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Regulation of Adenosine 3′,5′-cyclic Monophosphate (cAMP) Accumulation in UMR-106 Osteoblast-like Cells: Role of cAMP-phosphodiesterase and cAMP Efflux

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ABSTRACT. The present study aimed to define the role of adenosine 3′,5′-cyclic monophosphate (cAMP)-phosphodiesterase (PDE) activity and the possible involvement of cAMP efflux on parathyroid hormone (PTH)-stimulated intracellular cAMP accumulation in cultured osteoblast-like UMR-106 cells. Treatment of the cells with 10 nM PTH (1–84) rapidly increased the level of intracellular cAMP. PTH stimulation also increased the cAMP efflux rate. The efflux of cAMP could only account for a minor part of the decrease in intracellular cAMP. Six peaks of cAMP-hydrolyzing PDE activity were separated by Q-Sepharose chromatography. The first peak to elute was stimulated by Ca^{2+}/calmodulin and provided less than 2% of the total eluted cAMP-PDE activity. The second peak, providing less than 4% of the cAMP-PDE activity, was stimulated 3-fold by 4 μM cyclic GMP (cGMP) and was sensitive to the PDE2 isoenzyme-selective inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). The third peak, providing less than 10% of the cAMP-PDE activity, was insensitive to rolipram, EHNA, Ca^{2+}/calmodulin, and cGMP. Peaks 4, 5 and 6 were sensitive to rolipram ($IC_{50} < 0.1$ μM) and provided approximately 85% of the total cAMP-hydrolyzing activity. It is concluded that cAMP-PDE activity in UMR-106 cells plays a major role in the control of intracellular cAMP accumulation, whereas only moderate amounts of cAMP are extruded from the cells through cAMP efflux. The main cAMP-hydrolyzing PDE isozyme is cAMP-specific/rolipram-sensitive. Ca^{2+}/calmodulin-stimulated PDE, cGMP-stimulated PDE, and presently unidentified cAMP-specific/rolipram-insensitive PDE are also present in UMR-106 cells. BIOCHEM PHARMACOL **58**;8:1335–1340, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. osteoblast; UMR-106; osteosarcoma; cyclic AMP; parathyroid hormone; phosphodiesterase

Cells of the osteoblast lineage are central in the process of bone development, growth, and remodeling. The development of rapidly dividing osteoprogenitor cells into highly differentiated bone-forming osteoblasts is regulated by a variety of hormones and growth factors [1, 2]. PTH† has a well-known physiological role in bone metabolism and modulates the expression of several genes characteristic of the osteoblast phenotype [2–5]. Binding of PTH to its receptor causes activation of adenylate cyclase, the enzyme responsible for the formation of cAMP. The cAMP pathway is further transduced when cAMP binds to protein kinase A, which in its activated state phosphorylates target molecules in the cytosol [2].

A complex mechanism controlling the homeostasis of intracellular cAMP consists of cAMP-generating and -inactivating mechanisms. The degradation of cAMP to 5′AMP by the PDEs (EC 3.1.4.17) counteracts the generation of cAMP by the adenylate cyclases, and the cAMP signal is therefore not only dependent on the formation, but also on the degradation rate of cAMP (reviewed in Refs. 6 and 7). Mammalian cells contain multiple PDEs classified into seven isoenzyme families: Ca^{2+}/calmodulin-dependent PDE (PDE1), cGMP-stimulated PDE (PDE2), cGMP-inhibited PDE (PDE3), cAMP-specific (rolipram-sensitive) PDE (PDE4), cGMP-specific PDE (PDE5), retina cGMP-specific PDE (PDE6), and newly discovered cAMP-specific (rolipram-insensitive) PDE (PDE7). Most of these PDE families contain two or more closely related subfamilies. The exact identification and characterization of the PDEs is further complicated by splice variants that are generated by alternative splicing [6].

UMR-106 osteoblast-like osteosarcoma cells express features that are typical of mature osteoblasts: they respond to PTH by increasing intracellular cAMP, produce high levels of alkaline phosphatase and bone-specific type 1 collagen, and have receptors for 1 alpha, 25-dihydroxyvitamin D_3 [8].

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† *Abbreviations:* PTH, parathyroid hormone; cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; EHNA, erythro-9-[2-hydroxy-3-nonyl] adenine; HBSS, Hanks' balanced saline solution; and PIC, protease inhibitor cocktail.

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The effects of PTH on proliferation and differentiation are mainly mediated by cAMP in UMR-106 cells, and this signaling molecule thus plays a crucial role in the function of this cell line [5, 6, 8]. Although the importance of cAMP in osteoblast biology is well known, little attention has been given to the mechanisms that inactivate cAMP. There are no data that describe which PDE isozymes are present in osteoblasts, nor has the possible role of cAMP efflux as a cAMP-inactivating mechanism in osteoblasts been examined as yet. We described earlier that PTH increases total PDE activity in UMR-106 cells in a protein kinase A-dependent manner [9]. In the present study, we investigated which PDE isozymes take part in the hydrolysis of cAMP. The role of cAMP efflux on PTH-stimulated intracellular cAMP accumulation in UMR-106 cells was also studied.

MATERIALS AND METHODS

Reagents

Intact bovine PTH(1–84) and rabbit anti-cAMP antisera were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases. [^{125}I]cAMP (adenosine 3′,5′-cyclic phosphoric acid 2′-0-succinyl 3-[^{125}I]iodotyrosine methyl ester, 2000 Ci/mmol) and [^{3}H]cAMP ([8-^{3}H] adenosine 3′,5′-cyclic phosphate, 24 Ci/mmol) were from Amersham international. Ro 20-1724 (4-[3-butoxy-4-methoxybenzyl]-2-imidazolidine) was a gift from Roche. Rolipram (4-[3-{cyclopentyloxy}-4-methoxyphenyl]-2-pyrrolidinone) was from Calbiochem. EHNA was purchased from Biolog. Q-Sepharose High Performance was from Pharmacia. AG1-X8 was from Bio-Rad. All other chemicals were from Sigma.

Cell Culture

UMR-106 clonal rat osteosarcoma cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum supplemented with 50 IU/mL penicillin and 50 μg/mL streptomycin at 37° in 5% CO_2/95% air atmosphere on 100- and 60-mm plastic dishes. The medium was routinely changed twice a week.

Measurement of cAMP Accumulation

Cells were grown to confluence on 35-mm plastic dishes. Twenty hours before treatment with PTH and various compounds, the media was changed to Dulbecco's modified Eagle's medium with 0.1% BSA replacing fetal bovine serum. The cultures were washed with HBSS buffer containing 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM HEPES, 0.4 mM $CaCl_2$, pH 7.4. All incubations were carried out in HBSS buffer at 37°. In some experiments, Ro 20-1724 and probenecid (*p*-(dipropylsulfamyl)benzoic acid) were added 4 min prior to the treatments with PTH. For measurement of intracellular cAMP, the cultures were first washed twice with ice-cold HBSS and the cAMP then extracted from cells with 1 mL 96% ethanol at −18° for 3 hr. The extract was transferred to plastic tubes and evaporated in vacuum at 37°. The evaporated samples were then dissolved in 1 mL of assay buffer containing 0.05 M sodium acetate, pH 6.2. If necessary, the samples were further diluted with assay buffer, and the cAMP concentration was then determined by radioimmunoassay, with iodinated cAMP as tracer [10]. To measure cAMP efflux, 100 μL samples were withdrawn from the incubation buffer. The samples were instantly boiled for 1 min and assayed in triplicate by radioimmunoassay.

Q-Sepharose Chromatography

Five confluent cultures of UMR-106 cells, grown on 100-mm plastic dishes, were scraped in 6 mL ice-cold homogenization buffer (buffer A) containing 20 mM Bis–Tris, pH 6.5, 0.1% (v/v) Sigma PIC for mammalian cell and tissue, 0.5 mM EDTA, 5 mM benzamidine, and 5 mM mercaptoethanol. The scraped cells were then homogenized three times by ten strokes on ice with a Teflon/glass homogenizer. The homogenate was then centrifuged for 1 hr at 100,000 *g*. The supernatant (5 mL) was diluted with 20 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-μM syringe filter, and applied to a column (5 mL bed volume) of Q-Sepharose previously equilibrated with buffer B. After washing the column with 10 bed volumes, PDE activities were eluted with a 0.1–1.3 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 containing 50 μL 5% BSA and assayed for PDE activity as described below.

Assay of PDE Activity

PDE activity was assayed according to the method of Thompson and Appleman [11] in an incubation buffer containing 40 mM Tris–HCl, pH 8.0, 0.1% PIC, 0.05% BSA, 1 mM mercaptoethanol, 10 mM $MgCl_2$, and [^{3}H]cAMP (200,000 dpm/tube). A 50–150 μL sample was added to the incubation buffer to give a final reaction volume of 300 μL, and tubes were incubated at 34° for 15–60 min. The reaction was stopped by boiling tubes for 1 min. One hundred microliters of snake venom (*Crotalus atrox*) nucleotidase (1 mg/mL) was added, and the samples were further incubated at 34° for 10 min. Five hundred microliters of a 1:2 slurry of AG1-X8 anion exchange resin was then added, followed by 5 min of centrifugation at 6000 *g*. The radioactivity of the supernatant was counted.

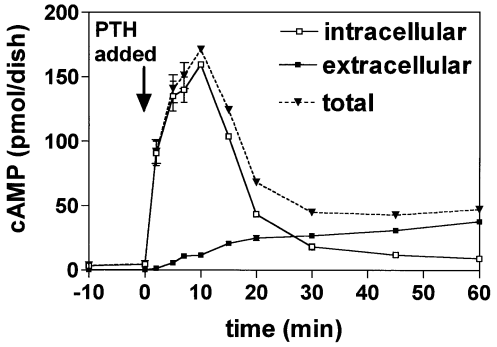

FIG. 1. Time–course of PTH-stimulated cAMP in UMR-106 cells. The cells were washed with HBSS buffer and incubated at 37° with 10 nM PTH for the indicated times. Samples from the buffer were withdrawn for measurement of released cAMP. Intracellular cAMP was extracted and assayed as described in the Methods section. Data presented represent means ± SEM.

Statistics and Determination of Kinetic Parameters

Experimental results are expressed as means ± SEM. Data were evaluated by analysis of variance and by students *t*-test, with Bonferroni adjustment when appropriate. Determination of K_m was calculated according to Torphy and Cieslinski [12]. The concentration of [^{3}H]cAMP was kept constant, whereas the concentration of unlabeled cAMP was varied. Corrections for the change in specific activity of the substrate were made, and the results were analyzed by non-linear regression analysis. Inhibition constants were also determined by non-linear regression.

RESULTS

PTH Effects on cAMP

Treatment of UMR-106 cells with 10 nM bovine PTH (1–84) resulted in a rapid increase in intracellular cAMP which peaked at 10 min after the onset of the stimulation. The subsequent decrease in intracellular cAMP from peak levels was also rapid, returning close to basal levels 10–20 min after the peak values had been reached (Fig. 1). In order to evaluate the role of cAMP efflux on PTH-stimulated intracellular cAMP levels, we measured the

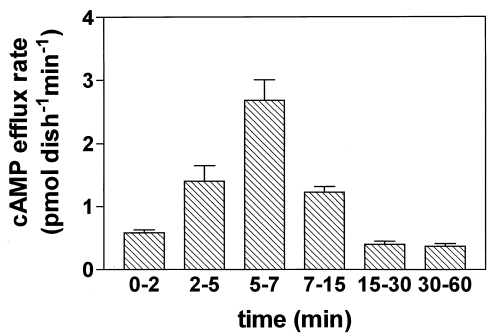

FIG. 2. The effect of PTH on cAMP efflux rate. UMR-106 cells were treated with 10 nM PTH at time 0. Released cAMP was assayed and corrected for the indicated time intervals. Data presented represent means ± SEM.

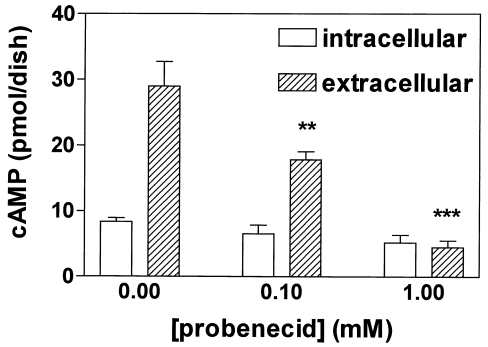

FIG. 3. The dose-dependent effect of probenecid on extracellular and intracellular cAMP levels. The cells were treated with indicated concentrations of probenecid as described in the Methods section and were then stimulated with 10 nM PTH for 45 min at 37°. Samples of intracellular and released cAMP were collected and assayed for cAMP concentration. **P < 0.05, ***P < 0.01 (students *t*-test). Data presented represent means ± SEM.

concentration of cAMP released into the medium. There was an increase in the medium cAMP efflux immediately after the addition of PTH, one that persisted for at least 60 min (Fig. 1). The rate of cAMP efflux first accelerated in parallel with the increase in intracellular cAMP, peaked between 5–7 min, and then slowed as the intracellular levels declined (Fig. 2). The cAMP efflux could be blocked up to 90% by probenecid, a non-selective antagonist of anion transport known to block cAMP efflux in several cell types [13–15] (Fig. 3). Although probenecid dose dependently blocked cAMP efflux, the intracellular cAMP accumulation, measured at 45 min after the onset of the PTH stimulation, was not increased by the probenecid treatment (Fig. 3). The effect of phosphodiesterase inhibition on intracellular cAMP was evaluated with the PDE4-selective inhibitor Ro 20-1724. The PTH-stimulated intracellular cAMP response was potentiated up to tenfold in the presence of 10 μM Ro 20-1724 (Fig. 4).

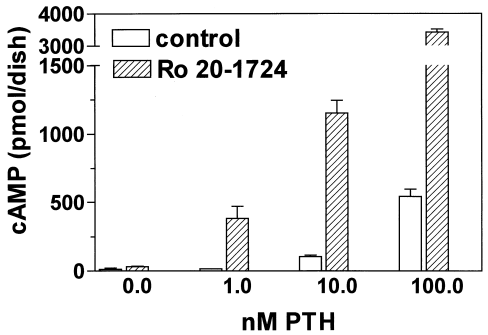

FIG. 4. The effect of PDE4-selective inhibitor Ro 20-1724 on PTH-stimulated intracellular cAMP accumulation. The cells were treated with 10 μM Ro 20-1724 and stimulated with indicated concentrations of PTH at 37° for 10 min. Intracellular cAMP was then extracted from the cells and assayed. Data presented represent means ± SEM. The PTH treatment was dose-responsive for both control and Ro 20-1724 treatment (P < 0.01, ANOVA).

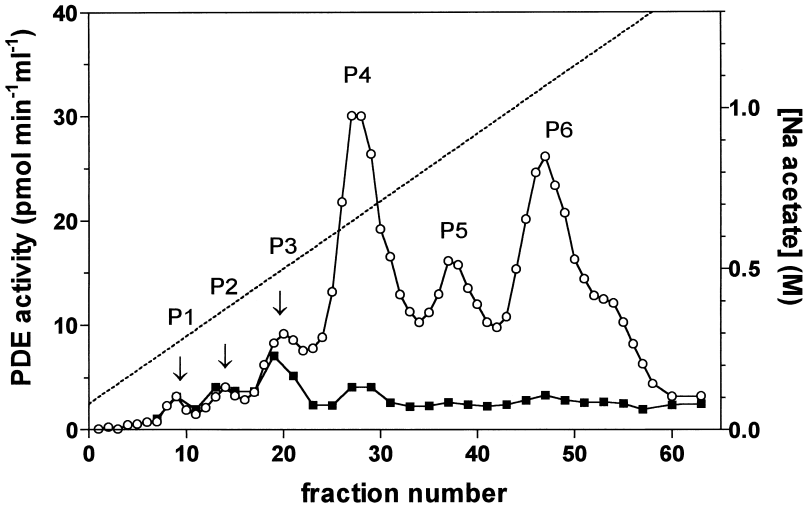

FIG. 5. Elution profile of PDE activity from UMR-106 cells separated by Q-Sepharose chromatography. Supernatant obtained from centrifuged cell extracts containing 4–5 mg protein was applied to a Q-Sepharose column and eluted with a linear gradient of sodium acetate (broken line). The eluted fractions were assayed for cAMP-PDE activity in the presence (filled squares) or absence (open circles) of 10 μM rolipram as described in the Methods section.

Isolation of PDE Isoenzymes in UMR-106 Cells

Six peaks of PDE activity, designated P1–P6, eluted on Q-Sepharose chromatography (Fig. 5). The addition of 50 U/tube calmodulin/1 mM $CaCl_2$ to the assay increased the rate of cAMP hydrolysis of P1, while the activity of P2 was potentiated by the addition of 4 μM cGMP (Fig. 6). The activity of this peak was sensitive to EHNA, an inhibitor of PDE2 (Table 1). The third peak (P3) was insensitive to cGMP, Ca^{2+}/calmodulin, EHNA, and rolipram (Fig. 6, Table 1). Peaks P4, P5, and P6 were all sensitive to rolipram, and had a high affinity for cAMP, suggesting that all three belong to the PDE4 isoenzyme family (Fig. 5 and Table 1).

DISCUSSION

PTH-stimulated cAMP in osteoblasts is controlled by several known mechanisms, among which are down-regulation of PTH receptors and modifications of postreceptor components [16, 17]. The intracellular cAMP signal can also be modified downstream of the cAMP generation by regulation of the rate of cAMP hydrolysis to 5′AMP [6, 7] and by removal of cAMP from the cell [13–15, 18, 19]. In the present study, the total PTH-stimulated cAMP (intracellular + extracellular) decreased rapidly after peak levels had been reached. The release of cAMP during the 60-min PTH stimulation was low compared to the initial intracellular stimulated concentrations. This suggests that cAMP inactivation is largely independent of cAMP efflux. Furthermore, the levels of intracellular cAMP were not increased by blocking the efflux of cAMP by probenecid. The efflux of cAMP by a probenecid-sensitive mechanism has been demonstrated in several cell types [13–15]. It has been suggested that the efflux mechanism could be important in the control of cyclic nucleotide levels in rat glial cells, bovine adrenal medullary cells, and platelets [13, 19, 20], although blocking the cAMP efflux apparently has little or no effect on intracellular cAMP in some cells [21]. Our results are basically in line with the latter findings, but show that the export of cAMP from UMR-106 cells is not a simple diffusional process.

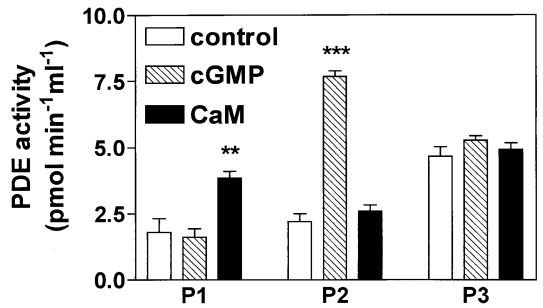

FIG. 6. The effect of Ca^{2+}/calmodulin (CaM) and cGMP on the cAMP-PDE activity of Q-Sepharose-separated peaks P1, P2, and P3. Peak fractions were pooled and assayed for cAMP-PDE activity with 1 mM Ca^{2+}/50 U/tube calmodulin and with 4 μM cGMP. Data presented represent means of three determinations ± SEM. **$P < 0.01$, ***$P < 0.001$ compared to control of each peak.

Of the total PDE activity eluted in Q-Sepharose chromatography, approximately 85% was sensitive to low concentrations of rolipram and insensitive to cGMP, in line with our earlier results indicating PDE4 as the main PDE in crude homogenates from UMR-106 cells [9]. The rolipram-sensitive cAMP-PDE eluted in three distinct peaks, and showed only slightly different affinity for cAMP and sensitivity to rolipram. It is known that PDEs belonging to this family of isoenzymes are generated from at least four separate genes [6]. Furthermore, distinct "short" and "long" splice variants of the PDE4 subfamilies are known [6]. Although not reported earlier, it is possible that the three

TABLE 1. Properties of cAMP-PDE peaks of UMR-106 cells eluted on Q-Sepharose

Peak	K_m (cAMP) (μM)	IC_{50} rolipram (μM)	IC_{50} EHNA (μM)	Elution of peak, Sodium acetate (M)	Activator
P1	—	>50	>50	0.25	calmodulin
P2	—	>50	3.4 ± 2.5	0.35	cGMP
P3	13.0 ± 5.8	>50	>50	0.45	—
P4	6.9 ± 2.5	0.1 ± 0.06	—	0.65	—
P5	5.3 ± 3.9	0.02 ± 0.01	—	0.80	—
P6	3.0 ± 1.7	0.03 ± 0.02	—	1.05	—

The data are expressed as means ± SEM. Experimental procedures are described in the Materials and Methods section.

peaks represent either products of different PDE4 genes or different splice products of the same gene. Molecular biological studies must be employed to clarify which of the several PDE4s are expressed in UMR-106 cells. Nevertheless, the PDE4 isoenzyme seems to be the main cAMP-hydrolyzing PDE in UMR-106 cells. Recently, it was reported that PDE4 inhibitors, i.e. denbufylline and rolipram, increase the formation of mineralized nodules *in vitro* [22]. Denbufylline also reduces Walker 256/S carcinoma-induced bone loss in rats [22]. These findings are interesting with regard to the physiological and pharmalogical importance of our findings. PDE4 inhibitors might reduce bone loss *in vivo* by inhibiting the activity of osteoblast PDE.

In the present study, less than 15% of the total PDE activity eluted was shown to be insensitive to rolipram. Of this activity, Ca^{2+}/calmodulin-PDE (PDE1) was present only in very low, albeit measurable activity. Our earlier results showing no PDE activation by Ca^{2+}/calmodulin in crude homogenates of UMR-106 cells [21] are not in conflict with the present data. The activation of a PDE that contributes less than 2% of total PDE activity is not likely to be detected in these crude samples. The second identified PDE activity was clearly stimulated by low concentrations of cGMP, suggesting that it belongs to the PDE2 isoenzyme family. This is also supported by the high sensitivity of P2 to the PDE2-selective inhibitor EHNA. The largest peak insensitive to rolipram (P3) has an apparent K_m for cAMP of 13 μM. This peak is insensitive to cGMP, indicating specificity for cAMP. A newly discovered cAMP-specific, rolipram-insensitive PDE isoenzyme family (PDE7) has been found in human tissues, most abundantly in skeletal muscle [23, 24]. This isoenzyme has a reported K_m of 0.2 μM, considerably lower than the PDE described in the present study. The PDE7 isoenzyme has also been found in several rat tissues [25], but because of the lack of selective inhibitors for the PDE7 isoenzyme family, the possible presence of PDE7 in peak P2 remains to be examined.

In conclusion, the present results indicate that cAMP release contributes only slightly to intracellular cAMP inactivation in UMR-106 osteoblast-like cells. PDE activity, mainly of the PDE4 isoenzyme, is likely to be the dominating cAMP-inactivating mechanism. In addition to PDE4, at least three distinct PDE isoenzymes can be found in UMR-106 cells.

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