Protein kinase A-dependent inhibition of alkaline phosphatase release by SaOS-2 human osteoblastic cells: studies in new mutant cell lines that express a cyclic AMP-resistant phenotype

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We have established mutant SaOS-2 cell lines that express a cyclic AMP (cAMP)-resistant phenotype to investigate the regulation and functional importance of orthophosphoric-monoester phosphohydrolase alkaline optimum (ALPase) in the action of parathyroid hormone (PTH). Cells were stably transfected with a plasmid that directs the synthesis of a mutant form of the type I regulatory subunit of protein kinase A (PKA) under the control of the metallothionein promoter. There was no significant difference between parental SaOS-2 cells and the mutant lines in the affinity or number of receptors for ^{125}I -Nle 8,18 Tyr 34 bPTH $_{1-34}$ NH $_2$, either in the absence or presence of Zn $^{2+}$. When cAMP-dependent gene transcription was examined using transient transfection with a somatostatin promoter-chloramphenicol acetyl transferase (CAT) reporter plasmid, CAT activity stimulated by human PTH and dibutyl cAMP (DBcAMP) was inhibited by >90% in the presence of Zn $^{2+}$ in the mutant cell lines. In contrast, activation by a phorbol ester of a pentameric collagenase promoter/CAT construct containing five tandem copies of the AP-1 response element (5x-TRE-CAT) was unaffected in Zn $^{2+}$ -treated mutant cells. The inhibitory actions of PTH and DBcAMP on ALPase release were blunted by

up to 80–90% in the mutant cell lines in the presence of Zn $^{2+}$; there were no significant differences in the magnitude of inhibitory effects between these agonists. We conclude that the inhibitory action of PTH on ALPase release in SaOS-2 cells is mediated via activation of PKA. These cAMP-resistant cell lines will be especially useful in elucidating signal transduction mechanism(s) for PTH in human osteoblastic cells.

Introduction

Although it has been suggested that alkaline phosphatase (orthophosphoric-monoester phosphohydrolase alkaline optimum [ALPase]) is involved in skeletal mineralization (Warner *et al.*, 1983; Register *et al.*, 1984, 1986; Register and Wuthier, 1984; McLean *et al.*, 1987; Genge *et al.*, 1988), the complete spectrum of actions of this enzyme in mineral ion homeostasis in osteoblasts remains unclear (Whyte, 1989). The human osteoblast-like cell line SaOS-2 (Ponten and Saksela, 1967; Murray *et al.*, 1987; Rodan *et al.*, 1987) contains high ALPase activity and constitutively releases the enzyme into the medium (Murray *et al.*, 1987; Fukayama and Tashjian, 1990; Nakamura *et al.*, 1988). We have shown previously that ALPase is functionally involved in the uptake of $^{45}\text{Ca}^{2+}$ stimulated by human parathyroid hormone (hPTH $_{1-34}$) in SaOS-2 cells (Fukayama and Tashjian, 1990). Treatment with PTH showed biphasic effects on the release of ALPase; first, the hormone transiently enhanced the release of ALPase, but then it inhibited release, by up to 50–70% of the control level, by 4 h. Although the inhibition of ALPase release was also observed when the cells were treated with vasoactive intestinal peptide (VIP, 10^{-8} M) and dibutyl cyclic AMP (DBcAMP, 1 mM) for 4 h, the precise mechanism of inhibition is unknown.

It has been shown that PTH acts via stimulation of adenylate cyclase to enhance production of cAMP and subsequent phosphorylation of regulatory proteins by protein kinase A (PKA)

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in osteoblastic cells (Chase and Aurbach, 1970; Dietrich *et al.*, 1976; Peck, 1979). However, several reports have indicated that PTH can activate other putative second messenger pathways, particularly the mobilization of intracellular calcium (Lowik *et al.*, 1985; Boland *et al.*, 1986; Reid *et al.*, 1987; Yamaguchi *et al.*, 1987, 1989; Civitelli *et al.*, 1988, 1990; Donahue *et al.*, 1988; Yamada *et al.*, 1989; Dunlay and Hruska, 1990). Although rapid calcium transients in response to PTH have not been observed in SaOS-2 cells (Boland *et al.*, 1986), we have found that PTH can stimulate acutely the uptake of $^{45}\text{Ca}^{2+}$ in these cells by a mechanism that, unlike adenylate cyclase activation, is not subject to homologous desensitization (Fukayama and Tashjian, 1990). These findings suggest that multiple intracellular second messenger systems may participate in the actions of PTH on target cells.

A particularly informative approach to investigating signal transduction mechanism(s) involved in PTH action is to study the functional consequence(s) of blocking directly the activation of PKA. We and others have previously described the use of mutant cell lines that express a cAMP-resistant phenotype after transfection with a mutant gene for the type I regulatory subunit of PKA (Bringhurst *et al.*, 1989; Wong and Schimmer, 1989; Wang and Ascoli, 1990; Segal and Pollock, 1990).

In this paper, we describe establishment of mutant human SaOS-2 cell lines stably transfected with a plasmid that directs the synthesis of a cAMP-resistant form of the type I regulatory subunit of PKA under the control of a metallothionein promoter. This construct permits regulated expression of the cAMP-resistant phenotype. We have used these mutants to demonstrate that inhibition by PTH and DBcAMP of the release of ALPase by SaOS-2 cells is dependent on activation of PKA.

Results

Screening of mutant cell lines

SaOS-2 cells contain high ALPase activity and constitutively release the enzyme into the culture medium (Fukayama and Tashjian, 1990). We have shown previously that the release is inhibited after 4 h of treatment with hPTH₁₋₃₄ (2.5×10^{-8} M), VIP (10^{-8} M), or DBcAMP (1 mM) (Fukayama and Tashjian, 1990). Because cAMP analogues such as DBcAMP or 8-bromo cAMP act primarily through PKA-activation (Krebs and Beavo, 1979; Edelman *et al.*, 1987), we used the inhibitory action of DBcAMP on ALPase re-

Table 1. Inhibition by DBcAMP of ALPase release by nontransfected control SaOS-2 cells and by four mutant cell lines

Cell type	ALPase release (% of control)	
	-Zn	+Zn
Untransfected control	32 ± 1	45 ± 3
pSV _{2neo} control	35 ± 2	51 ± 4
REV AB clones		
Ca#2A	66 ± 5	97 ± 6
Ca#6-4	51 ± 5	92 ± 3
Ca#4A	89 ± 5	106 ± 12

Cells were preincubated in serum-free, phenol red-free medium for 24 h in the absence or presence of ZnCl_2 (25 μM). They were then incubated without or with DBcAMP (1 mM) in the continued absence or presence of ZnCl_2 (25 μM) for 4 h. Medium ALPase activity was measured as described in Materials and methods. Each value gives the means $\pm$ SE (for 6 dishes) of the percentage of control ($\pm\text{Zn}$) enzyme activity (range, 53–144 nmol/min per well) observed in the DBcAMP-treated cultures.

lease to identify mutant cell lines (Table 1). In parental (nontransfected) SaOS-2 cells, DBcAMP inhibited the release of ALPase to 30–40% of control values, both in the absence or presence of Zn^{2+} . Similar results were obtained in control SaOS-2 cells transfected only with the selection plasmid pSV_{2neo}. Inhibition of alkaline phosphatase release by DBcAMP was only slightly less in Zn-treated parental cells than in untreated parental cells. In contrast, the inhibitory effect of DBcAMP on ALPase release was markedly blunted or eliminated in three mutant cell lines (Ca#2A, Ca#6-4, Ca#4A) in the presence of Zn^{2+} , consistent with the hypothesis that PKA activation by DBcAMP was substantially blocked in these mutant cells. In one line Ca#4A, the inhibitory effect of DBcAMP was blocked almost completely even in the absence of Zn^{2+} .

Characterization of mutant cell lines

We examined PTH binding and cAMP responsiveness stimulated by hPTH₁₋₃₄ and VIP in these mutant cell lines. Curves for displacement by hPTH₁₋₃₄ of ^{125}I -Nle PTH binding to receptors on the mutant SaOS-2 cells were similar to those for the nontransfected controls when studied in the absence of Zn^{2+} (Figure 1); specific and nonspecific binding to parental SaOS-2 and mutant Ca#4A and Ca#6-4 cell strains were 8.2 and 1.9%, 8.4 and 1.4%, and 7.8 and 1.5%, respectively, with half-displacement of

Figure 1. PTH binding in nontransfected control SaOS-2 cells (SaOS-2) and in two mutant cell lines (Ca#4A and Ca#6-4). PTH binding was determined by incubating cells with ^{125}I -(Nle⁸,Nle¹⁸,Tyr³⁴)-bPTH₁₋₃₄-NH₂ in the absence or presence of various concentrations of bPTH₁₋₃₄ for 4 h at 15°C. Each point gives the means $\pm$ SE of triplicate samples. Where brackets are not shown, the SE was within symbol size.

tracer at about 3×10^{-9} M for each cell type. Pretreatment with Zn^{2+} did not significantly alter PTH binding in either parental or mutant Ca#4A cells (Figure 2).

cAMP production in response to both hPTH₁₋₃₄ and VIP also was well maintained in the mutant cell lines (Table 2).

Figure 2. PTH binding in nontransfected control SaOS-2 cells (left) and in the mutant cell line Ca#4A (right). PTH binding was determined by incubating with ^{125}I -(Nle⁸,Nle¹⁸,Tyr³⁴)-bPTH₁₋₃₄-NH₂ in the absence or presence of various concentrations of bPTH₁₋₃₄ for 4 h at 15°C without or with 24-h pretreatment with ZnCl_2 (100 μM). Specific and nonspecific (observed in presence of 10^{-8} M hPTH₁₋₃₄) binding was 13.6 and 2.1% (-Zn), and 11.1 and 3.3% (+Zn) in SaOS-2, and 7.5 and 1.0% (-Zn), and 7.5 and 1.4% (+Zn) in Ca#4A, respectively. Each point gives the means $\pm$ SE of triplicate samples. Where brackets are not shown, the SE was within symbol size.

Table 2. Effects of hPTH₁₋₃₄ and VIP on the accumulation of cAMP in nontransfected control SaOS-2 cells and three mutant cell lines

Treatment	Medium cAMP (pmol/well)	
	-Zn	+Zn
SaOS-2		
Control	0.12 $\pm$ 0.09	0.03 $\pm$ 0.02
hPTH ₁₋₃₄	33 $\pm$ 1.8	38 $\pm$ 2.1
VIP	14 $\pm$ 0.9	12 $\pm$ 0.3
Ca#2A		
Control	0.18 $\pm$ 0.09	0.77 $\pm$ 0.47
hPTH ₁₋₃₄	10 $\pm$ 2.0	20 $\pm$ 5.0
VIP	13 $\pm$ 1.7	16 $\pm$ 1.1
Ca#6-4		
Control	1.9 $\pm$ 0.62	0.11 $\pm$ 0.10
hPTH ₁₋₃₄	17 $\pm$ 0.6	34 $\pm$ 2.4
VIP	8.4 $\pm$ 1.7	14 $\pm$ 2.0
Ca#4A		
Control	1.1 $\pm$ 0.90	2.9 $\pm$ 0.30
hPTH ₁₋₃₄	31 $\pm$ 1.5	42 $\pm$ 3.1
VIP	12 $\pm$ 1.1	24 $\pm$ 1.9

Cells were preincubated in serum-free, phenol red-free medium for 12 h. Then the cells were incubated without or with ZnCl_2 (25 μM) for 12 h. Medium cAMP concentrations were determined by RIA 30 min after addition of hPTH₁₋₃₄ (2.5×10^{-8} M) or VIP (10^{-8} M) in the presence of 0.1 mM IBMX. Each value gives the means $\pm$ SE for triplicate dishes.

The consequence of the REV AB mutant phenotype on PKA and PKC activities was examined by measuring regulation of chloramphenicol acetyl transferase (CAT) reporter expression in cells that were transfected transiently with the somatostatin (SST)-CAT reporter plasmid (for PKA) or with a 5x-TPA-responsive element (TRE)-CAT plasmid (for protein kinase C [PKC]). Treatment with hPTH₁₋₃₄ (2.5×10^{-8} M) resulted in a twofold or greater increase above basal levels in PKA-dependent gene regulation, both in the absence or presence of Zn^{2+} , in the nontransfected control SaOS-2 cells (Table 3). In the mutant cell line Ca#4A, however, PTH-stimulation of SST-CAT activity was nearly absent in uninduced cells ($-\text{Zn}^{2+}$) and was totally abolished in the presence of Zn^{2+} , despite enhanced cAMP production (Table 2). Likewise, DBcAMP (0.5 mM) did not enhance SST-CAT activity in Ca#4A cells. Expression of transfected 5x-TRE-CAT was stimulated three- to fivefold by 200 nM 12-O-tetradecanoyl phorbol 13-acetate (TPA) in the absence or presence of Zn^{2+} , presumably via activation of PKC (Table 3). In sharp contrast to the PKA-dependent SST promoter, no inhibition of 5x-TRE regulation was observed, either in control SaOS-2 cells or in the mutant Ca#4A cell line.

Effects of PTH on the release of ALPase

The availability of mutant SaOS-2 cell lines that express a cAMP-resistant phenotype, especially in the presence of Zn^{2+} , permitted us to determine the role of PKA in the actions of $hPTH_{1-34}$ and DBcAMP on the release of ALPase. Treatment with maximally effective concentrations of DBcAMP or $hPTH_{1-34}$ resulted in a decrease in ALPase release to 60–30% of control values in the absence of Zn^{2+} in nontransfected control SaOS-2 cells (Figure 3). The inhibitory effect was still clearly observed in the presence of Zn^{2+} in these cells, although the magnitude of the inhibition was somewhat reduced, as noted above. In the two mutant cell lines, inhibition of ALPase release by $hPTH$ or DBcAMP in the absence of Zn^{2+} was comparable with that observed in nontransfected cells. However, the inhibitory effect was almost completely blocked (90%) after Zn^{2+} pretreatment of these mutant cell lines.

We next examined the effect of $hPTH_{1-34}$, DBcAMP, $hPTH_{53-84}$, and ionomycin on the release of ALPase in nontransfected control SaOS-2 cells (Figure 4). Both $hPTH_{1-34}$ (10^{-8} M) and DBcAMP (0.5 mM) inhibited the release of ALPase by up to 50%. The combination of $hPTH_{1-34}$ (10^{-8} M) and DBcAMP (0.5 mM) did not result in any further inhibition of ALPase release. Treatment with $hPTH_{53-84}$ (10^{-8} M), which does not stimulate cAMP production (or activate PKA) but does stimulate $^{45}Ca^{2+}$ influx

Figure 3. Actions of DBcAMP and $hPTH_{1-34}$ on the release of ALPase by nontransfected control SaOS-2 cells and two mutant cell lines Ca#4A and Ca#6-4. Cells were preincubated in serum-free, phenol red-free medium for 24 h in the absence or presence of $ZnCl_2$ (25 μ M). They were then incubated without or with $hPTH_{1-34}$ (2.5×10^{-8} M) or DBcAMP (1 mM) in the absence or presence of $ZnCl_2$ (25 μ M) for 4 h. Medium ALPase activity was measured as described in Materials and methods and expressed as percentage of control (100%) for each cell line. Each bar gives the means $\pm$ SE for triplicate cultures. This experiment was repeated nine times with similar results.

in these cells (data not shown), did not inhibit the release of ALPase. Furthermore, treatment with ionomycin (200 nM), which is known to increase cytosolic-free calcium in these cells (Boland *et al.*, 1986), was completely without effect on the release of ALPase.

Table 3. Protein kinase A- and protein kinase C-dependent gene regulation in nontransfected control and mutant SaOS-2 cells

Treatment	CAT expression (fold stimulation)			
	SST-CAT		5x-TRE-CAT	
	-Zn	+Zn	-Zn	+Zn
SaOS-2				
$hPTH_{1-34}$	1.8 $\pm$ 0.20	2.9 $\pm$ 0.83	—	—
DBcAMP	3.1 $\pm$ 0.71	5.4 $\pm$ 1.6	—	—
TPA	—	—	3.1 $\pm$ 0.03	4.8 $\pm$ 0.21
Ca#4A				
$hPTH_{1-34}$	1.4 $\pm$ 0.16	0.8 $\pm$ 0.08	—	—
DBcAMP	1.2 $\pm$ 0.18	0.9 $\pm$ 0.03	—	—
TPA	—	—	4.8 $\pm$ 1.0	5.1 $\pm$ 0.58

Cells were preincubated in MEM⁺ for 24 h (SaOS-2) or 10 h (Ca#4A) in the absence or presence of $ZnCl_2$ (100 μ M). Then the cells were transfected transiently with the SST-CAT reporter plasmid or the 5x-TRE-CAT plasmid as described in Materials and methods. After transfection, cells were incubated in MEM⁺ without or with $ZnCl_2$ (100 μ M) for 14 h (SaOS-2) or 12 h (Ca#4A) in the absence or presence of $hPTH_{1-34}$ (2.5×10^{-8} M), DBcAMP (0.5 mM), or TPA (200 nM). CAT activity was then measured as described in Materials and methods and expressed as fold stimulation over that observed in untreated controls.

Figure 4. Actions of hPTH₁₋₃₄, DBcAMP, hPTH₁₋₃₄ + DBcAMP, hPTH₅₃₋₈₄, and ionomycin on the release of ALPase by SaOS-2 cells. SaOS-2 cells were preincubated in serum-free, phenol red-free medium for 18 h. They were then incubated without or with hPTH₁₋₃₄ (10^{-8} M), DBcAMP (0.5 mM), hPTH₁₋₃₄ (10^{-8} M) + DBcAMP (0.5 mM), hPTH₅₃₋₈₄ (10^{-8} M), or ionomycin (2×10^{-7} M) for 4 h. Medium ALPase activity was measured as described in Materials and methods and expressed as percentage of control (100%). Each bar gives the means $\pm$ SE for six cultures. Control ALPase activity was 472 nmol/min mg protein.

Discussion

In this paper, we have described the establishment and characterization of several mutant human osteoblast-like cell lines that express a cAMP-resistant phenotype. It is well accepted that cAMP acts through activation of PKA (Krebs and Beavo, 1979; Edelman *et al.*, 1987) even though it has been suggested that some actions of cAMP analogues may not be PKA mediated (Nakamura and Gold, 1987; Light *et al.*, 1989). We first identified the mutant cell lines by examining the action of DBcAMP on ALPase release in the absence and presence of Zn^{2+} . One of these mutant cell lines (Ca#4A) appeared to express the cAMP-resistant phenotype constitutively (in the absence of Zn^{2+}). The Ca#2A and Ca#6-4 cell lines exhibited nearly normal phenotype in the absence of Zn^{2+} but consistently exhibited cAMP resistance in the presence of Zn^{2+} . These results are consistent with Zn^{2+} -regulated expression of REV AB in these two mutant cell lines, resulting in the inducible blockade of PKA by depletion of wild-type PKA holoenzyme, as previously reported in mutant UMR 106-01 rat osteosarcoma cells (Bringham *et al.*, 1989). Additional evidence for the inhibition of PKA activation in the mutant cell lines was obtained by transient transfection with a SST-CAT reporter plasmid that contains a func-

tional cAMP-response element (Montminy *et al.*, 1986; Montminy and Bilezikjian, 1987). The SST-CAT activity stimulated by hPTH₁₋₃₄ and DBcAMP was essentially completely inhibited in the presence of Zn^{2+} in the mutant cell lines but not in nontransfected control SaOS-2 cells. In contrast, no inhibition was observed in PKC-dependent gene regulation as evaluated by TPA stimulation of 5x-TRE-CAT in these cells (Miksic *et al.*, 1986; Angel *et al.*, 1987), showing that the inhibition of PKA was not nonspecific. Indeed, PKC activity stimulated by TPA sometimes was modestly enhanced in the mutant cell lines. These findings support the specificity of the mutant phenotype for the cAMP-dependent signal pathway and highlight the extent of the PKA blockade within these mutant cells.

The availability of these cAMP-resistant cells provided an opportunity to examine directly the role of cAMP in ALPase regulation by PTH. The results indicate that this action of PTH (and probably VIP) is mediated by activation of PKA, and the virtually complete blockade of this effect of the hormone in the Zn -induced mutant cells suggests that other putative second messengers probably play little or no role. Additional evidence for the cAMP/PKA mechanism was the finding of no significant difference in the magnitude of inhibition of ALPase release induced by hPTH₁₋₃₄ alone, DBcAMP alone, or the combination of hPTH₁₋₃₄ and DBcAMP in nontransfected control SaOS-2 cells. If the inhibitory action of PTH involved some cAMP-independent mechanism(s), the combined treatment of hPTH₁₋₃₄ plus DBcAMP would have been greater than either treatment alone. Incubation with hPTH₅₃₋₈₄, which does not stimulate cAMP production or PKA activation but does stimulate $^{45}Ca^{2+}$ uptake in SaOS-2 cells, did not inhibit the release of ALPase, arguing against the possibility that the action of hPTH₁₋₃₄ to stimulate acutely the uptake of $^{45}Ca^{2+}$ previously reported by us in these cells (Fukayama and Tashjian, 1990) is responsible for inhibition of ALPase. Furthermore, treatment with ionomycin, which is known to release acutely calcium from intracellular calcium stores in these cells (Boland *et al.*, 1986) was without effect on the release of ALPase, indicating that rapid calcium transients are not likely to inhibit the release of ALPase. Taken together, these data are consistent with the hypothesis that the inhibitory action of hPTH₁₋₃₄ on the release of ALPase is dependent on activation of PKA. If non-cAMP-associated pathways are involved in the inhibition of ALPase release by PTH, their actions must depend in some way on PKA.

It is of interest that the characteristics of PTH-receptor binding in the PKA-deficient mutant cells are indistinguishable from normal SaOS-2 cells, which we have also found in REV mutant UMR 4-7 rat osteosarcoma cells (Abou-Samra *et al.*, 1991). These findings suggest that PTH-receptor expression may not be strongly influenced by ambient PKA activity in unstimulated osteoblastic cells. The suggestion of enhanced cAMP accumulation after agonist exposure in the PKA-blocked mutant cells (Table 2) is also intriguing and might be explained by loss of some degree of basal cAMP-dependent restraint on receptor/cyclase coupling. Effects of PKA blockade on phosphodiesterase activity are unlikely because the concentrations of isobutyl methylxanthine (IBMX) used inhibit cAMP degradation maximally in these cells (Fukayama and Tashjian, unpublished data).

In summary, we have established mutant subclones of SaOS-2 human osteoblastic cells that express constitutive or inducible resistance to cAMP. As shown here for regulation of alkaline phosphatase release, these cells should prove especially useful in further investigations on the functional roles of different signaling systems (e.g., cAMP or inositol lipid pathways) that are regulated by PTH in osteoblastic cells.

Materials and methods

Materials

Culture media and sera for cell culture were purchased from GIBCO (Grand Island, NY), and tissue culture plasticware was obtained from Becton Dickinson Co. (Oxnard, CA). Synthetic porcine VIP (lots 76F-0632 and 18F-01711), IBMX, the cAMP standard, and (Bu)₂cAMP(DBcAMP; lots 49F-7270, 67F-7296, and 89F-7180) were purchased from Sigma Chemical Co. (St. Louis, MO). TPA was purchased from LC Services Corp. (Woburn, MA). hPTH₁₋₃₄ was from Peninsula Laboratories, Inc. (Belmont, CA; lot 011237) or from Bachem Inc. (Torrance, CA; lot WD-930). (Nle^{8,18}, Tyr³⁴) bovine PTH₁₋₃₄-NH₂ (Nle PTH) was purchased from Bachem Inc. (custom synthesized product). A plasmid (mt REV AB) that directs synthesis of a mutant cAMP-resistant form of type I regulatory subunit of PKA (Woodford *et al.*, 1989) was kindly provided by Dr. G. S. McKnight (University of Washington, Seattle, WA). A cAMP-responsive somatostatin promoter-chloramphenicol acetyl transferase (CAT) reporter plasmid incorporating the cAMP-responsive element TGACGTCA within a 5' regulatory segment of the rat somatostatin gene ($\Delta(-71)$ SST-CAT) (Montminy *et al.*, 1986; Montminy and Bilzikjian, 1987) was kindly provided by Dr. Mark Montminy (The Salk Institute, La Jolla, CA). A phorbol ester-responsive CAT vector, containing a pentameric tandem repeat of the human collagenase AP-1 response element ATGAGTCAG upstream of the CAT gene (5x-TRE-CAT) was kindly provided by Drs. Peter Angel and Michael Karin (University of California, San Diego, CA) (Angel *et al.*, 1987). Labeled tracer for the radioimmunoassay (RIA) of cAMP, (¹²⁵I) cAMP 2'-O-succinyl-(iodotyrosine methyl ester) cAMP, and anti-3',5', cyclic AMP-bovine serum albumin serum was obtained from ICN ImmunoBiologicals (Lisle, IL).

Cell culture

The clonal human osteoblast-like line SaOS-2 (Ponten and Saksela, 1967; Murray *et al.*, 1987; Rodan *et al.*, 1987; Hohmann and Tashjian, 1984) was originally obtained from Dr. E. Lloyd (Argonne National Laboratory, Argonne, IL) with the permission of Dr. Jorgen Fogh (Sloan-Kettering Memorial Cancer Center, New York, NY).

Cells were cultured at 37°C as monolayers in Eagle's Minimum Essential Medium supplemented (vol/vol) with 10% fetal bovine serum (hereafter MEM⁺) in a humidified atmosphere of 95% air and 5% CO₂. For the assay of cAMP and ALPase release, cells were preincubated in serum-free, phenol red-free MEM (hereafter MEM⁻) for 12–24 h.

In preliminary experiments, we found that the maximally effective nontoxic concentrations of ZnCl₂ for induction of the cAMP-resistant phenotype were 100 and 25 μ M in MEM⁺ and MEM⁻, respectively.

Transfection of DNA

For DNA transfection by electroporation, trypsinized cells were washed and resuspended (20×10^6 /ml) in 120 mM potassium glutamate, 5 mM ATP, 7 mM magnesium acetate, 4.3 mM glucose, 17 mM K piperazine-*N,N'*-bis(2-ethanesulfonic acid (pH 6.9), 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. The cells were cotransfected with 1 μ g of selection plasmid pSV_{2neo} and a 10- to 20-fold excess of the mt REV AB expression vector using a Bio-Rad apparatus (Bio-Rad, Richmond, CA) (1500 V; 25 μ F). Some transfections were performed by the calcium phosphate transfection method (Gorman *et al.*, 1982). In our experience, calcium phosphate transfection was more efficient than electroporation in obtaining mutant cell lines that expressed a cAMP-resistant phenotype. Transfected cells were cultured in MEM⁺ containing 600 μ g/ml G418 (Geneticin, GIBCO). Surviving colonies were subcultured in the continued presence of G418.

For transient transfections, subconfluent SaOS-2 cells in six-well 35-mm plastic dishes were preincubated in *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid-adjusted MEM⁺ (pH 7.1) for 2–3 h, the appropriate plasmid (30 μ g/dish) was added to each well, and cells were subjected to a 3-min glycerol shock after 4 h. After washing with phosphate-buffered saline, MEM⁺ or serum-free medium (MEM⁻) was added without or with appropriate stimulators as described in Results.

CAT assay

For CAT measurement, cells were scraped into 40 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM NaCl, 1 mM EDTA, pH 7.4, centrifuged at $10\,000 \times g$ for 5 min, resuspended in 0.25 M Tris buffer, and subjected to three cycles of freeze-thawing. CAT activities in cell lysates were determined from conversion of (¹⁴C)chloramphenicol to butyryl (¹⁴C)chloramphenicol using a simple phase-extraction separation (Seed and Sheen, 1988).

Other methods

The concentration of cAMP secreted into the medium was measured by RIA (Hohmann and Tashjian, 1984; Fukayama and Tashjian, 1989a,b). For the PTH radioreceptor assays, Nle PTH was radioiodinated and purified by high-performance liquid chromatography (Shigeno *et al.*, 1988). Confluent SaOS-2 cells in 24-well plastic dishes were used for the radioreceptor assay (Yamamoto *et al.*, 1988). Medium ALPase activities were determined by measuring the release of p-nitrophenol from p-nitrophenyl-phosphate spectro-

tometrically (405 nm) at 37°C (Fukayama and Tashjian, 1990). Protein concentrations were determined by the Bio-Rad method (according to the manufacturer's procedure). All results were expressed as the means $\pm$ SE. Statistical significance was analyzed by Student's *t* test.

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