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Inorganic Phosphate Inhibits Growth of Human Osteosarcoma U2OS Cells Via Adenylate Cyclase/cAMP Pathway

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Abstract In order to elucidate how phosphate regulates cellular functions, we investigated the effects of inorganic phosphate (Pi) on adenylate cyclase (AC)/cyclic AMP (cAMP) axis. Here we describe that Pi treatment of human osteosarcoma U2OS cells results in a decrease of both intracellular cAMP levels and AC activity, and in a cell growth inhibition. The phosphate-triggered effects observed in U2OS cells are not a widespread phenomenon regarding all cell lines, since other cell lines screened respond differently to parallel Pi treatments. In U2OS cell line, the AC activity/cAMP downregulation is accompanied by significant variations in the levels of some membrane proteins belonging to the AC system. Remarkably, the above effects are blunted by pharmacological inhibition of sodium-dependent phosphate transport. Moreover, 8-Br-cAMP and other cAMP-elevating agents, such as IBMX and forskolin, interestingly, prevent the cell growth inhibition in response to phosphate. Our results enforce the increasing evidences of phosphate as a signaling molecule, identifying in U2OS cell line the AC/cAMP axis, as a novel-signaling pathway modulated by phosphate to ultimately affect cell growth. J. Cell. Biochem. 98: 1584–1596, 2006.

Key words: phosphate; adenylate cyclase; cAMP; growth inhibition; osteosarcoma cells

Inorganic phosphate (Pi) is essential for all living cells. Central role by phosphate in bone mineralization where its local availability is a prerequisite for normal hydroxyapatite formation is well known [Bellows et al., 1992]. Increasing evidences indicate that Pi is also required in events other than mineralization, proposing phosphate itself as a global signaling molecule capable of modulating multiple cellular functions [Conrads et al., 2005]. Increased phosphate has been linked to the nuclear export of the osteoblast transcription factor Runx2/Cbfa1 [Fujita et al., 2001] in MC3T3-E1 cells. Moreover, phosphate has been shown to upregulate the phosphate transporter Glvr-2 in rat bone marrow stromal cells [Wada et al., 2004]. Additionally, selective activation of the extracellular signal-regulated kinase (ERK1/2) signaling pathway is required for the phosphate-induced stimulation of osteopontin expression in MC3T3-E1 cells [Beck and Knecht, 2003]. Finally, a microarray study has identified a number of genes regulated by Pi treatment in MC3T3 cells [Beck et al., 2003]. The products of the gene downregulated by phosphate represent almost exclusively extracellular matrix proteins; on the other hand, the genes upregulated by phosphate are much more varied ranging from transcriptional regulators to signal transduction molecules. Moreover, the ability of phosphate to alter gene expression is not restricted to bone forming cells. Regulation of osteopontin expression by phosphate has been demonstrated in NIH3T3 fibroblasts, too [Beck et al., 2000]. Phosphate has also been linked to the regulation of Cbfa-1 and osteocalcin in human smooth muscle cells [Jono et al.,

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2000], to the regulation of phosphate transporter-II in the murine kidney [Kido et al., 1999], to PTH induction and secretion in parathyroid tissue in vivo and in vitro [Silver et al., 2002]. As far as phosphate-mediated biological phenotypes concerned, the critical role by phosphate on osteoblast differentiation is undoubted [Beck, 2003; Conrads et al., 2005]. Also relevant is the phosphate role on chondrocyte maturation [Wuthier, 1993]. Additionally, phosphate, in a calcium dependent manner, has been shown to cause apoptosis in both primary human osteoblasts and MC3T3-E1 cells [Meleti et al., 2000; Adams et al., 2001]. Phosphate-induced apoptosis has also been described to occur in chondrocytes [Mansfield et al., 2001], whereas a proliferative effect by phosphate has been depicted in osteoblasts through an insulin-like growth factor-1 dependent mechanism [Kanatani et al., 2002]. A role by phosphate on cell growth control is also suggested by a study showing that in normal 3T3 fibroblasts, phosphate intracellular levels are cell growth regulated with significant changes depending on cell density, whereas in polyoma virus transformed counterpart ones, are not [Gray et al., 1976]. It is commonly believed that phosphate must enter the cell to affect cell functions. The main mechanism for Pi entry into the cell is via a family of Na-dependent phosphate transporters, subdivided into three groups, based in part on tissue specificity [Caverzasio et al., 1996; Takeda et al., 2000]. Osteoblasts and chondrocytes differently express mainly two isoforms (Pit-1 and Pit-2) of the type III transporters [Kavanaugh and Kabat, 1996]. Expression of a type III phosphate transporter has also been detected in human osteosarcoma cell lines [Palmer et al., 1997]. Phosphate entry into the cell is a regulated event. In fact, a number of agents, including parathyroid hormone, insulin like growth factor-1, platelet derived growth factor, epinephrine, fluoride, calcium, basic fibroblast growth factor, Pi, that promote phosphate entry into the cell via these transporters, have been identified [Caverzasio et al., 1996; Suzuki et al., 2001; Wada et al., 2004; Zoidis et al., 2004]. Moreover, overexpression of a type II phosphate transporter has been found by serial analysis of gene expression as one of novel human ovarian cancer-specific transcripts [Rangel et al., 2003].

Adenylate cyclase (AC) is the plasma membrane-bound enzyme that converts ATP to the second messenger cyclic AMP (cAMP). cAMP signaling is involved in the regulation of multiple cellular functions ranging from short-term effects, like metabolic processes or contractile function of the heart [Soeder et al., 1999], to long-term effects, like control of cell proliferation, differentiation, and apoptosis [Dumont et al., 1989; Dhanasekaran and Prasad, 1998; Smit et al., 1998]. Demonstration that the main control of cellular cAMP concentration lies at the level of its synthesis has focused attention on the comprehension of the molecular mechanisms regulating AC activity [Hanoune et al., 1997].

So far, ten mammalian isoforms of the AC enzyme have been cloned [Hanoune et al., 1997]. Activity of the different AC isoforms is controlled mainly by association with subunits of heterotrimeric GTP-binding proteins (G proteins) that mediate the effects of numerous hormones, neurotransmitters or sensory stimuli by coupling transmembrane receptors to AC enzyme itself [Dhanasekaran and Gutkind, 2001].

A recent study in MC3T3 cells, by a combined proteome and microarray investigation, has identified a great number of proteins, including the AC, isoform VI, whose abundance is regulated by Pi treatment [Conrads et al., 2005].

We knew by our previous recent work [Naviglio et al., 2004] that human osteosarcoma U2OS cells exhibit an easily detectable AC activity with functional GPCRs/G-proteins/AC pathways, making them a useful experimental model to study AC complex. Otherwise, U2OS cells have been recently used to explore an inverse relationship between malignancy of human osteosarcoma and expression and activity of liver/bone/kidney (L/B/K) alkaline phosphatase (ALP) tissue non-specific enzyme, a member of a large family of cell surface glycoproteins that catalyze the hydrolysis of phosphomonoesters with release in extracellular environment of Pi [Zucchini et al., 2004]. Although numerous functions have been proposed for ALP enzymes [Whyte, 1994], increasing locally the Pi levels is very likely the most important one [Beck, 2003].

With also this in mind, in order to elucidate how phosphate regulates cellular functions, we analyzed in human osteosarcoma U2OS cell line the effects of intracellular phosphate increase on AC/cAMP pathway and on cell growth.

Here we describe that in U2OS cells, treatment with Pi results in both a reduced cAMP

biosynthetic activity by AC enzyme with a parallel decrease of intracellular cAMP content, and in a cell growth inhibition, whereas other cell lines screened behave differently in response to Pi. We also show that the AC activity/cAMP content reduction is consistently accompanied by relevant changes in the levels of some membrane proteins belonging to the AC system. Importantly, the above effects are prevented by pharmacological inhibition of Na-dependent phosphate transport. Remarkably, we also provide evidence that the phosphate-induced AC/cAMP downregulation mediates the cell growth inhibition in response to phosphate.

MATERIALS AND METHODS

Materials

[α-^{32}P] ATP (800 Ci/mmol) was obtained from New England Nuclear (NEN). All cell culture materials were from Gibco-Life Technologies. Sodium phosphate mono and dibasic, foscarnet (phosphonoformic acid or PFA), sodium sulfate, 8-Br-cAMP, forskolin, isoproterenol, epinephrine, and GTP were purchased from Sigma. Antibodies anti-cyclin A, anti-AC catalytic subunits (pan-AC), anti-Gα_s, anti-G$\alpha_{i\text{-}1}$, anti-β were obtained from Santa Cruz Biotechnology. Anti-tubulin antibodies were obtained from Oncogene.

Cell Culture

All cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere. Human osteosarcoma U2OS cells, human osteosarcoma Saos cells, mouse NIH 3T3 fibroblasts, rat cardiomyoblast H9c2 cells, human epidermoid cancer KB cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Murine calvaria-derived MC3T3-E1 osteoblast-like cells were cultured in α-MEM containing 10% FBS supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. All cell lines were obtained from the American Type Culture Collection (ATCC). Pi was used in the form of $NaPO_4$, pH 7.4 [Kanatani et al., 2002; Beck and Knecht, 2003]. Typically, cells, cultured in 10% serum-containing medium, were split (5×10^5/10 cm plate, for biochemical experiments; 5×10^4/well in 6-well plates, for biological experiments). After 24 h, the cells were washed with phosphate-buffered saline (PBS) and incubated with fresh medium, enriched or not (control, [Pi]-1 mM) of Pi (time 0). Then, cells were grown for the times indicated in Results, with medium changes every 24 h, again enriched or not of Pi.

Preparation of Cell Lysate or Membranes

Cell extracts were prepared from monolayer cultured cells by discarding the medium, washing the cells twice with cold PBS pH 7.3, scraping, collecting, and centrifuging cells at 1,200 rpm for 10 min, discarding the supernatant, resuspending the cell pellets in 0.4–1 ml of lysis Buffer (50 mM Tris-HCl, pH 7.5, 0.33 M sucrose, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml pepstatin A, 1 mM PMSF) and disrupting the cells in a tight-fitting glass Dounce with 30 strokes. The obtained total homogenate was recovered; some aliquots were taken for protein quantification, some others were diluted in 4× Laemmli buffer, boiled and stored as samples for Western blotting analysis, while the larger amount was used for membrane preparation. After centrifuging the total homogenate at 2,000 rpm in an Eppendorf microcentrifuge for 10 min at 4°C, the supernatant was further centrifuged at 14,000 rpm for 20 min at 4°C with the same micro centrifuge and the obtained pellets (membranes) were resuspended in 50 mM Tris-HCl, pH 7.5, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml pepstatin A, 1 mM PMSF. Aliquots of these membrane preparations were taken for protein concentration, while the larger amount was used as enzymatic source for testing AC activity (see below).

Protein concentration was determined according to Bradford [Bradford, 1976].

Adenylate Cyclase Assay and cAMP Measurement

The AC assay was performed according to a modification of a published radiometric procedure [Salomon et al., 1974]. Membrane preparations (as above) were used as enzymatic source and incubated for 10 min at 30°C in a medium (final volume 100 μl) including 0.5 mM ATP, [α-^{32}P]ATP (1.5×10^6 cpm), 24 mM $MgCl_2$, 0.05 mg/ml CPK, 0.01 M creatine phosphate, 1 mg/ml aminophylline, 1 mg/ml BSA, 22.5 mM Tris-HCl, pH 7.4. Reactions were initiated by adding the membranes (~50 μg) to the incubation medium and stopped by adding 100 μl of ice-cold solution containing 17.5 mM Tris-HCl, pH

7.5, 0.04 M ATP, 0.0125 M cAMP and [^{3}H]cAMP (20,000 cpm), the latter being used to assess the efficiency of isolation procedure. The cAMP was isolated by sequential chromatography on a Dowex cation-exchange resin and aluminum oxide. Each enzymatic determination was performed in triplicate. Measurement of cAMP was performed as described previously [Pagano et al., 2004].

Immunodetection of Proteins

Proteins from cell preparations were separated by SDS–PAGE and transferred onto nitrocellulose sheets (Schleicher & Schuell) by a Mini Trans-Blot apparatus (BioRad). II goat anti-rabbit or anti-mouse antibodies, conjugated with horseradish peroxidase (BioRad), were used as a detection system (ECL) according to the manufacturer's instructions (Amersham-Pharmacia).

Immunofluorescence and DNA Synthesis Analysis

For immunofluorescence analysis, cells were seeded onto glass coverslips and cultured as described in *Cell culture* section. The DNA synthesis was assayed by 2 h-pulse with 100 μM BrdU (Boehringer Mannheim). After in vivo labeling, cells on coverslips were fixed and permeabilized as reported [Castoria et al., 2001]. The BrdU incorporation into newly synthesized DNA was assessed according to the same report using diluted (1:10 in PBS) mouse anti-BrdU monoclonal antibody (clone BMC9318 from Boehringer). Nuclei were stained with Hoechst 33258 (Sigma) at a final concentration of 1 μg/ml. After extensive washings in PBS, the coverslips were inverted and finally mounted in Mowiol (Calbiochem). Slides were analyzed by fluorescence microscopy (Leica DM LB) and images processed using IM1000 software (Leica).

Analysis of Apoptosis

The frequency of hypoploid cells (sub-G1 cells) was assessed by studying the cell cycle after fixation overnight at 4°C with 70% ice-cold methanol/PBS followed by staining with a solution containing PI (50 μg/ml) and RNase A (0.5 mg/ml), and analyzed with a Becton Dickinson FACScan cytofluorometer.

Statistical Analysis

The data represent the means ± SD (standard deviation) obtained from three independent experiments. Each experimental point was performed in triplicate. The means were compared using analysis of variance (ANOVA) plus Bonferroni's *t*-test. The *P* values less than 0.05 was considered significant.

RESULTS

Treatment With Inorganic Phosphate of U2OS Cells Results in a Time-Dependent and a Dose-Dependent Downregulation of Adenylate Cyclase Activity

In Figure 1 an AC activity assay is depicted. U2OS cells were treated or not with 10 mM Pi containing medium during a time course (from 6 to 48 h). The Pi concentration, 10 mM, we chose to treat cells, was the same used in a recent microarray study that has identified a discrete set of genes up- and downregulated by treating MC3T3-E1 osteoblasts with 10 mM Pi for 72 h [Beck et al., 2003]; moreover, in MC3T3-E1 cells has also been shown that 10 mM Pi treatment, during a time course from 15 min up to 32 h, activates biphasically the extracellular signal regulated kinase (ERK1/2) signaling pathway [Beck and Knecht, 2003]. Otherwise, our preliminary observations indicated that treatment with 10 mM Pi was accompanied in U2OS neither by obvious cellular toxic effects nor by relevant increased cell death.

Figure 1 indicates that AC activity appears decreased in 12, 24, 48 h Pi treated cells versus control ones; particularly, 12 h treated cells exhibit a dramatically lower (almost of 50%; $P < 0.001$) AC activity, while no obvious changes

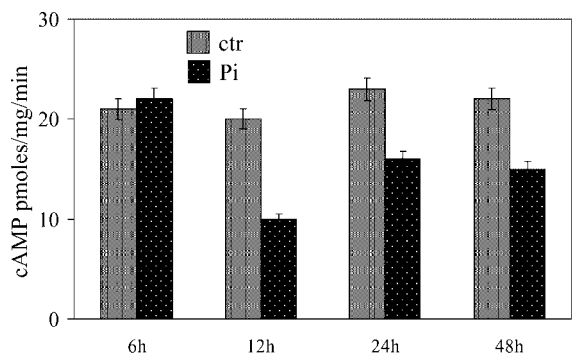

Fig. 1. Effect of phosphate on AC activity. Time course experiment. U2OS cells were treated or not with 10 mM Na-phosphate (Pi) for 6, 12, 24, and 48 h. Fifty micrograms of membrane preparations were used for AC activity assay. Values are means ± SD of triplicate samples of three experiments. *P* values of 12, 24, and 48 h treated versus control groups were: <0.001, <0.05, <0.001, respectively.

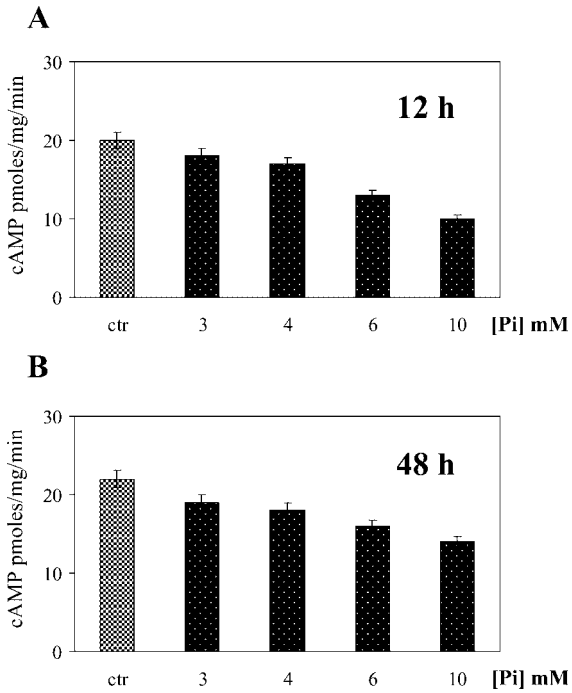

Fig. 2. Effect of phosphate on AC activity. Dose-response experiment. U2OS cells were treated or not with increasing concentrations (3–10 mM) of Na-phosphate (Pi) for 12 and 48 h. Fifty micrograms of membrane preparations were used for AC activity assay. Values are means ± SD of triplicate samples of three experiments. *P* values of 3, 4, 6, and 10 mM Pi treated versus control groups were: <0.05, <0.01, <0.001, <0.001, respectively, for both times.

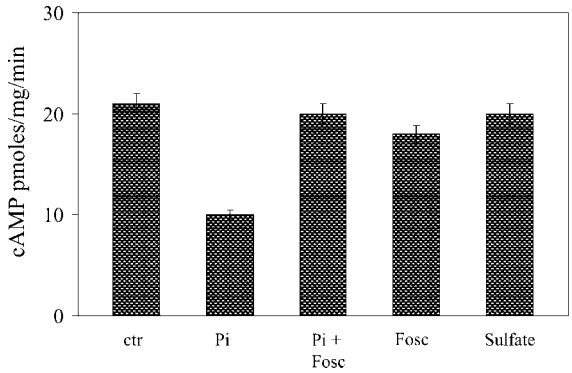

Fig. 3. Effect of sulfate on AC activity and of foscarnet on phosphate-induced AC activity decrease. U2OS cells were treated or not for 12 h with either 10 mM Na-phosphate (Pi), or both 10 mM Na-phosphate and 300 μM foscarnet (Pi + fosc), or 300 μM foscarnet (fosc) alone, or 10 mM Na-sulfate (*sulfate*). Fifty micrograms of membrane preparations were used for AC activity assay. Values are means ± SD of triplicate samples of three experiments. *P* values of Pi and fosc treated versus control groups were: <0.001, <0.05, respectively.

could be seen in 6 h Pi treated versus untreated ones.

Since 10 mM Pi concentration is almost tenfold higher than that found in plasma, we decided to evaluate whether less elevated Pi concentrations, more similar to those around the extracellular environment, were also effective to affect AC activity. To do this, we performed dose-response experiments. In Figure 2, it is shown that the exposure of cells to increasing Pi concentrations (3–10 mM) caused a dose-dependent AC activity inhibition with minimal effective concentration at 3 mM ($P < 0.05$), both in cells exposed to Pi for 12 and 48 h.

Adenylate Cyclase Activity Downregulation Occurring in U2OS Cells Upon Inorganic Phosphate Treatment is Depending on Phosphate Entry and is Not a Widespread Phenomenon Regarding All Cells

To investigate whether the effects of Pi treatment on AC activity were due to phosphate "as molecule" rather than to any less specific change in ionic concentration in the environment of the cells, we looked also at the impact on AC activity of treatment with equimolar concentrations of sulfate salt [Beck and Knecht, 2003]. No obvious consequences on AC activity were seen in 10 mM sulfate treated versus control untreated U2OS cells (Fig. 3).

Since previous findings established that phosphate must enter the cell to affect cellular functions [Beck, 2003], in order to determine whether the AC activity decrease observed upon Pi treatment was depending on phosphate intracellular entry, we examined the effects of phosphate on AC activity in the presence of foscarnet (phosphonoformic acid or PFA), a largely used phosphate transport inhibitor [Loghman-Adham, 1996; Beck et al., 2000; Mansfield et al., 2001], added to 10 mM Pi containing culturing medium. Figure 3 also shows that foscarnet treatment prevents almost completely the effects of elevated levels of Pi on AC activity. To note, foscarnet alone treated cells also show a little decreased AC activity, very likely, because of a described direct effect of foscarnet on AC catalytic subunit itself [Kudlacek et al., 2001].

Moreover, to extend the relationship between Pi treatment and AC activity, AC assays from U2OS and many other cell lines were performed. In Figure 4, it is shown that parallel Pi treatments of various cell lines produce

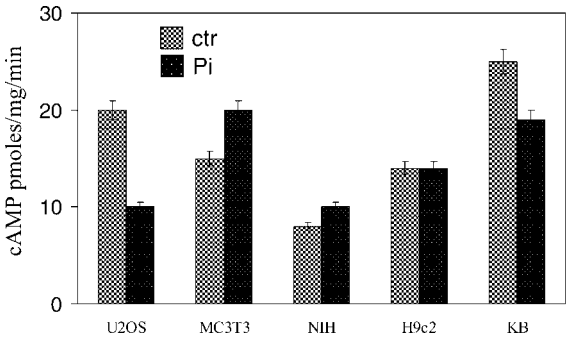

Fig. 4. Effect of phosphate on AC activity from various cell lines. U2OS, MC3T3-E1, NIH3T3, H9c2, KB cell lines were treated or not for 12 h with 10 mM Na-phosphate (Pi). Fifty micrograms of membrane preparations were used for AC activity assay. Values are means ± SD of triplicate samples of three experiments. *P* values of treated versus control U2OS, MC3T3-E1, NIH3T3, KB cells were: <0.001, <0.001, <0.01, <0.001, respectively.

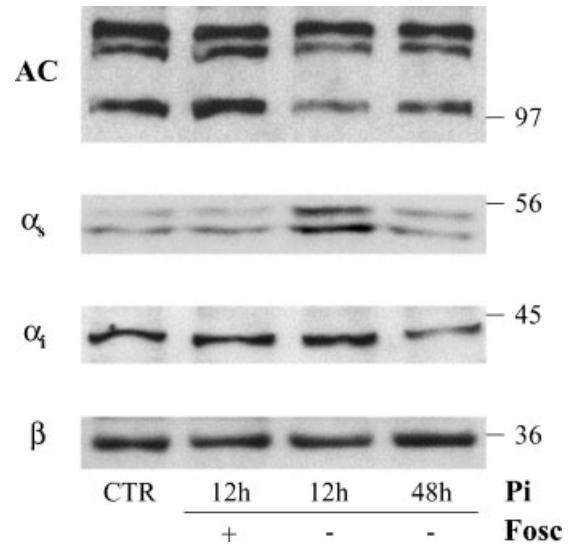

Fig. 5. Effects of phosphate on levels of proteins related to AC complex. U2OS cells were treated or not (*ctr*) with 10 mM Na-phosphate (Pi) for 12 and 48 h, or with both 10 mM Na-phosphate (Pi) and 300 μM foscarnet (fosc) for 12 h. Thirty micrograms of membrane preparations were subjected to SDS–PAGE and blotted with antibodies against the indicated proteins. The image is representative of three-immunoblotting analysis from three different membrane preparations with similar results.

different effects on AC activity depending on different cell sources screened.

Overall, the above data indicate that: (1) Phosphate intracellular entry is an event necessary for phosphate-induced AC activity decrease in U2OS. (2) Pi treatment affects AC activity, very likely, by triggering signaling to AC depending on time at and on cell types in which treatment occurs.

Adenylate Cyclase Activity Downregulation Occurring in U2OS Cells Upon Inorganic Phosphate Treatment Reflects Alteration of Function of Both AC Catalytic Subunit Itself and G-Proteins, and is Accompanied by a Decrease of Intracellular cAMP Levels

To investigate the molecular basis of AC activity variations in Pi treated cells, we examined whether the AC activity downregulation was paralleled by a modified concentration of protein components of the AC systems. To do this, we performed immunological detection by Western-blotting analysis of some proteins related to AC complex (Fig. 5). In U2OS membrane preparations (the same used for AC activity testing), we found that Pi treatment caused a relevant decrease in AC catalytic subunit(s) signal, revealed using a pan-AC antibody (i.e., an antibody broadly reactive with several membrane-bound isoforms of AC family), more evident at 12 h point, and pronounced differences in signals of both stimulatory, $G\alpha_s$, and inhibitory, $G\alpha_{i-1}$, subunits of heterotrimeric G proteins. These differences basically resulted in an increased ratio between $G\alpha_s/G\alpha_{i-1}$ protein levels. No significant variations in the levels of β subunit could be detected. Interestingly, as for the AC activity case, such Pi-induced protein levels alterations were prevented by the treatment with the phosphate transport inhibitor, foscarnet.

To prove the correlation between the Pi-induced AC system protein levels changes and AC activity, we treated cells with Pi and then tested AC activity in basal conditions and also in the presence of AC activity modulators added in vitro to the incubation mixture (Table I, panel A). The level of forskolin stimulated AC activity from Pi treated cells was much lower than that from untreated ones. Since forskolin maximally stimulates AC activity mainly interacting with and directly activating the catalytic component of AC system [Taussig and Gilman, 1995], the evidence of significant differences in forskolin-stimulated AC activity suggests that Pi treatment is very likely affecting, at least, AC catalytic subunit itself. Moreover, when AC activity was tested in the presence of GTP, it also reached lower levels, but with a higher degree of stimulation, in Pi treated cells than in control untreated ones. Since GTP (or its

TABLE I. Effects of Phosphate on Basal, GTP-Stimulated, Isoproterenol-Stimulated, Epinephrine-Stimulated, Forskolin-Stimulated AC Activities, and on Intracellular cAMP Content

	Levels of AC activity (cAMP pmoles/mg protein/min)			
	12 h		48 h	
	ctr	Pi	ctr	Pi
A				
Basal	20 ± 0.8	10 ± 0.3	22 ± 0.6	15 ± 0.3
GTP	29 ± 1.1	22 ± 0.6	30 ± 1.2	27 ± 0.9
Iso	44 ± 1.8	31 ± 1.1	42 ± 2.0	39 ± 1.3
Epi	37 ± 1.3	26 ± 0.9	37 ± 1.2	33 ± 1.2
Forskolin	700 ± 28	480 ± 15	680 ± 29	570 ± 18
B				
pmoles of intracellular cAMP level/dish	1.3 ± 0.04	0.7 ± 0.02	1.4 ± 0.06	0.9 ± 0.03

U2OS cells were treated or not with 10 mM Na-phosphate (Pi) for 12 and 48 h. A: Fifty micrograms of membrane preparations were used for AC activity assay. 10^{-5} M GTP, 10^{-7} M isoproterenol (*Iso*), 10^{-7} M epinephrine (*Epi*), 10^{-5} M Forskolin were used in vitro at the indicated final concentration. Values are means ± SD of triplicate samples of three experiments. B: cAMP intracellular levels have been extracted and measured as indicated in Materials and Methods. Values are means ± SD of triplicate samples of three experiments.

analogs) is normally used to modulate AC catalytic subunit via G protein activation [Neer, 1995], the higher degree of stimulation of basal AC activity by GTP in Pi treated cells suggests that the contribute of the "tonic" positive regulation exerted by G protein on AC enzyme is increased in Pi treated versus untreated cells.

We then investigated on receptor-mediated AC activity. In Table I, panel A, it is also shown that the AC activity is stimulated by the presence of isoproterenol both in Pi treated and untreated cells, with levels lower and a degree of stimulation higher in Pi treated versus untreated cells, indicating that β-AR/$G\alpha_s$/AC pathway is functional not only in control U2OS cells, but also in Pi treated ones. The higher degree of stimulation of basal AC activity by isoproterenol in Pi treated cells suggests that the contribution of the "tonic" positive regulation exerted by β-AR/$G\alpha_s$ to AC enzyme is increased in Pi treated versus untreated cells. We also investigated on epinephrine-modulated AC activity. Epinephrine is known to affect AC activity potentially via both α-AR/$G\alpha_i$ and β-AR/$G\alpha_s$ pathways depending on its concentration and on cell type involved [Brodde and Michel, 1999; Heubach et al., 2004]. However, in human osteosarcoma cells epinephrine and norepinephrine have been described to increase intracellular levels of cAMP [Bjurholm et al., 1992; Moore et al., 1993]. Accordingly, what we found was that the AC activity is stimulated by the presence of epinephrine both in control and in Pi treated U2OS cells, with levels lower and a degree of stimulation higher in Pi treated versus untreated cells (Table I, panel A). The higher degree of stimulation of basal AC activity by epinephrine in Pi treated cells suggests that the ratio between the "tonic" positive and negative regulation exerted by epinephrine via β-AR/$G\alpha_s$ and/or α-AR/$G\alpha_i$ to AC enzyme is increased in Pi treated versus untreated cells. Finally, we extracted and measured the intracellular cAMP in control and in Pi treated cells (Table I, panel B). Pi treatment results in a large decrease of cAMP content.

Synthetically, results enclosed in Table I indicate that AC activity is basically decreased, but more strongly stimulated by G-proteins, in Pi treated compared to control untreated U2OS cells. The above AC activity variations are in good agreement with the modifications of protein levels of AC catalytic, $G\alpha_s$ $G\alpha_i$ subunits depicted in Figure 5. Altogether, the immunological and biochemical data indicate that in U2OS cells, the increase of intracellular phosphate concentration affects the function of both AC catalytic subunit itself and of heterotrimeric G-proteins and underline that AC system is clearly amenable to be modulated by phosphate, and, remarkably, that the phosphate-induced AC activity decrease is accompanied by a parallel reduction of intracellular cAMP levels.

Inorganic Phosphate Treatment Negatively Affects Growth of U2OS Cells

On the basis of the obtained AC/cAMP results, we decided to evaluate the biological consequences of Pi treatment on U2OS cells, speculating on the idea that phosphate-induced AC/cAMP downregulation could affect cell growth. To note, although U2OS are transformed cells, lacking of G1 checkpoint function, they are currently used to test the effects of acute alteration of critical cellular components on cell growth [Naviglio et al., 1998; Krutzfeldt et al., 2005; Novy et al., 2005]. To test the effects of phosphate on cell growth, we treated cells with either 3 mM or 10 mM Pi containing medium for 48 and 72 h; cell proliferation and DNA synthesis were then monitored by cell counting and by BrdU incorporation, respectively. The expression of the cell cycle regulator, cyclin A protein, was also checked. Figure 6 provides evidence that Pi treatment has an inhibitory effect on the growth of U2OS cells. Panels A and B show that in Pi treated cells, BrdU incorporation was largely (almost of 50%) reduced compared to that observed in control untreated cells. In panel C, it is shown that from Pi treated plates, clearly less cells were recovered, although no relevant increase of apoptosis was detected, as assessed by the lack of the appearance of sub-G1 hypoploid cells by FACS analysis (data not shown). Moreover, it also appears in panel C that Pi treated cells were growing at a significant lower rate compared to that of control cells. For example, the percent increase between the two time points, 48–72 h, is +94% (180×10^3 vs. 350×10^3) for control cells, +53% (130×10^3 vs. 200×10^3) for 10 mM Pi treated cells. So, although still proliferating, Pi treated cells appear significantly growth inhibited.

Finally, in panel D, it is shown that in Pi treated cells, accordingly, levels of cyclin A

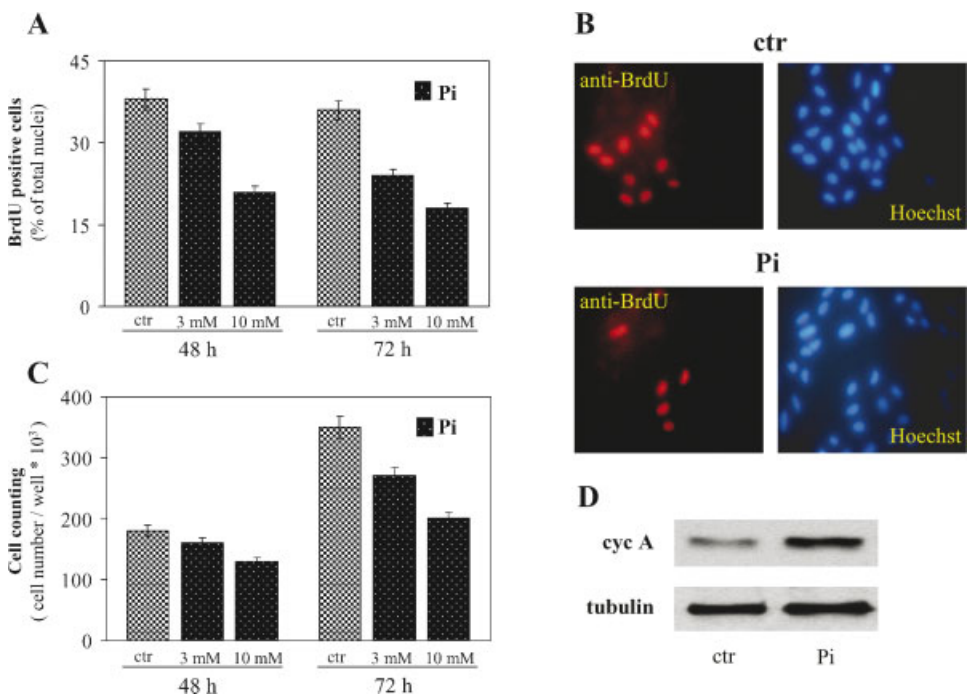

Fig. 6. Effect of phosphate on the growth of U2OS cells. **A**: U2OS cells on coverslips were treated or not either with 3 mM or 10 mM Na-phosphate (Pi) for 48 and 72 h. After in vivo labeling with 100 μM BrdU, the cells were fixed, permeabilized, and stained for BrdU incorporation. Several coverslips were analyzed. DNA synthesis was calculated by the formula: percentage of BrdU-positive cells = (number of BrdU-positive cells/number of total nuclei) × 100. Data represent the average of three independent experiments. The means and SEM are shown. **B**: Representative images of DNA synthesis in U2OS cells treated (*lower parts*) or not (*upper parts*) with 10 mM Na-phosphate (Pi) for 48 h. *Left parts* show the staining obtained with the anti-BrdU antibody. Nuclear staining is shown in the *right parts* (*Hoechst*). **C**: U2OS cells, seeded in 6-well plates (50×10^3/well), were treated or not either with 3 or 10 mM Na-phosphate (Pi) for 48 and 72 h. Cell proliferation was determined by cell counting. Data represent the average of three independent experiments. The means and SEM are shown. **D**: Thirty micrograms of total protein extracts from control and 48 h, 10 mM Pi treated cells were subjected to SDS–PAGE and blotted with antibodies against the indicated proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

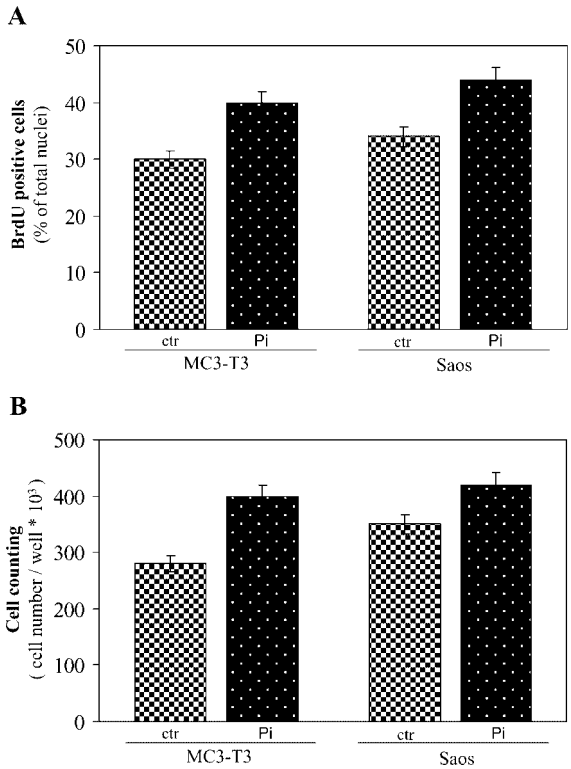

Fig. 7. Effect of phosphate on the growth of MC3-T3 and Saos cells. **A**: MC3-T3 and Saos cells on coverslips were treated or not with 10 mM Na-phosphate (Pi) for 72 h. After in vivo labeling with 100 μM BrdU, the cells were fixed, permeabilized, and stained for BrdU incorporation. Several coverslips were analyzed. DNA synthesis was calculated by the formula: percentage of BrdU-positive cells = (number of BrdU-positive cells/number of total nuclei) × 100. Data represent the average of two independent experiments. The means and SEM are shown. **B**: MC3-T3 and Saos cells, seeded in 6-well plates (50×10^3/well), were treated or not with 10 mM Na-phosphate (Pi) 72 h. Cell proliferation was determined by cell counting. Data represent the average of two independent experiments. The means and SEM are shown.

protein, the required cell-cycle regulator for the onset of DNA replication was decreased. We also evaluated, in parallel experiments, the effects of 10 mM Pi on BrdU incorporation and on proliferation of osteoblastic MC3T3-E1 cells and of human osteosarcoma Saos cells. We found that 10 mM Pi, stimulates growth of MC3T3-E1 cells, as described to occur with less elevated Pi concentrations [Kanatani et al., 2002], and, surprisingly, also of Saos cells (Fig. 7).

Altogether, these data indicate that Pi treatment negatively affects growth of U2OS cells and, remarkably, that growth responses to Pi are cell type dependent.

The Inhibitory Effect by Inorganic Phosphate Treatment on U2OS Cell Growth is Prevented by Inhibiting Phosphate Entry and is Counteracted by Increasing Intracellular cAMP Levels

Above, we have reported that pharmacological inhibition of phosphate transport prevents the AC activity decrease and the AC protein levels changes by Pi treatment (Figs. 3 and 5). To see whether also the growth inhibition of U2OS cells observed upon Pi treatment was depending on its intracellular entry, we looked at the effects by phosphate transport inhibitor, foscarnet, on phosphate-induced cell growth inhibition. Figure 8 shows that foscarnet treatment prevents, almost completely, the inhibitory effects by Pi treatment on cell proliferation (panel A) and on DNA synthesis (panel B). On the other hand, since cAMP signaling is largely known to mediate cell growth responses in many cell systems, we asked whether the phosphate-triggered AC/cAMP downregulation was involved in cell growth inhibition observed in U2OS cells in response to Pi treatment. To approach this question, we performed cotreatments with intracellular cAMP elevating agents in control untreated and Pi treated U2OS cells, to compare the cell growth effects in Pi treated cells, containing lowered cAMP levels, to those in cells treated both with cAMP elevating agents and Pi, in which cAMP levels were just up-maintained.

Figure 8, panel A, shows that 8-Br-cAMP cotreatment of Pi treated cells results in an increase of cell counts compared to Pi treated alone ones, suggesting that the upregulation of cAMP content contributes to restore the U2OS cell growth inhibited by Pi treatment. Remarkably, 8-Br-cAMP cotreatment of Pi treated cells also results in restoring BrdU incorporation, reaching levels similar even higher, to those of control untreated cells. Similar results were also obtained upon treatment with cAMP phosphodiesterases inhibitor, IBMX, and with forskolin (data not shown).

Altogether, the above data indicate that in U2OS cells: (1) Phosphate intracellular entry is an event necessary for phosphate-induced growth inhibition. (2) Increasing intracellular cAMP levels counteracts the phosphate-induced growth inhibition.

DISCUSSION

In this study, we report that in human osteosarcoma U2OS cell line, treatment with

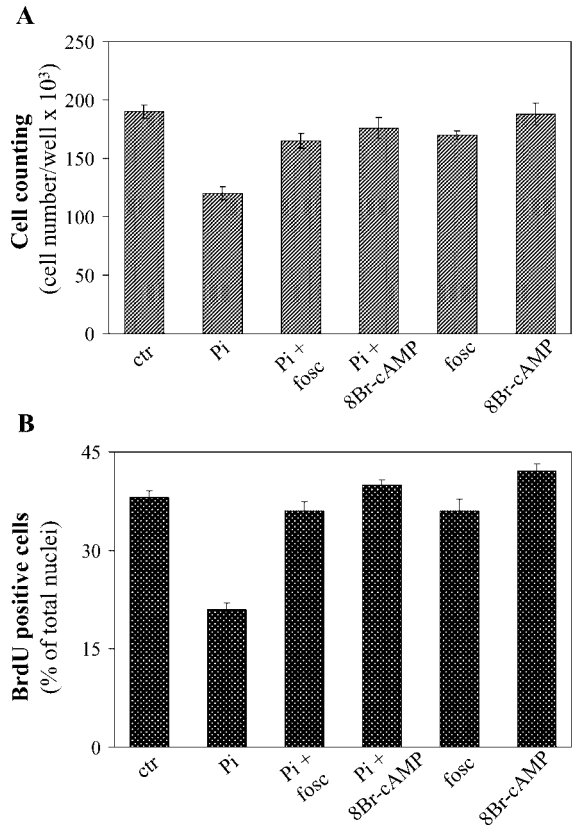

Fig. 8. Effects of 8-Br-cAMP and of foscarnet on the phosphate-induced growth inhibition of U2OS cells. **A**: U2OS cells, seeded in 6-well plates (50×10^3/well), were treated or not with 10 mM Na-phosphate (Pi), or 300 μM foscarnet (fosc), or 0.5 mM 8-Br-cAMP, or both 10 mM Na-phosphate and foscarnet (Pi + fosc), or both 10 mM Na-phosphate and 8-Br-cAMP (Pi + 8-Br-cAMP) for 48 h. Cell proliferation was determined by cell counting. Data represent the average of three independent experiments. The means and SEM are shown. **B**: U2OS cells on coverslips were treated or not with 10 mM Na-phosphate (Pi), or 300 μM foscarnet (fosc), or 0.5 mM 8-Br-cAMP, or both 10 mM Na-phosphate and foscarnet (Pi + fosc), or both 10 mM Na-phosphate and 8-Br-cAMP (Pi + 8-Br-cAMP) for 48 h. DNA synthesis by BrdU incorporation was determined as indicated in Figure 6. Data represent the average of three independent experiments. The means and SEM are shown.

Pi causes both a reduced cAMP biosynthetic activity by AC enzyme with a parallel decrease of intracellular cAMP content, and a cell growth inhibition. The above effects are blunted by pharmacological inhibition of Na-dependent phosphate transport. Remarkably, we also describe that the cell growth inhibition in response to phosphate is almost completely prevented by upregulating intracellular cAMP levels, either with 8-Br-cAMP or stimulating adenylate catalytic subunit with forskolin or inhibiting cAMP phosphodiesterases with IBMX, strongly suggesting that the phosphate-induced AC/cAMP downregulation mediates such growth inhibition.

A large number of phosphate-triggered molecular changes have been described in many cell types, so far; notwithstanding, the mechanisms by which an increase in intracellular phosphate levels affect gene transcription and protein function are not completely known and some understanding is just emerging. Studies using calcium chelators, and calcium channel blockers suggest that phosphate is neither acting by sequestering available calcium pools nor by producing an influx of calcium through traditional calcium channels, although intracellular calcium may be required [Beck, 2003].

To date, no other studies have demonstrated the consequences of increasing the intracellular concentrations of phosphate on AC/cAMP pathway. We have been able to detect in U2OS cells a phosphate-triggered decrease of AC activity, accompanied by significant variations in protein levels of some component of AC complex. Our biochemical data are consistent with AC activity/cAMP downregulation being mainly dependent on variations of such protein levels, although the way by which phosphate affects them has not been yet characterized and is actually under our investigation. It is important to note that the AC/cAMP levels changes occurring in U2OS cells in response to Pi are not an universal effect regarding all cell lines screened, suggesting that phosphate might differently be involved in modulation of cAMP depending pathways in different cell types. Whether systematically in osteoblastic cell types opposite effects on AC/cAMP levels could correlate with opposite effects on cell growth in response to Pi (i.e., cAMP decrease = antiproliferative, and viceversa) has not been fully investigated in our study, but it should be unlikely, also considering our observations on Saos cells (See below).

That cAMP, via either PKA dependent or PKA independent pathways, could mediate the phosphate-induced growth inhibition of U2OS cells is in agreement with the known action of cAMP on cell growth in bone cells [Fujita et al., 2002; Stork and Schmitt, 2002; Cheung et al., 2005].

Particularly, it has been shown that in MC3T3-E1 cells increased cAMP levels decrease the stability of cbfa1 protein, a transcription factor known to be a master regulator of osteoblastic differentiation [Tintut et al.,

1999]. Whether in U2OS cells the decreased cAMP levels in response to phosphate could result in increased cbfa1 protein level has not been approached in our study. However, we checked by immunoblotting analysis, the expression of osteopontin, a downstream cbfa1-regulated gene product, and at least after 72 h, 10 mM Pi treatment, time and dose described to be enough to upregulate osteopontin protein [Beck and Knecht, 2003], no obvious changes occurred (data not shown).

Although further investigation is needed to define the molecular mechanism(s) by which in phosphate treated U2OS cells that cAMP-mediated growth inhibitory effect occurs, our feeling is that the decreased proliferation is not a downstream effect related to changes of function of U2OS cells being driven toward differentiation, but an early event related to a direct effect of cAMP on the proliferation machinery with a mechanism, very likely, requiring p53 action, since in human osteosarcoma cell line Saos, lacking of p53 function, phosphate does cause a cAMP reduction (data not shown) but does not a decrease of cell growth.

Interestingly, our finding that phosphate has an antiproliferative effect on U2OS cells is opposite to that observed in other cellular models [Kanatani et al., 2002], and is according to a recent one [Zucchini et al., 2004] indicating an inverse relationship between malignancy of human osteosarcoma and expression and activity of liver/bone/kidney (L/B/K) ALP tissue non-specific enzyme, whose main action is to increase locally the Pi concentration in extracellular environment. Intriguingly, in U2OS cells, the antitumorigenic effect by both Pi treatment and ALP upregulation, strongly suggests that alteration of such phosphate-mediated pathway(s) could contribute to the development of some malignant bone diseases.

In conclusion, data here presented enforce the increasing evidences of phosphate as a signaling molecule, identifying in U2OS the AC/cAMP axis, as a novel signaling pathway modulated by phosphate to ultimately affect cell growth, and suggest that a pharmacological intervention targeting AC/cAMP axis might be useful in such bone proliferation diseases.

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