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Regulation of Alkaline Phosphatase Activity by p38 MAP Kinase in Response to Activation of Gi Protein-Coupled Receptors by Epinephrine in Osteoblast-Like Cells*

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ABSTRACT

The signaling mechanisms responsible for the regulation of alkaline phosphatase (ALP) activity by exogenous factors in osteoblast-like cells remain poorly understood. Among various agents, epinephrine was recently found to increase ALP activity in differentiating MC3T3-E1 cells by stimulating α1 adrenergic receptors coupled to Gi proteins. In the present study, we investigated the role of both ERK2 and p38 mitogen-activated protein (MAP) kinases in mediating this response in MC3T3-E1 cells. Our results indicate that both MAP kinases are transiently stimulated by epinephrine in differentiating cells via a pertussis toxin sensitive mechanism. The role of each MAP kinase pathway in mediating the stimulation of ALP activity by epinephrine was investigated using specific inhibitors. The MEK inhibitor PD98059, blocked ERK2 activity induced by epinephrine but had no effect on the stimulation of ALP activity. In contrast, low concentrations of SB203580, a specific inhibitor of the p38 MAP kinase, completely blunted this cellular response. However, this inhibitor had no influence on the stimulation of ALP activity induced by ascorbic acid. In conclusion, the results of this study suggest distinct roles for ERK and p38 MAP kinase pathways in regulating activity of MC3T3-E1 osteoblastic cells. The ERK pathway is likely involved in the control of cell proliferation whereas the p38 MAP kinase pathway regulates ALP activity in response to activation of Gi protein-coupled receptors. (*Endocrinology* **140:** 3177–3182, 1999)

THE TISSUE nonspecific alkaline phosphatase (ALP), expressed in bone, liver, and kidney, plays an important role for the mineralization of bone, as suggested by studies of human hypophosphatasia, in which a deficiency of ALP is associated with defective bone mineralization (1, 2). The gene encoding ALP has been cloned (3), and its inactivation by homologous recombination in mice has confirmed a critical role for this enzyme in the mineralization of skeletal tissues (4). In agreement with this functional aspect, ALP is predominantly expressed at late stages of embryogenesis in skeletal tissues, mainly in bone forming cells (5) and *in vitro* studies have shown that expression of ALP increases with the differentiation of osteoblastic cells (6). In addition, a number of factors have been shown to regulate ALP activity in bone forming cells. Among them, epinephrine was recently found to enhance DNA synthesis in growing and ALP activity in differentiating MC3T3-E1 osteoblast-like cells (7). Both responses were sensitive to pertussis toxin (PTX), an uncoupler of GTP binding protein (G protein-coupled) of the Gi family, suggesting that this type of G protein is probably involved in mediating these cellular responses. The signaling mechanism involved in the regulation of ALP activity by G proteins, in particular Gi and Gq, in bone forming cells is unknown. Recent information suggest that signaling through G protein-coupled receptors implicates several mitogen activated protein (MAP) kinases including ERK, p38 MAPK and c-*Jun* NH2-terminal protein kinase (JNK) (8), which could be involved in the regulation of the differentiation of some cell types (9–13). The potential role of these different MAP kinases in controlling osteoblastic cell proliferation and differentiation is poorly understood. The aim of this study was to investigate the role of ERK2 and p38 MAPK in the regulation of ALP activity induced by activation of Gi protein-coupled receptors. Studies were performed in MC3T3-E1 cells using epinephrine as a potent agonist of this type of receptors in this cell line (7).

The results indicate that the stimulation of ALP activity induced by epinephrine in differentiating cells is preceded by a rapid and transient activation of both ERK2 and p38 MAPK. The specific MEK inhibitor (PD98059) had no effect on ALP stimulation induced by epinephrine in differentiating cells but could blunt the proliferative effect of this catecholamine in growing cells. The selective p38 MAPK inhibitor (SB 203580), however, completely blocked the increase in ALP activity induced by epinephrine, suggesting an important role for the p38 MAPK pathway in the regulation of ALP activity by agonists of Gi protein-coupled receptors in osteoblast-like cells.

Materials and Methods

Chemicals

Cell culture reagents were purchased from Flow Laboratories (ICN Biochemicals, Inc., Costa Mesa, CA) and FCS was from Gibco Life Technologies Ltd. (Paisley, UK). Epinephrine and collagenase type I were obtained from Sigma Chemical Co. (St. Louis, MO). 2′-amino-3-methoxyflavone (PD98059) was bought from Calbiochem-Novabiochem (La Jolla, CA). 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyri-

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dyl)imidazole (SB203580) was kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). ^{3}H-Thymidine was obtained from Amersham International plc (Little Chalfont, UK). All reagents for SDS-PAGE were from Bio-Rad Laboratories, Inc. (Munich, Germany). All other chemicals were purchased from standard laboratory suppliers and were of the highest purity available.

Cell culture

The murine calvaria-derived MC3T3-E1 osteoblast-like cells were grown in α-minimum essential medium (α-MEM) containing 10% FCS, 1% (vol/vol) nonessential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin. Subcultures were obtained once a week by removing the cells from the dish using 0.1% collagenase and 0.25% trypsin in a Ca- and Mg-free Earle's salt solution containing 0.02% EDTA. All cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2-95% air and the culture medium was changed every 2–3 days. With these experimental conditions, the cells reached confluency after 4 days. For analysis of cell proliferation, cells were seeded into 24-well plates (4×10^4 cells/well) and analyzed after 6 days culture (growing cells). For analysis of ALP and MAP kinases activities, cells were seeded into 6-well plates (16×10^4 cells/well) and 75 cm^2 flasks (14×10^5 cells/flask), respectively, and analyzed after 12 days in culture (differentiating cells).

Cell proliferation

After 5 days, the culture medium containing 10% FCS was replaced with α-MEM containing 2% FCS. Cell were pretreated with PD98059, a specific MEK inhibitor, or its vehicle for 1 h. They were then exposed to epinephrine or vehicle for 24 h for analysis of DNA synthesis or 48 h for cell replication in presence of the inhibitor. DNA synthesis was assessed by ^{3}H-thymidine incorporation during the last 6h of incubation in α-MEM containing 0.243 μg/ml of thymidine. Cell replication was determined by cell counting (Coulter counter).

Alkaline phosphatase activity

After 11 days, the culture medium of MC3T3-E1 cells was replaced with α-MEM containing 2% FCS for 24 h. The cells were pretreated either with the ERK kinase inhibitor PD 98059 or the p38 MAPK inhibitor SB 203580 for 1 h before exposure to epinephrine. They were then harvested in 1 ml of 0.2% Nonidet P-40 by scraping with a rubber policeman and disrupted by brief sonication. After centrifugation at 1,500 × *g* for 5 min, ALP activity was measured in the supernatant by the method of Lowry *et al.* (14).

Determination of ERK2 and p38 MAP kinase tyrosine phosphorylation

MC3T3-E1 cells, cultured in 75 cm^2 flasks and prepared as described above for ALP activity studies, were treated with agents in 15 ml α-MEM containing 2% FCS. At the end of the incubation period, cells were frozen in liquid nitrogen and lysed at 4 C in 3 ml of lysis buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1.0 mM phenylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na_3VO_4, 10 nM calyculin A, 50 nM myclocystin LR and 1% NP-40. Equal amounts of proteins, determined by Coomassie Plus Protein Assay Reagent (Pierce Chemical Co.), were immunoprecipitated with the agarose-conjugated antiphosphotyrosine [anti-Tyr(P)] monoclonal antibody (mAb), 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Anti-Tyr(P) immunoprecipitates or biotinylated molecular weight standards (MW) were fractionated by reducing SDS-PAGE on 6–10% acrylamide gradient gels and transferred to Immobilon P membranes according to the manufacturer's instructions (Millipore Corp., Bedford, MA). The membrane was then blocked for 4 h at room temperature in TBST, 1% BSA, and immunoblotted with either rabbit polyclonal anti ERK2 or anti p38 MAP kinase antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands and biotinylated MW standards were visualyzed by ECL Western blotting technique (Amersham International plc, Little Chalfont, UK) using horseradish peroxidase-labeled secondary antibodies or streptavidin conjugates. The intensity of the blots was quantified by densitometry (300 A Computing densitometer; Molecular Dynamics, Inc., Sunnyvale, CA).

Statistical analysis

Results of cell proliferation and ALP activity are expressed as mean ± SEM. A one-way ANOVA for multiple comparison was used for statistical analysis. A difference between experimental groups was considered to be significant when the probability value was less than 5.0%.

Results

Analysis of the ERK pathway

As expected from the known functional role of ERK in mediating cell proliferation induced by growth factors (15), PD98059, a specific ERK1/2 kinase inhibitor (16), dose dependently blunted the proliferative effect of epinephrine in growing MC3T3-E1 cells (Table 1). In addition to its effect on cell proliferation, the ERK pathway has also been suggested to regulate cell differentiation (9–13). We thus investigated whether this pathway was also involved in the stimulation of ALP activity induced by epinephrine. We first tested whether ERK is stimulated by epinephrine in differentiating MC3T3-E1 cells, which is the experimental condition previously used to describe stimulation of ALP activity by this catecholamine (7). As shown in Fig. 1, epinephrine induced a rapid and transient increase in tyrosine phosphorylation of ERK2, which was maximal after 2- to 5-min exposure (5.4-fold). ERK1, which is also recognized by the polyclonal ERK2 antibody used in this study, was not detected in most experiments and was thus not considered in this analysis. Pretreatment of the cells with 0.5 μg/ml PTX for 24 h reduced both the basal level and the extent of ERK2 phosphorylation induced by exposure to epinephrine. The absolute change in

TABLE 1. Effect of the ERK kinase inhibitor PD98059 on DNA synthesis and cell proliferation induced by epinephrine in MC3T3-E1 cells

PD98059 (μM)	Incorporation of ^{3}H-Thymidine (cpm/well)		Cell proliferation (number/well × 10^{-2})	
	Vehicle	Epinephrine (10 μM)	Vehicle	Epinephrine (10 μM)
0	3697 ± 211	22797 ± 2502[a]	11616 ± 62	16000 ± 93[a]
5	3236 ± 74	12161 ± 1303[b]	11568 ± 87	14264 ± 240[b]
15	3192 ± 98	4642 ± 492[b]	11024 ± 125	11368 ± 193[b]
25	3022 ± 126	3562 ± 269[b]	10304 ± 127	10444 ± 249[b]
50	2329 ± 28	2326 ± 123[b]	10264 ± 143	10104 ± 102[b]

MC3T3-E1 cells were preincubated with various concentrations of PD98059 in α-MEM medium containing 2% FCS for 1 h before addition of epinephrine or vehicle for 24 h for the determination of DNA synthesis and 48 h for the measurement of cell number.

Data are mean ± SEM of three determinations of a representative out of two experiments.

[a] $P < 0.01$ compared with vehicle (ANOVA Scheffe's F test).

[b] $P < 0.01$ compared with control cells not treated with PD98059 (ANOVA Scheffe's F test).

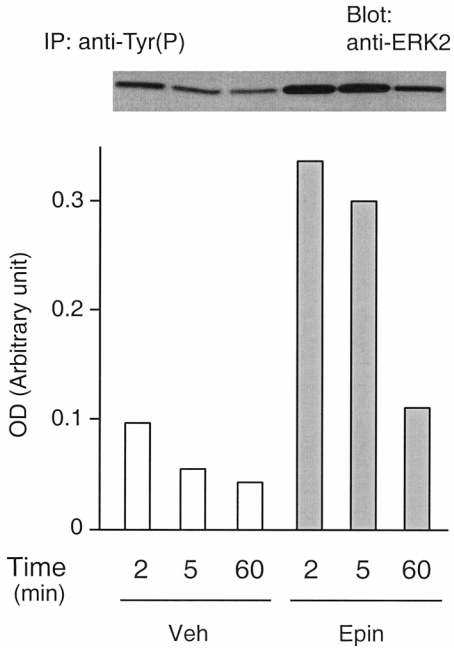

FIG. 1. Effect of epinephrine on changes in tyrosine phosphorylation of ERK2 MAP kinase in MC3T3-E1 cells. Osteoblast-like cells were incubated for the indicated periods with 10 μM epinephrine (Epin) or vehicle (Veh). Then, they were rapidly frozen in liquid nitrogen and lysed at 4 C. Equal amounts of proteins were used to immunoprecipitate tyrosine phosphorylated proteins with agarose-conjugated anti-PTyr mAb 4G10. Proteins were then separated by SDS-PAGE on a 6–10% acrylamide gradient gel, transferred to Immobilon P membranes, immunoblotted with rabbit polyclonal anti-ERK2 antibody, and detected by ECL method using horseradish peroxidase-labeled secondary antibodies. IP, immunoprecipitation; O.D., optical density. Data shown are derived from a representative out of three similar experiments.

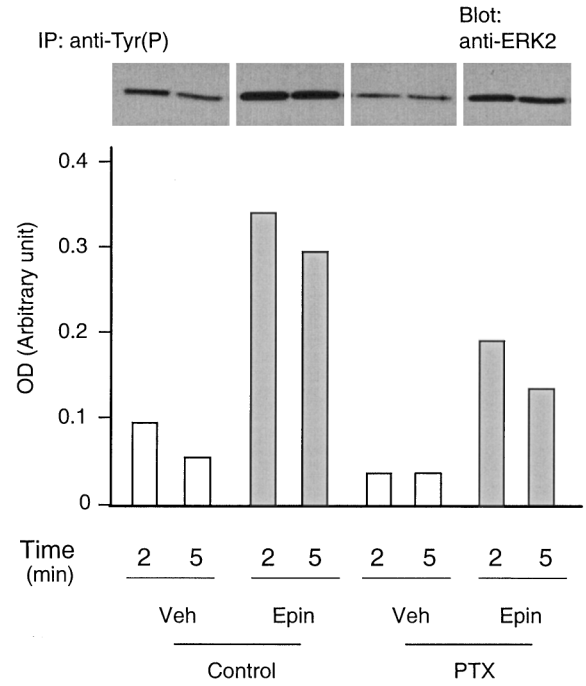

FIG. 2. Effect of pertussis toxin (PTX) on changes in ERK2 tyrosine phosphorylation induced by epinephrine in MC3T3-E1 cells. Osteoblast-like cells were preincubated with or without 0.5 μg/ml PTX for 24 h before exposure to 10 μM epinephrine (Epin) or vehicle (Veh) for the indicated time. Analysis of ERK2 tyrosine phosphorylation was then performed as described in the legend of figure 1. Data shown are derived from a representative out of three similar experiments.

ERK2 phosphorylation induced by epinephrine was reduced by 37 and 59.7% compared with control groups (Fig. 2) for the 2- and 5-min time point respectively. This finding suggests that, like the increase in ALP activity (7), the stimulation of the ERK2 pathway by epinephrine is mediated in part by activation of a Gi protein. This association prompted us to investigate whether this MAP kinase was involved in the regulation of ALP activity induced by epinephrine. As expected, PD98059 dose dependently blunted the tyrosine phosphorylation of ERK2 induced by epinephrine (Fig. 3, *left panel*). However, it did not reduce the effect of epinephrine on ALP activity (Fig. 3, *right panel*) suggesting that the ERK2 pathway is probably not involved in mediating this cellular response.

Analysis of the p38 MAPK pathway

Evidences have recently been provided that, in addition to ERK1/2, other MAP kinases such as the p38 MAPK can be stimulated by activation of G protein-coupled receptors (17, 18). Changes in the tyrosine phosphorylation of this MAP kinase in response to epinephrine was therefore also investigated in differentiating MC3T3-E1 cells. Reblotting of the membrane used for detection of ERK2 (Fig. 2) with a specific p38 MAPK antibody indicated that, in addition to increasing ERK2 activity, epinephrine also enhanced p38 MAPK tyrosine phosphorylation in differentiating MC3T3-E1 cells (Fig. 4). This response, like ERK2 activation, was maximal after 2–5 min epinephrine exposure (2.7-fold) and sensitive to PTX, suggesting that activation of Gi proteins probably leads to the stimulation of both ERK2 and p38 MAPK pathways in these cells. To determine whether p38 MAPK mediates the effect of epinephrine on ALP activity, we used the specific p38 MAPK inhibitor SB203580 (19). As shown in Fig. 5, this compound dose dependently reduced both basal levels and the stimulation of ALP activity induced by epinephrine with a complete inhibition at 10 μM. This inhibitor had, however, no effect on changes in ALP activity induced by ascorbic acid (Fig. 5), a well described differentiating factor for osteoblasts.

Discussion

The signaling mechanisms responsible for the regulation of ALP activity by exogenous factors in osteoblast-like cells remain poorly understood. Essentially, protein kinase A has been shown to mediate the stimulation of ALP activity in response to PTH and PTH-related peptide (20, 21). Except this regulatory mechanism, little is known about cellular events in osteoblasts specifically involved in the regulation of the activity of this essential enzyme for the mineralization of the bone matrix. Results presented in this study provide first evidences that the p38 MAP kinase is another signaling pathway involved in the regulation of ALP activity in osteoblastic cells. In differentiating MC3T3-E1 cells, the stimulation of the p38 MAPK by epinephrine is mediated by a Gi

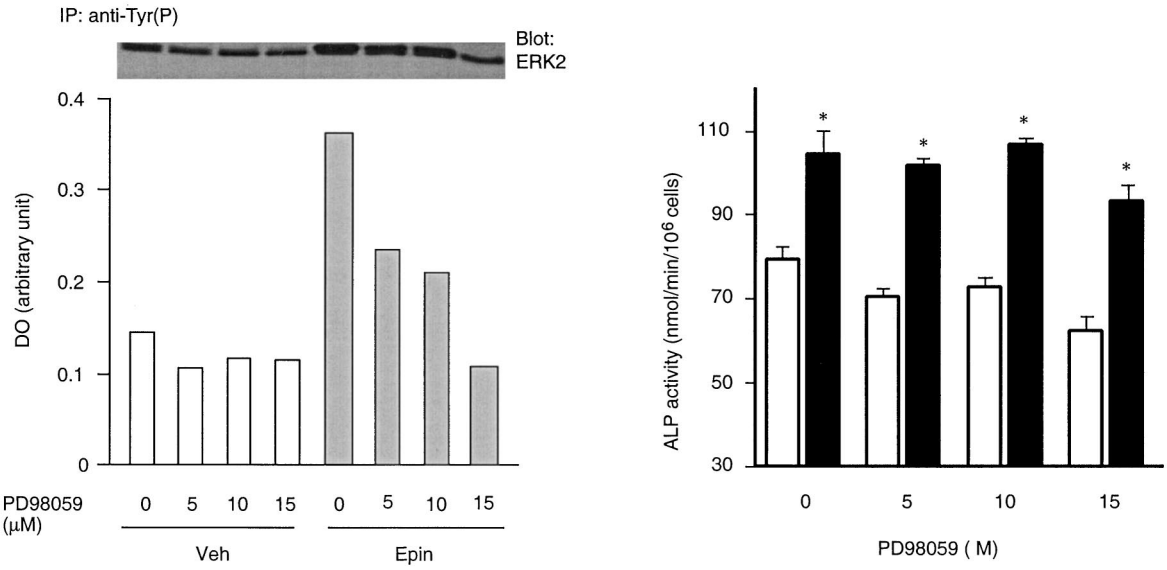

FIG. 3. Effect of PD98059 on ERK2 tyrosine phosphorylation and ALP activity induced by epinephrine. MC3T3-E1 cells were pretreated with various concentrations of PD98059 for 1 h. Then, they were exposed to 1 μM epinephrine (Epin, ■) or vehicle (Veh, □) during either 2 min for the determination of ERK2 tyrosine phosphorylation (*left panel*) or 48 h for the measurement of ALP activity (*right panel*). Data shown on ERK2 are derived from a representative out of two experiments. Data on ALP activity are mean ± SEM of four determinations of a typical experiment. *, $P < 0.001$ compared with vehicle (ANOVA Scheffé's F test).

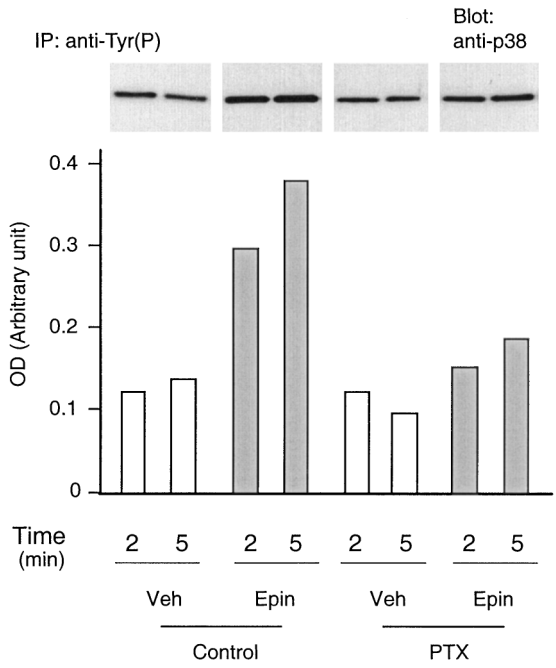

FIG. 4. Effect of epinephrine and PTX on changes in p38 MAP kinase tyrosine phosphorylation in MC3T3-E1 cells. Osteoblast-like cells were preincubated with or without 0.5 μg/ml PTX for 24 h before exposure to 10 μM epinephrine (Epin) or vehicle (Veh) for the indicated time. Then, they were rapidly frozen in liquid nitrogen and lysed at 4 C. Equal amounts of proteins were used to immunoprecipitate tyrosine phosphorylared proteins with agarose-conjugated anti-Ptyr mAb 4G10. Proteins were then separated by SDS-PAGE on a 6–10% acrylamide gradient gel, transferred to Immobilon P membranes, immunoblotted with rabbit polyclonal anti p38 MAP kinase antibody and detected by ECL method using horseradish peroxidase-labeled secondary antibodies. Data shown are derived from a representative out of three similar experiments.

protein-coupled receptor. The signaling mechanism responsible for this stimulation is unknown and is the subject of further investigation. Data shown in Fig. 5 on the change in baseline ALP activity induced by the p38 MAP kinase inhibitor suggest that, in addition to mediating epinephrine-effect, p38 MAP kinase is probably also involved in the regulation of ALP activity in relation with the development of MC3T3-E1 cell differentiation. In this experiment, the change in baseline ALP activity observed with SB203580 is unlikely to be due to cell toxicity because the stimulation of this enzyme activity induced by ascorbic acid, a potent inducer of mesenchymal cell differentiation, was not affected by this inhibitor. This latter observation is also of interest regarding the mechanism by which ascorbic acid influences ALP activity. In MC3T3-E1 cells, it has been suggested that the stimulation of ALP activity by ascorbic acid involves collagen and the $\alpha 2\beta 1$ integrin receptor (22) suggesting the implication of signaling events triggered by this extracellular matrix sensing mechanism. Data shown in Fig. 5 clearly indicate that p38 MAP kinase is not the mediator of this cellular response and suggest that there exist at least two distinct signaling processes in bone forming cells for the regulation of ALP activity. To our knowledge, a specific role of the p38 MAPK pathway in the regulation of differentiation in other cell types has not yet been reported. Whether this biological activity is preferentially expressed in osteoblastic cells is an interesting issue that remains to be considered. Initially, p38 and JNK have been described to control apoptosis and other stress responses in mammalian cells (23, 24). More recently, however, evidences that p38 can regulate other cellular functions than apoptosis have been provided. For instance, p38 has been reported to regulate glucose transport in mammalian cells (25) and collagenase expression in human squamous carcinoma cells (26) and fibroblasts (27).

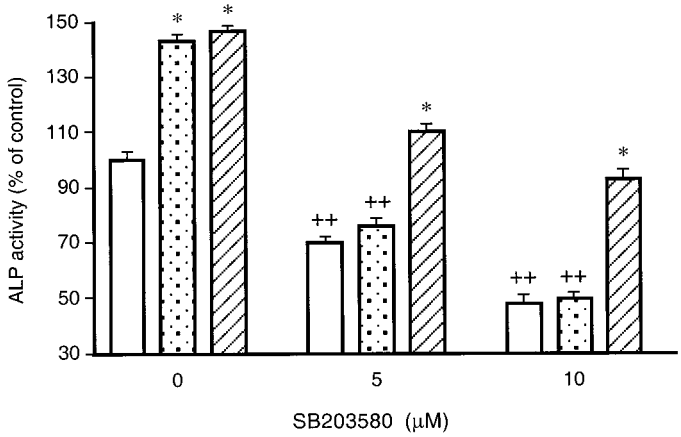

FIG. 5. Effect of SB203580 on ALP activity induced by either epinephrine or ascorbic acid in MC3T3-E1 cells. Osteoblast-like cells were pretreated with various doses of SB203580 for 1 h. Then, they were exposed to either 1 μM epinephrine (⬚) or ascorbic acid (▨) or to vehicle for 48 h before the determination of ALP activity. Data are mean ± SEM of four determinations of typical experiments. *, $P <$ 0.001 compared with vehicle (ANOVA Scheffé's F test). 2 + $P < 0.001$ compared with the corresponding value without SB203580 pretreatment (ANOVA Scheffé's F test).

Results of this study also indicate that the ERK pathway does not play a significant role in controlling ALP activity in response to epinephrine in MC3T3-E1 cells. It is well documented that this MAP kinase is important for osteoblastic cell proliferation induced by growth factors (28–31). As expected, this is also the case for the mitogenic response induced by epinephrine recently described in growing MC3T3-E1 cells (7) (Table 1). It is intriguing to note, however, that a stimulation of ERK2 is still detected is differentiating MC3T3-E1 cells (Fig. 1) in which the proliferative effect of epinephrine is barely expressed (7). This observation suggest that, in addition to cell replication, the ERK2 pathway might control other cellular functions in MC3T3-E1 cells. This finding also point to the possibility that, in response to a variety of growth factors, each MAP kinase pathway may have distinct biological activities in osteoblastic cells.

In our recent study describing the effect of epinephrine on MC3T3-E1 cells, the stimulation of ALP activity induced by this catecholamine was not associated with changes in other parameters of osteoblastic cell differentiation such as the rate of collagen synthesis or osteocalcin production (7), and therefore our study does not provide information about the role of ERK2 and p38 MAP kinase for the regulation of other parameters of differentiating osteoblast-like cells. Further investigations are in progress to determine the importance of these MAP kinase pathways for the normal development of osteoblastic cells, in particular the role of p38 MAP kinase and JNK for controlling the biological activities of differentiating bone forming cells.

In conclusion, the results of the present study suggest for the first time distinct roles of ERK and p38 MAPK pathways in regulating MC3T3-E1 osteoblastic activity in response to activation of Gi protein-coupled receptors. The ERK pathway is involved in controlling proliferation and probably in other function of differentiating osteoblastic cells in response to activation of this type of receptors, whereas the p38 MAP kinase pathway regulates ALP activity. Whether these or other MAPK pathways are also involved in the regulation of other function in osteoblastic cells is an interesting issue which remains to be investigated.

Acknowledgments

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