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Inactivation of Atrial Natriuretic Factor-Stimulated Cyclic Guanosine 3′,5′-Monophosphate (cGMP) in UMR-106 Osteoblast-like Cells

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ABSTRACT. Previous studies have suggested a role of cyclic guanosine 3′,5′-monophosphate (cGMP) in the differentiation and proliferation of osteoblasts. We studied the effect of ANF (atrial natriuretic factor) on intracellular cGMP accumulation, cGMP efflux, and cGMP–phosphodiesterase (PDE) activity in UMR-106 osteoblast-like cells. ANF rapidly increased both intracellular cGMP and cGMP efflux. ANF-stimulated intracellular cGMP peaked at 2 min in the absence and at 10 min in the presence of 0.25 mM 3-isobutyl-1-methylxanthine. Probenecid, an antagonist of anion transport, blocked the efflux of cGMP (IC_{50} = 0.1 mM), ruling out simple diffusion as a mechanism of the efflux. cGMP–PDE activity was increased threefold in crude homogenates from ANF-treated cells (IC_{50} = 23 nM). ANF-evoked stimulation of cGMP–PDE activity was reached simultaneously with the peak in intracellular cGMP. Separation of the PDEs by Q-Sepharose chromatography revealed three cGMP-hydrolyzing peaks. The first peak was sensitive to the PDE5 (cGMP-specific PDE) isoenzyme-selective inhibitor zaprinast (IC_{50} = 0.45 μM). The second peak was stimulated fourfold by the addition of calcium/calmodulin, indicating the presence of PDE1. The third peak was sensitive to the PDE2 (cGMP-stimulated PDE) isoenzyme-selective inhibitor 9-[2-hydroxy-3-nonyl]adenine (EHNA) (IC_{50} = 3 μM), and was activated by over 300% in the presence of 4 μM cGMP. Our results show that ANF-stimulated cGMP is released from UMR-106 cells by a probenecid-sensitive mechanism. ANF also stimulates cGMP hydrolysis by activating cGMP–PDE activity. Three distinct cGMP-hydrolyzing PDEs, namely PDE5, PDE1, and PDE2, are present in the studied cells. BIOCHEM PHARMACOL **59**;9:1133–1139, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. osteoblast; UMR-106; cGMP; phosphodiesterase; atrial natriuretic factor; bone

During differentiation, pre-osteoblastic cells acquire structural and functional properties characteristic of bone-forming osteoblasts. Each step of the differentiation is mediated by expression of a cascade of genes in response to a series of intracellular signals. ANF† is a peptide hormone mainly synthesized and released into the blood by cardiocytes. Several tissue types have specific receptors for ANF [1], and the binding of ANF to its receptor leads to increased formation of intracellular cGMP. Both normal osteoblasts and osteosarcoma cells have been shown to have receptors for ANF [2, 3]. A number of studies present evidence that cGMP regulates the proliferation and differentiation of osteoblasts [3–5] and reduces PTH- and prostaglandin E_2-induced resorption of bone [6, 7]. The control of intracellular levels of cGMP could subsequently be of importance in the regulation of bone metabolism. In mammalian cells, intracellular cGMP levels are generated by the action of membrane-bound natriuretic peptide receptor guanylate cyclase [1] and by soluble guanylate cyclase activated by nitric oxide [8]. The inactivation of cGMP is in turn accomplished by export of cGMP outside the cell and by the action of the PDEs (EC 3.1.4.17) that hydrolyzes cGMP to its inactive form 5′-GMP. The PDEs consist of 10 families of isoenzymes (PDE1–PDE10) that differ in tissue distribution, substrate specificity, and regulatory properties [9–14].

In the present study, we examined the inactivation of ANF-stimulated cGMP in the phenotypically osteoblast-like UMR-106 cell line. In addition, the cGMP-hydrolyzing PDE isoenzymes present in the cells were identified.

MATERIALS AND METHODS

Reagents

Rat ANF (1–28) was from Calbiochem-Novabiochem. ^{125}I-cGMP (guanosine 3′,5′-cyclic phosphoric acid 2′-0-

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† *Abbreviations:* ANF, atrial natriuretic factor; cGMP, cyclic guanosine 3′,5′-monophosphate; PTH, parathyroid hormone; PDE, cyclic nucleotide phosphodiesterase; HBSS, Hanks' balanced saline solution; EHNA, 9-[2-hydroxy-3-nonyl]adenine; IBMX, 3-isobutyl-1-methylxanthine; PIC, protease inhibitor cocktail; cAMP, cyclic adenosine 3′,5′-monophosphate; and CNP, C-type natriuretic peptide.

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succinyl 3-[^{125}I]iodotyrosine methyl ester, 2000 Ci/mmol), [^{3}H]cGMP ([8-^{3}H]guanosine 3′,5′-cyclic phosphate, 10 Ci/mmol), [^{3}H]cAMP ([8-^{3}H]adenosine 3′,5′-cyclic phosphate, 26 Ci/mmol), and Q-Sepharose High Performance were from Amersham Pharmacia Biotech. AG1-X8 anion-exchange resin was from Bio-Rad. EHNA was from Biolog. All other chemicals were from Sigma.

Cell Culture

UMR-106 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 plastic Petri dishes. The medium was routinely changed twice a week.

Measurement of cGMP Accumulation

Cells were grown to confluence on 35-mm plastic Petri dishes. Twenty hours before treatment with ANF and various compounds, the medium was changed to Dulbecco's modified Eagle's medium, fetal bovine serum being replaced by 0.1% BSA. 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, IBMX, probenecid (*p*-[dipropylsulfamoyl] benzoic acid), EHNA, and zaprinast were added 4 min prior to the treatments with ANF. For measurement of intracellular cGMP, the experiments were terminated by washing twice with ice-cold HBSS. The cGMP was 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 cGMP concentration was then determined by radioimmunoassay with ^{125}I-cGMP as a tracer [15]. To measure cGMP efflux, 100-μL samples were withdrawn from the incubation buffer, instantly boiled for 1 min, and assayed by radioimmunoassay.

Preparation of Cell Homogenates

UMR-106 cells grown on 60-mm plastic Petri dishes were treated with ANF in HBSS buffer at 37°. At the end of the experiments, the cells were washed twice with HBSS and harvested with a cell scraper into 2 mL ice-cold 40 mM Tris–HCl, pH 8.0, containing 0.1% PIC and 5 mM mercaptoethanol. The harvested cells were then homogenized by ten passages through a Teflon/glass homogenizer on ice and centrifuged at 800 *g* for 5 min. The cGMP–PDE activity of the supernatant was assayed as described below.

Q-Sepharose Chromatography

Five confluent cultures of UMR-106 cells grown on 100-mm plastic Petri dishes were washed twice with HBSS buffer and harvested with a cell scraper into 6 mL ice-cold homogenization buffer (buffer A) containing 20 mM Bis-Tris, pH 6.5, 0.1% (v/v) PIC, 0.5 mM EDTA, 5 mM benzamidine, and 5 mM mercaptoethanol. The harvested cells were then homogenized by ten passages on ice with a Teflon/glass homogenizer. The homogenate was then centrifuged for 1 hr at 100,000 *g*. Five milliliters of the supernatant 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 High Performance previously equilibrated with buffer B. After the column was washed with 10 bed volumes, the PDE activities were eluted with a 0.1–1.0 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 either cGMP–PDE or cAMP–PDE activity as described below.

Assay of PDE Activity

PDE activity was assayed according to the method of Thompson and Appleman [16] in an incubation buffer containing 40 mM Tris–HCl, pH 8.0, 0.1% PIC, 0.05% BSA, 1 mM mercaptoethanol, 10 mM MgCl, and [^{3}H]cGMP (0.25 μM) or [^{3}H]cAMP (0.5 μM). A 50–150-μL sample was added to the incubation buffer to give a final reaction volume of 300 μL, and the mixture was incubated at 34° for 30–60 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 a 5-min centrifugation at 6000 *g*. The radioactivity of the supernatant was counted.

Statistics

The data were evaluated by Student's *t*-test (for comparison between two means) or analysis of variance (ANOVA, for comparison between more than two means). If a statistically significant difference was observed after ANOVA, the difference between two means was further tested by Student's *t*-test, with Bonferroni correction. Half-maximal inhibition (EC_{50}) was determined by non-linear regression.

RESULTS

Both intracellular and released cGMP levels were increased by treating UMR-106 cells with 30 nM ANF (Fig. 1A). The effect of PDE inhibition on cGMP levels was examined

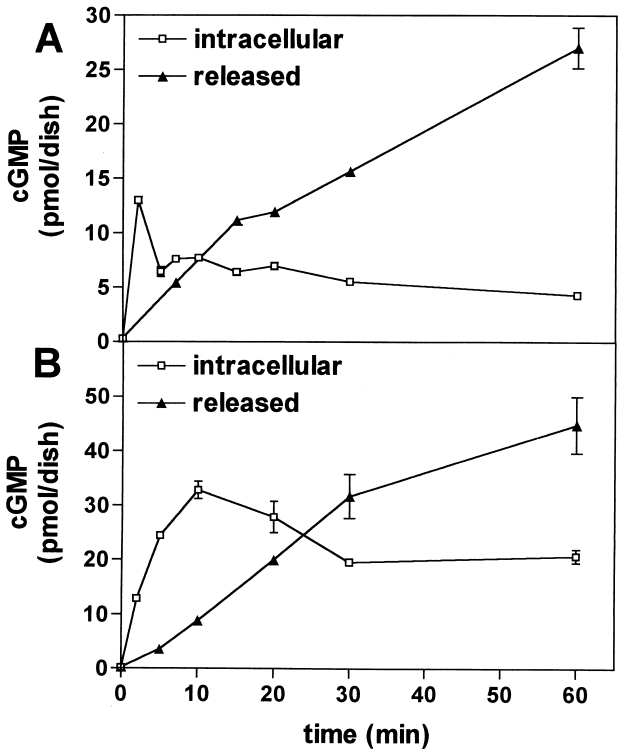

FIG. 1. Time–courses of the ANF effect on intracellular and released cGMP levels. UMR-106 cells were stimulated at 37° with 30 nM ANF in the absence (A) and presence (B) of 0.25 mM IBMX. Samples from indicated time-points were assayed in triplicate by radioimmunoassay. The results given represent the means ± SEM.

by adding 0.25 mM of the non-specific PDE inhibitor IBMX to the incubation buffer (Fig. 1B). The mean efflux rate of cGMP, measured within the first 60 min of ANF stimulation, was increased almost 2-fold by the IBMX treatment (control = 0.45 ± 0.06 pmol/min/dish, IBMX = 0.75 ± 0.12 pmol/min/dish, $P = 0.018$). The intracellular cGMP response was also modulated by IBMX. The peak of the ANF response was reached at 2 min in the absence of IBMX, but in presence of IBMX the peak was delayed to 10 min (Fig. 1, A and B). The corresponding cGMP peak levels were increased from 13.1 ± 0.9 pmol/dish to 31.5 ± 2.4 pmol/dish by the IBMX treatment ($P < 0.001$). The role of PDE inhibition by IBMX on total (intracellular + released) cGMP is shown in Fig. 2. The rate of total cGMP accumulation within the first 20 min of ANF stimulation was more than 2-fold in the presence of IBMX (control = 1.20 ± 0.08 pmol/min/dish, IBMX = 2.41 ± 0.23 pmol/min/dish, $P < 0.001$). During the later phase of ANF stimulation (20–60 min), the rate of total cGMP accumulation was the same in both control and IBMX-treated cells (control = 0.39 ± 0.08 pmol/min/dish, IBMX = 0.42 ± 0.06 pmol/min/dish, $P = 0.62$). ANF stimulated both intracellular and released cGMP dose dependently (Fig. 3), and there was a correlation between the intracellular and released amounts of cGMP (Fig. 3, insert). Probenecid, which is a non-selective antagonist of anion transport, has

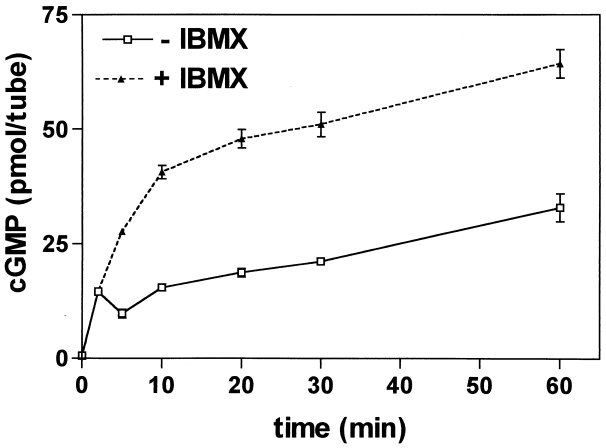

FIG. 2. Time–courses of ANF-stimulated total (intracellular and released) cGMP levels in the presence and absence of 0.25 mM IBMX. UMR-106 cells were stimulated at 37° with 30 nM ANF in the absence and presence of 0.25 mM IBMX. Samples were then assayed in triplicate by radioimmunoassay. Intracellular and released cGMP for the indicated time-points were added to give total accumulated cGMP. The results given represent the means ± SEM. $P < 0.001$, ANOVA (control vs IBMX treatment).

been shown to block the release of cyclic nucleotides from a variety of animal cells [17–20]. Treatment of UMR-106 cells with probenecid dose dependently inhibited the ANF-stimulated extracellular accumulation of cGMP (IC_{50} = 0.1 mM, Fig. 4).

cGMP–PDE activity in crude homogenates prepared from cells treated by ANF was increased in a dose-responsive manner (EC_{50} = 23 nM), but cAMP–PDE activity was not affected by the ANF treatment (Fig. 5). Stimulation of both cGMP–PDE-activity and intracellular cGMP was induced by similar concentrations of ANF (Figs.

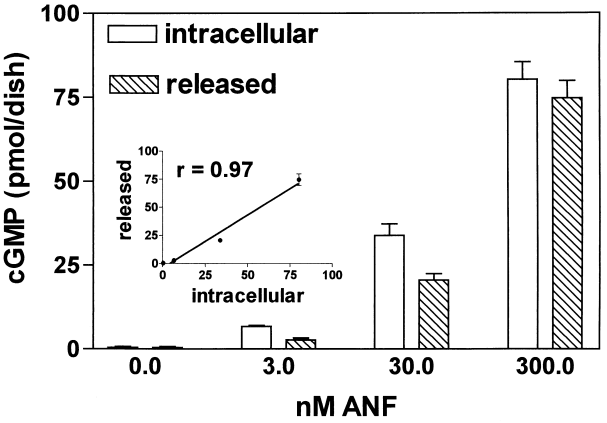

FIG. 3. Dose–response of ANF on intracellular and released cGMP. UMR-106 cells were stimulated at 37° with indicated concentrations of ANF for 10 min in the absence of IBMX. Samples were then assayed by radioimmunoassay. The dose–response was significant for both intracellular and released cGMP ($P < 0.001$, ANOVA). A correlation exists between the intracellular and secreted cGMP (insert). The results given represent the means ± SEM of triplicate determinations.

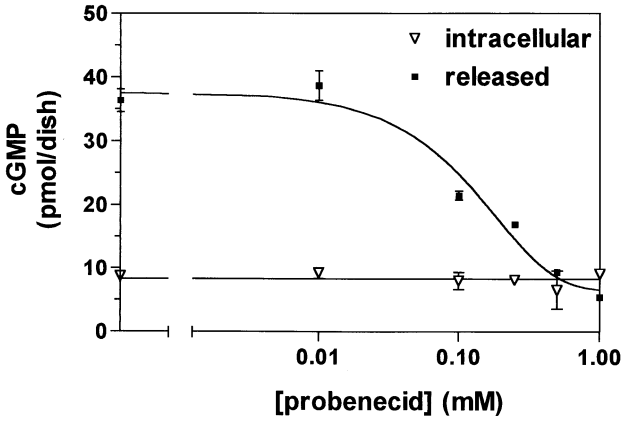

FIG. 4. Concentration-dependent probenecid-mediated inhibition of ANF-stimulated cGMP release. UMR-106 cells were stimulated with 30 nM ANF, in the absence of IBMX, with indicated concentrations of probenecid, at 37°. At 30 min, the incubation was stopped as described in the Methods section. The released and intracellular cGMP was assayed by radioimmunoassay. The given results represent means ± SEM of triplicate determinations.

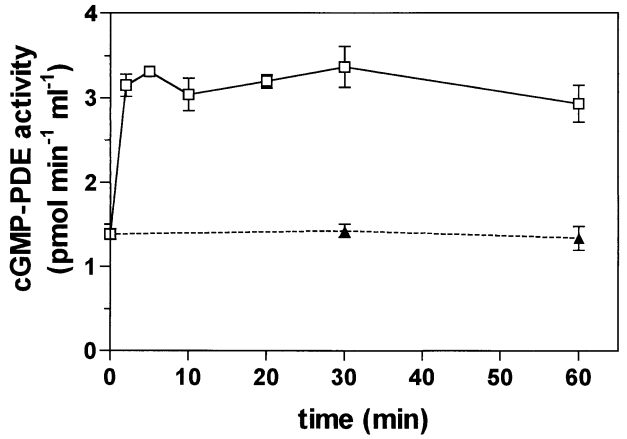

FIG. 6. Time–course of ANF stimulation of cGMP–PDE. UMR-106 cells were incubated for the indicated times at 37°. Control cells (filled triangles), and ANF-treated cells (open squares) were harvested, homogenized, and assayed for cGMP–PDE-activity. The given results represent means ± SEM of triplicate determinations.

3 and 5). The time–response of ANF-stimulated PDE activity and ANF-stimulated intracellular cGMP also coincided, both reaching maximal levels within 2 min (Figs. 1A and 6). 8-Bromo-cGMP (0.5 mM), a cGMP analogue which activates protein kinase G, did not mimic the cGMP–PDE stimulating effects of ANF (data not shown).

PDE activities separated on Q-Sepharose chromatography by a 0.1–1.0 M sodium acetate gradient revealed three peaks hydrolyzing cGMP (Fig. 7), eluting at 0.2, 0.3, and 0.35 M sodium acetate, respectively. The peaks were identified by modulation of the hydrolytic activity by calmodulin and cGMP, and by sensitivity to isoenzyme-selective inhibitors. The first peak, which was designated G1, showed high sensitivity to the PDE5-selective inhibitor zaprinast (IC_{50} = 0.45 μM), but not to EHNA, a PDE2-selective inhibitor (Fig. 8). The activity of the second peak, designated G2, was stimulated 4-fold in the presence of 20 U/tube calmodulin/10 μM $CaCl_2$, indicating the presence of PDE1. The third peak (G3) was, in contrast to G1, sensitive to EHNA (IC_{50} = 3 μM), but not to zaprinast (Fig. 8). The activity of G3 was also stimulated more than 3-fold by 4 μM cGMP, confirming the presence of PDE2 in this peak (Fig. 7).

Fractions eluting at higher sodium acetate concentrations than peak G2 did not contain any cGMP-hydrolyzing

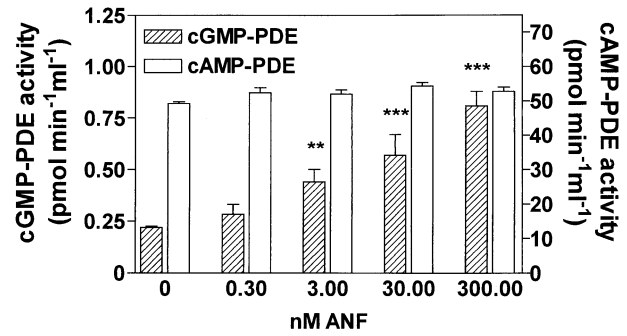

FIG. 5. Concentration-dependent ANF stimulation of PDE activity in UMR-106 cells. The cells were incubated for 15 min at 37° with the indicated concentrations of ANF and then harvested, homogenized, and assayed for cGMP–PDE and cAMP–PDE activity. The given data represent means ± SEM of triplicate determinations. The stimulation was dose-responsive for cGMP–PDE ($P < 0.001$, ANOVA), but not for cAMP–PDE ($P = 0.24$, ANOVA). **$P = 0.002$, ***$P = 0.001$ compared to control (Student's *t*-test).

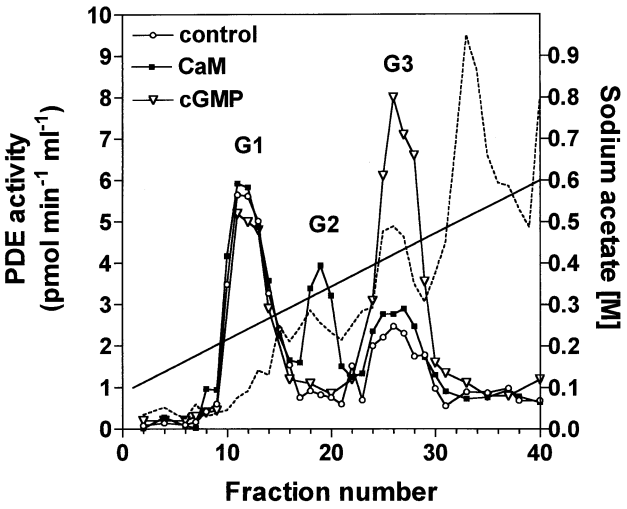

FIG. 7. Effect of calmodulin and cGMP on the Q-Sepharose elution profile of cGMP–PDE activity present in UMR-106 cells. Extracts of UMR-106 cells prepared as described in the Methods section were applied to a 5-mL column of Q-Sepharose and eluted by a 0.1–1.0 M linear sodium acetate gradient (straight line). Fractions were collected and assayed for cGMP–PDE activity in the presence and absence of 20 U/tube calmodulin/10 μM $CaCl_2$ and in the presence and absence of 4 μM cGMP. cAMP–PDE activity was also assayed (broken line).

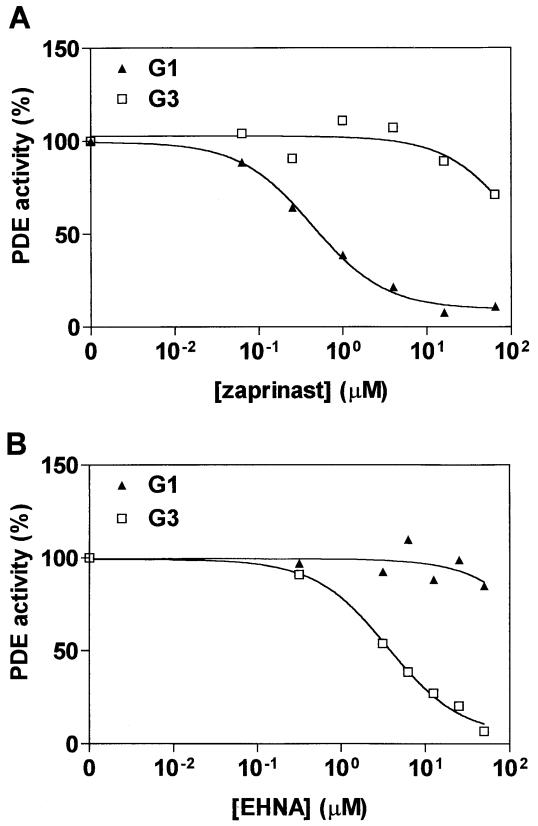

FIG. 8. Inhibition of the Q-Sepharose-separated cGMP–PDE peaks G1 and G3 by the PDE5 isoenzyme inhibitor zaprinast (A) and by the PDE2 isoenzyme inhibitor EHNA (B). Pooled peak fractions were assayed for cGMP–PDE activity with indicated concentrations of zaprinast and EHNA in the presence of 4 μM cGMP. The given results represent the means of two determinations.

peaks (fractions 41 to 70 not shown). The cAMP hydrolytic activity of the fractions was also assayed. Of the three cGMP-hydrolyzing peaks, only peak G1 did not show any cAMP hydrolytic activity (Fig. 7). Following the identification of EHNA- and zaprinast-sensitive PDE activity in the chromatograms, we wanted to test the effect of these inhibitors on ANF-stimulated intracellular cGMP accumulation in the cells. Both of these compounds significantly potentiated ANF-stimulated cGMP accumulation, and combined use of 30 μM EHNA and 10 μM zaprinast had a similar effect on intracellular cGMP as 500 μM IBMX (Fig. 9).

DISCUSSION

A number of reports suggest an important role of cGMP in bone cell metabolism. Fletcher *et al.* [2] identified high-affinity receptors for ANF and reported a several hundred-fold stimulation of intracellular cGMP levels by ANF in both normal osteoblasts from rat calvaria and in rat UMR 106-01 osteoblast-like cells. Sodium nitroprusside, a nitric oxide donor that elevates intracellular cGMP, increased alkaline phosphatase and osteocalcin gene expression in mouse calvarial osteoblasts [5] and decreased PTH- and calcitriol-induced bone resorption in rat calvarial bone cells [7]. In MC3T3-E1 clonal osteoblasts, CNP, also acting via cGMP, increased mRNA steady-state levels of alkaline phosphatase, osteocalcin, and type I collagen, which are all well-known markers of osteoblastic differentiation [3]. Suda *et al.* [21] found that both ANF and CNP dose dependently stimulate cGMP and CNP dose dependently decreases DNA synthesis in the same cell line. A recent study reported CNP-stimulated bone growth of fetal mouse tibias in organ culture [22]. The effect of CNP was mimicked by a cGMP analogue and inhibited by a natriuretic receptor antagonist. Although ANF seems to have no major direct effect on bone resorption of fetal rat bone organ cultures, it seems to act as a modulator of prostaglandin E_2-mediated bone resorption [6].

Animal cells have two mechanisms by which inactivation of intracellular cGMP can take place. cGMP can either be transported out of the cell or hydrolyzed to the inactive form 5′-GMP by PDEs. The efflux of cyclic nucleotides from animal cells has previously been shown to be an energy-dependent [18] and unidirectional movement, sensitive to prostaglandin A_1 and to probenecid [17–20]. Our present results clearly show that the cGMP efflux rate in UMR-106 cells is dependent on the ANF-stimulated intracellular concentration of cGMP. Additionally, the efflux mechanism of cGMP is sensitive to probenecid, ruling out the possibility of the efflux's being a simple diffusional process. Within the first 20 min of ANF stimulation, the PDEs seem to have a greater role than the cGMP efflux in intracellular cGMP. During this time of ANF stimulation, the phosphodiesterase inhibitor IBMX causes a large increase in total accumulated cGMP. After 20 min of ANF stimulation, the rate of total cGMP accumulation is the same both in the presence and absence of IBMX,

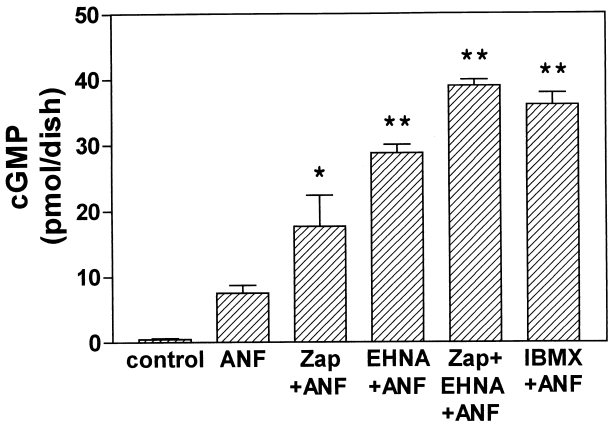

FIG. 9. Effect of zaprinast (Zap, 10 μM), EHNA (30 μM), and IBMX (500 μM) on ANF-stimulated cGMP accumulation in UMR-106 cells. The intracellular cGMP concentration was assayed by radioimmunoassay. The given results represent the means ± SEM of triplicate determinations. *$P < 0.05$, **$P < 0.01$ compared to ANF stimulation without PDE inhibitors (Students *t*-test).

indicating a more important role of the outward cGMP movement in the later phase (20–60 min) of the stimulation. Nevertheless, PDE activity seems to be responsible for removal of at least half of the total cGMP accumulated during the first 60 min of ANF stimulation.

We described earlier a cAMP/protein kinase A-mediated rapid stimulation of cAMP–PDE activity by PTH in homogenates from UMR-106 cells [23]. In this study, over 90% of the total cAMP hydrolytic activity was shown to be inhibited by low concentrations of the PDE4-selective inhibitor RO 20-1724. In a more recent paper, where we studied the role of PDE and cAMP efflux in PTH-stimulated cAMP accumulation, we could confirm PDE4 as the main cAMP-hydrolyzing PDE family [24]. In the same study, we found that PDE1, PDE2, and a currently unidentified cAMP-specific PDE also hydrolyze cAMP in UMR-106 cells.

Of the three cGMP–PDE peaks separated on Q-Sepharose, the first to elute has properties characteristic of the PDE5 (cGMP-specific) isoenzyme; it is sensitive to low concentrations of zaprinast and does not hydrolyze cAMP. The second eluted peak has characteristics typical of the PDE1 isoenzyme. It is undetected in the absence of calcium/calmodulin and, in contrast to the first peak, hydrolyzes both cAMP and cGMP. The presence of PDE1 activity is in line with our earlier study [24], where we found a calcium/calmodulin-stimulated cAMP hydrolytic peak that eluted at the same sodium acetate concentration as peak G2 in the present study. The third peak isolated is sensitive to low concentrations of EHNA and highly inducible by low concentrations of cGMP, properties characteristic of the PDE2 (cGMP-stimulated) isoenzyme. This peak hydrolyzes both cGMP and cAMP, and corresponds to the cAMP-hydrolyzing PDE2 activity that we identified earlier [24].

An elevation in intracellular cGMP should in theory increase the activity of PDE2 in the cells. We could, however, not detect an effect of ANF on cAMP hydrolysis of UMR-106 homogenates. A similar lack of effect of cGMP on cAMP hydrolysis in homogenates from osteoblast-like MC3T3-E1 cells has been reported [25]. If the cAMP-specific PDE isoforms are as dominant in MC3T3-E1 as in UMR-106 cells [23, 24], these results are not surprising. A cGMP-mediated activation of PDE2, that constitutes only a small part of the total cAMP–PDE activity would easily be masked by the other cAMP-hydrolyzing PDE isoenzymes. In contrast to its role in cAMP–PDE activity, PDE2 seems to be one of the main cGMP-hydrolyzing PDEs. This explains why an increase in the activity of cGMP–PDE, but not cAMP–PDE, can be seen in the cells following stimulation by ANF.

There are two proposed ways in which cGMP can activate PDE: by direct binding of cGMP to regulatory parts of the PDE and by protein kinase G-mediated phosphorylation of the PDE [1, 9]. In the present study, the cGMP analogue 8-bromo-cGMP, which does not stimulate PDE but is a potent activator of protein kinase G [26, 27], was without effect on the cGMP–PDE activity. This lack of effect suggests that the presently reported ANF stimulation of cGMP–PDE activity is mediated by direct binding of cGMP to PDE2, rather than by protein kinase G-mediated phosphorylation. The simultaneous increase in intracellular cGMP and cGMP–PDE activation also suggests that this is the case.

In conclusion, we have demonstrated that cGMP is extruded from UMR-106 cells by a probenecid-sensitive mechanism that in part could be responsible for the inactivation of ANF-stimulated intracellular cGMP. However, cGMP–PDE activity seems to be responsible for the inactivation of at least half of the generated cGMP. ANF stimulates cGMP–PDE activity in the cells, most likely by increasing intracellular cGMP and independently of protein kinase G. In addition, three peaks hydrolyzing cGMP have been separated and identified as PDE5, PDE1, and PDE2.

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