primer sequences used were designed as described previously, except primer pairs PDE4A1 and 4A7 which were designed for the present study with Primer3 software.

were optimised for the number of PCR cycles, and the amount of total RNA applied to the reactions for the PDE subtypes/isoforms and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an external standard. Human brain RNA and a cocktail of RNA from rat spleen, brain, kidney and heart was used to prepare cDNAs used as positive controls. Negative controls included samples in which cDNA synthesis was performed in the absence of reverse transcriptase. Reactions in which no cDNA was added were also performed to check for possible non-specific products. 10 μl of the PCR-amplified products were analysed on 1.5% agarose gels containing ethidium bromide. Gel results were quantified by AlphaDigiDoc™ System 1000 (Alpha Innotech Corporation). The results were analysed as a ratio of integrated optical density of the PDE subtypes/isoforms to GAPDH.

2.8. Statistical analysis

The results presented represent the means ± S.E.M. The data was analyzed using either ANOVA or unpaired *t*-tests with GraphPad® Software.

3. Results

3.1. cAMP-phosphodiesterase activity in MG-63 and SaOS-2 cells

Three main cAMP-PDE activity peaks were identified in MG-63 cells after Q-sepharose ion-exchange chromatography (Fig. 1). The first peak (peak 1, fractions 20–28) consisted of calmodulin stimulated PDE1 activity, sensitive to 8-MMX. This peak was also sensitive towards

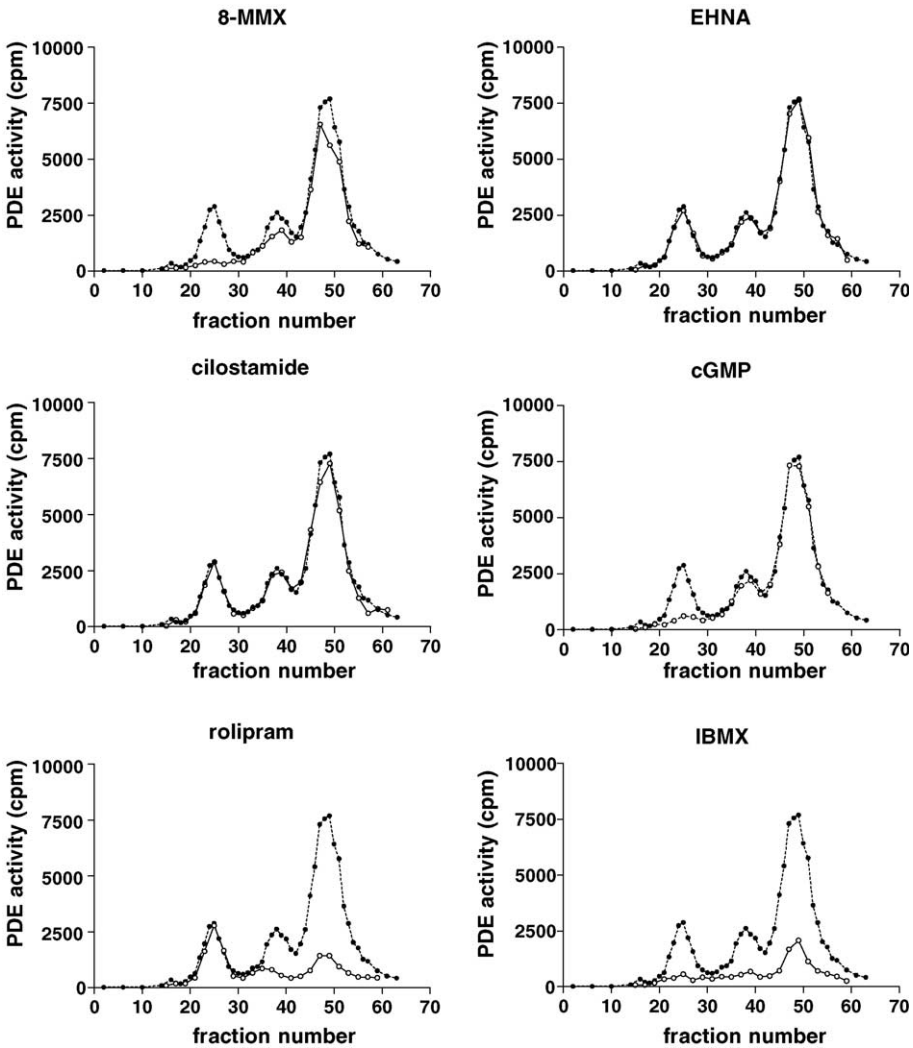

Fig. 1. Q-sepharose elution profile of cAMP-PDE activity present in MG-63 cells. Extracts of MG-63 cells prepared as described in Section 2 were applied to a 5 mL column of Q-sepharose and eluted by a linear gradient of sodium acetate (0.05–1.2 M). Fractions were collected and assayed for cAMP-PDE activity in the presence (open circles) or absence (filled circles) of PDE inhibitors and cGMP. The concentrations of the inhibitors were: 8-MMX, 25 μM; EHNA, 10 μM; cilostamide, 5 μM; rolipram, 10 μM and IBMX, 50 μM. The concentration of cGMP was 5 μM.

Table 3
Relative PDE1 and PDE4-activity in MG-63 and SaOS-2 cell lysates (%)

	Total	PDE1	PDE4
MG-63	100 ± 0.9	32 ± 3.6	63 ± 5.4**
SaOS-2	100 ± 2.9	56 ± 5.5*	21 ± 0.5

The PDE activities were determined using 20 μM 8-MMX (PDE1 selective inhibitor) and 10 μM rolipram (PDE4 selective inhibitor).
* $P < 0.05$.
** $P < 0.01$ significant differences (Student's *t*-test) between the cell lines.

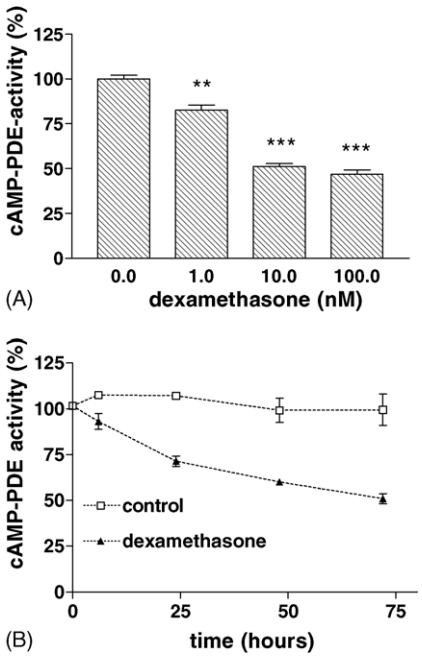

Fig. 2. Effect of Dex on cAMP-PDE activity of MG-63 cells. (A) Dose–response of Dex on cAMP-PDE activity. The cells were treated for 48 h with the indicated concentrations of Dex and then harvested, homogenized, and assayed for cAMP-PDE activity. (B) Time–response for Dex treatment on cAMP-PDE activity. The cells were treated for the indicated times with 25 nM Dex and then harvested, homogenized and assayed for cAMP-PDE activity. The given results represent means ± S.E.M. of triplicate determinations (** $P < 0.01$ and *** $P < 0.001$).

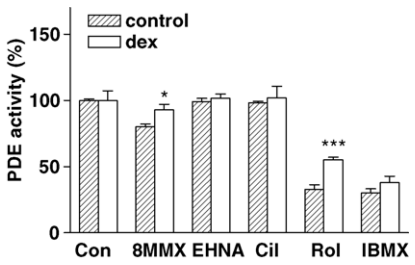

Fig. 3. Effect of Dex on sensitivity of homogenates of MG-63 cells towards PDE inhibitors. The cells were treated for 48 h with 50 nM Dex. The cells were then homogenized, and assayed for PDE activity in the presence of the different PDE inhibitors. The concentrations of the inhibitors were: 25 μM 8-MMX; 10 μM EHNA; 5 μM cilostamide (Cil); 10 μM rolipram (Rol); 50 μM IBMX.

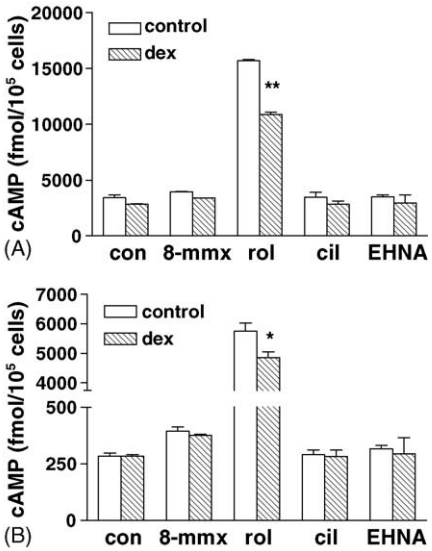

Fig. 4. Effect of Dex treatment on forskolin stimulated cAMP accumulation in MG-63 (A) and SaOS-2 (B) cells. The cells were pretreated for 48 h with 50 nM Dex, then stimulated with forskolin and PDE inhibitors for 5 min. The concentrations of the inhibitors were: 25 μM 8-MMX, PDE1 selective inhibitor; 10 μM rolipram (Rol) PDE4 selective inhibitor; 5 μM cilostamide (Cil), PDE3 selective inhibitor; EHNA, 10 μM, PDE2 selective inhibitor. The given results represent means ± S.E.M. of triplicate determinations (* $P < 0.05$ and ** $P < 0.001$).

cGMP which can be explained by the dual-substrate nature of PDE1. Also IBMX but neither EHNA, cilostamide or rolipram affected this peak. The two following activities (peaks 2 and 3), eluting at fractions 32–42 and 44–58, contained rolipram sensitive PDE4 activity (Fig. 1). EHNA, cilostamide and cGMP did not reduce the activity of these two peaks. The peaks 2 and 3 were also sensitive towards IBMX. 8-MMX had a mild effect on the activity of peaks 2 and 3 possibly due to a non-specific inhibition. IBMX inhibited the activity of all fractions. The relative PDE1 and PDE4 activities of MG-63 and SaOS-2 cell

Table 4
Effect of rolipram on forskolin-stimulated cAMP accumulation in MG-63 and SaOS-2 cells

MG-63	0.35 ± 0.035	0.57 ± 0.032**	0.46 ± 0.026	10.80 ± 1.73**
SaOS-2	0.29 ± 0.018	0.54 ± 0.044**	0.73 ± 0.042***	17.45 ± 1.74***
Forskolin	−	+	−	+
Rolipram	−	−	+	+

The cells were challenged with forskolin (20 μM) with or without the PDE4 selective inhibitor rolipram (10 μM) for 5 min and assayed for cAMP levels by radioimmunoassay as described in Section 2. The data shown are expressed as pmol cAMP/plate and show the means ± S.E.M. of triplicate determinations.
** $P < 0.01$.
*** $P < 0.001$ as compared to no treatment (Student's *t*-test).

homogenates were also assayed. MG-63 cells contained proportionally higher levels of PDE4 activity as compared with SaOS-2 cells (Table 3).

3.2. Effect of dexamethasone on phosphodiesterase activity

Treatment of MG-63 cells with Dex significantly decreased the cAMP-PDE activity in a dose- and time-dependent manner (Fig. 2A and B). The effect of Dex on the cAMP-PDE activity was detectable within a 24 h treatment. After a 48 h treatment the cAMP-PDE activity was reduced by up to 50%, with the half-maximal inhibition of the cAMP-PDE activity being 5 nM. Treatment with 100 nM Dex for 48 h did not affect the cGMP-PDE activity in MG-63 cells (control = 3014 ± 187 cpm, Dex = 2894 ± 134 cpm), indicating that Dex specifically affects cAMP-PDE-activity. The effect of Dex on the cAMP-PDE-activity of SaOS-2 cells (data not shown) was less pronounced, probably due to the lower PDE4 activity in these cells (Table 3). The sensitivity of homogenates towards the PDE-inhibitors EHNA and cilostamide was not affected by Dex treatment. However, homogenates of the Dex treated cells were, as expected, less sensitive towards rolipram. The Dex treatment also resulted in homogenates slightly less sensitive towards 8-MMX (Fig. 3).

3.3. Effect of PDE inhibitors on intracellular cAMP levels

Stimulation with forskolin or rolipram separately only moderately increased the cAMP levels in the cells. Simultaneous treatment with rolipram and forskolin resulted in a 20-fold and 60-fold increases in cAMP levels in MG-63 and SaOS-2 cells, respectively (Table 4). Treatment with Dex did not increase cAMP levels in either cell type. However, the Dex treatment significantly reduced the potency of rolipram to increase forskolin-stimulated cAMP response (Fig. 4).

3.4. Effect of dexamethasone on PDE subtype mRNA expression

Expression of the following cAMP-PDE subtypes were detected by reverse transcriptase PCR: PDE1A, PDE1C, PDE2A, PDE3A, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B, PDE8A, PDE10A and PDE11A

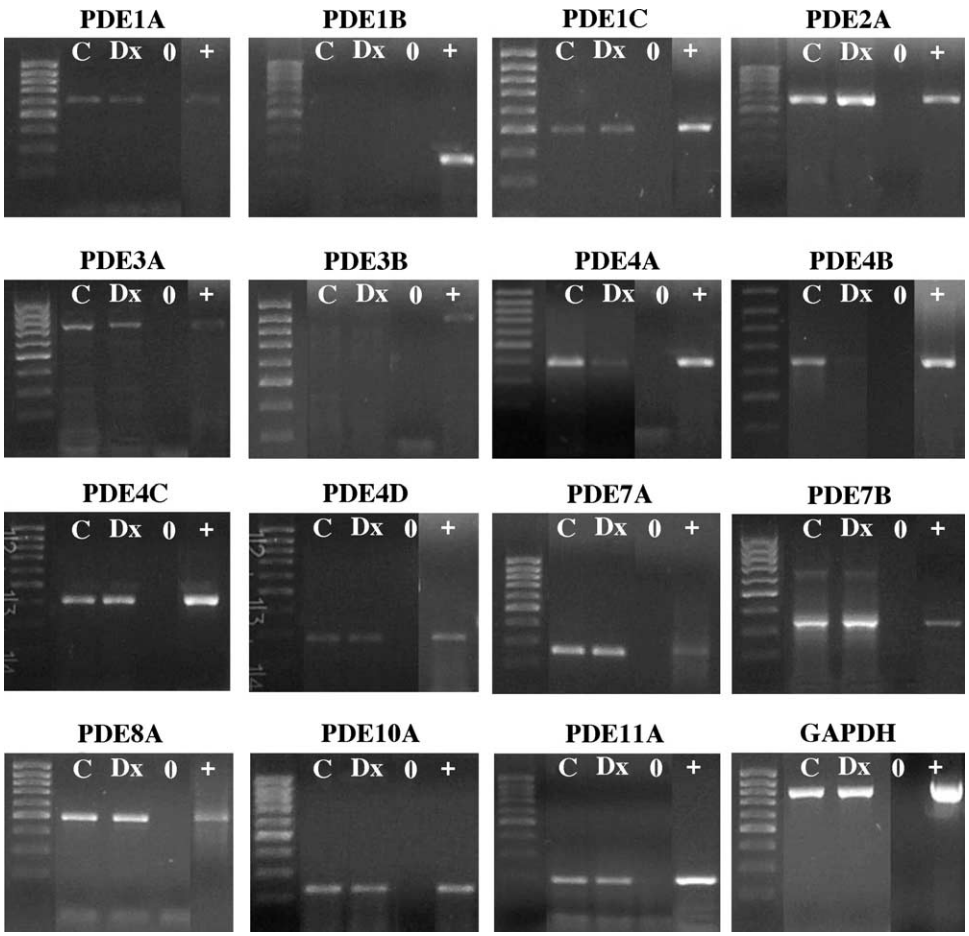

Fig. 5. Expression of, and Dex effects on PDE subtype mRNA transcripts in MG-63 cells. mRNA transcripts were assayed by RT-PCR after 24 h Dex treatment (1 μM). Lanes from reverse transcriptase-free reactions (0) and positive controls (human brain cDNA) are also shown.

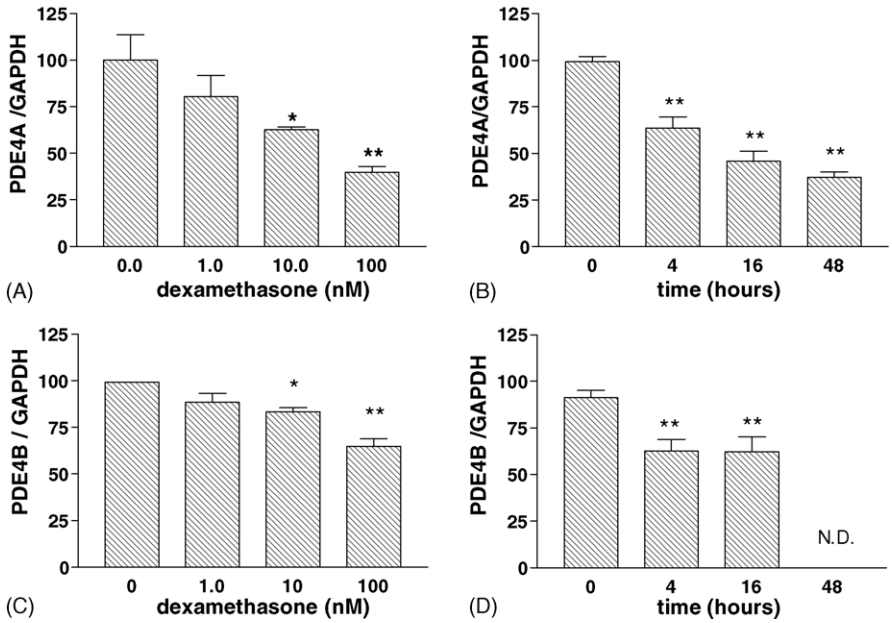

Fig. 6. Dexamethasone dose– and time–response for PDE4A (A and B) and PDE4B (C and D) mRNA expression in MG-63 cells. Dexamethasone decreases PDE4A and 4B mRNA expression, both dose- and time-dependently. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

(Fig. 5). Of these subtypes Dex reduced the mRNA of PDE4A and PDE4B subtypes in MG-63 (Figs. 5 and 6) and SaOS-2 cells (data not shown). The decrease in PDE4 mRNA was dose- and time dependent, with the half-maximal inhibition being 5–10 nM Dex, with significant effects seen within 4 h of treatment. Further analysis revealed a reduction in the mRNA of the PDE4A4 and PDE4B1 isoforms (Figs. 7 and 8). Transcription of PDE4A1, 4A7, 4A10, 4B2 and 4B3 was also evident but we could not detect any reduction by Dex treatment in these isoforms.

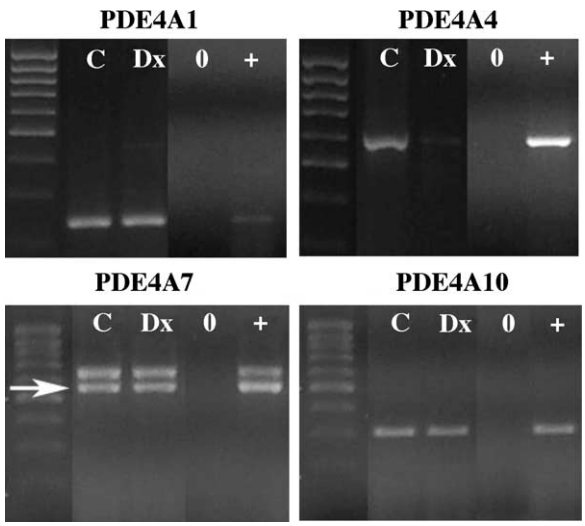

Fig. 7. Expression and effect of Dex on the expression of PDE4A isoforms in MG-63 cells. Lanes from reverse transcriptase-free reactions (0) and positive controls (+) from reactions with human brain cDNA are also shown.

4. Discussion

PDE4 is the most diverse of the PDE families; 4 genes, and at least 15 different isoforms have been found. Perhaps because of this diversity and the promising role of PDE4 selective inhibitors as therapeutic agents [31], this enzyme is among the best characterized of the PDE families. The present study, showing high levels of PDE4 activity in the human osteosarcoma cells is in agreement with our earlier reports were the main cAMP-hydrolyzing PDE activity in UMR-106 rat osteosarcoma cells was shown to consist of PDE4 [18,32]. In the present study, we could detect a substantial decrease in the cAMP-specific PDE activity after treatment with Dex. By using selective PDE inhibitors the Dex treated cells were shown to contain PDE activity that was less sensitive to the PDE4 selective inhibitor rolipram, indicating a reduced proportional presence of PDE4. In line with these results, Dex also decreased the effect of rolipram on cAMP accumulation. The Dex treatment alone did not affect the intracellular cAMP levels, but taking into account that PDE4 is the most abundant PDE type in both MG-63 and SaOS-2 cells, the reduction of cAMP response in the Dex treated cells might better describe the effect of an altered PDE profile of the cells (shift towards less PDE4 activity). In addition, a previous study with SaOS-2 cells has presented similar results showing that Dex alone does not affect cAMP levels [33]. The observed effects on cAMP-PDE activity and cAMP accumulation were accompanied by a decrease in the mRNA levels of PDE4A4 and PDE4B1 isoforms. The presented results thereby support the hypothesis that PDEs could play a role in Dex mediated effects on cAMP

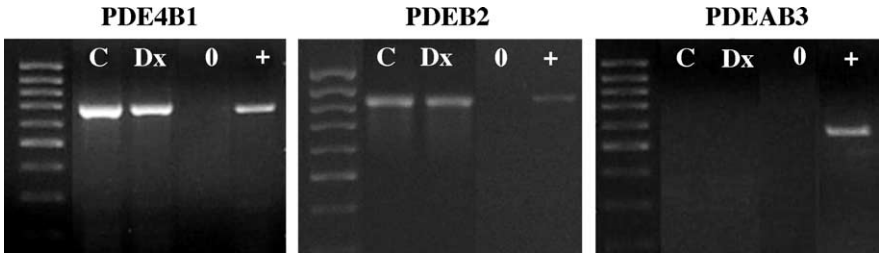

Fig. 8. Expression and effect of Dex on the expression of PDE4B isoforms in MG-63 cells. Lanes from reverse transcriptase-free reactions (0) and positive controls (+) from reactions with human brain cDNA are also shown.

signaling in osteoblasts. A multitude of cellular mechanisms that are modified by Dex are also likely to be involved in determining the levels of cAMP. One of the main effects of glucocorticoids is to inhibit prostaglandin synthesis by acting on COX-2 (cyclooxygenase-2). The reduction of PGE_2 secretion that follows by this inhibition could affect the cAMP signaling and is one of the possible mechanisms producing the effects in the present study. This is an interesting possibility, considering that PGE_2 is known to have anabolic effects on bone [34].

Several recent studies suggest a role of PDE4 in the regulation of bone metabolism. In rats, inoculated with Walker 256/S carcinoma to induce an artificial osteoporotic state, bone loss was reduced by over 50% after a two-week treatment with the PDE4 selective inhibitor denbufylline [35]. Rolipram, and two novel PDE4 inhibitors XT-44 and XT-611, have been shown to increase mineralized nodule formation [36] and PGE_2-mediated anabolic effects [37] in rat bone marrow culture. XT-44 also increases the bone mineral density of ovariectomized rats. Furthermore, Kinoshita et al. [38] showed that a five-week treatment of mice with rolipram or by a non-selective inhibitor, pentoxifylline significantly increased both cortical and cancellous bone mass. Horiuchi et al. [39] reported that daily injections of pentoxifylline enhance bone formation on bone morphogenic protein (BMP) impregnated collagen disks, implanted into the back muscles of mice. Pentoxifylline also promotes the osteoblastic differentiation of ST2 mouse bone marrow-derived stromal cells, although a recent report suggests that the effects of pentoxifylline are protein kinase A-independent [40].

One of the most severe side effects of long-term treatment with glucocorticoids is a rapid induction of bone-loss followed by osteoporotic fractures. The mechanism causing this common and clinically relevant side effect is not completely clear. Glucocorticoids have been shown to decrease the formation of new osteoblasts, modulate type 1 collagen synthesis, and affect the transcription of osteopontin, fibronectin, bone sialoprotein and a number of local growth factors [4,5]. Glucocorticoids have been shown to increase cAMP-levels induced by PTH [41,42]. These effects have also been reported in MG-63 [11] and SaOS-2 cells [33], but the increased cAMP-response seems to be linked to an increase in PTH receptors. In ROS 17/2.8 osteoblast-like cells the PTH receptor number was increased 10-fold with a simultaneous reduction of cAMP generation per receptor by 80% [43]. Increased responsiveness to PTH and increased adenylate cyclase activity has been suggested as possible routes in the glucocorticoid induced bone-loss [11,40–43]. Our results suggest an additional mechanism by which glucocorticoids could be involved in bone loss.

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