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DIRECT INVOLVEMENT OF cAMP-DEPENDENT PROTEIN KINASE IN THE REGULATION OF ALKALINE PHOSPHATASE ACTIVITY BY PARATHYROID HORMONE (PTH) AND PTH-RELATED PEPTIDE IN OSTEOBLASTIC UMR-106 CELLS

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The present study was performed to characterize the participation of parathyroid hormone (PTH)- and PTH-related peptide (PTHrP)-responsive dual signal transduction systems [cAMP-dependent protein kinase (PKA) and Calcium/protein kinase C (Ca/PKC)] in the regulation of alkaline phosphatase (ALP) activity in osteoblastic osteosarcoma cells (UMR-106). Both human (h) PTH-(1-34) and hPTHrP-(1-34) at 10^{-8}M stimulated ALP activity to the similar degree. Dibutyryl cAMP (dbcAMP) (10^{-5}, 10^{-4}M) and Sp-diastereoisomer of adenosine cyclic 3', 5'-phosphorothioate (Sp-cAMPS), a direct stimulator of PKA (10^{-4}M) also stimulated its activity. Phorbor 12-myristate 13-acetate (PMA), an activator of PKC, (10^{-7}, 10^{-6}M) did not affect its activity, while calcium ionophores, A23187 and ionomycin (10^{-7}, 10^{-6}M) inhibited it. Although Rp-diastereoisomer of adenosine cyclic 3', 5'-phosphorothioate (Rp-cAMPS), a direct inhibitor of PKA, (10^{-4}M) did not affect ALP activity by itself, it significantly antagonized not only Sp-cAMPS-induced increase in ALP activity, but also PTH- and PTHrP-induced one. The present study first indicated that the activation of PKA was directly involved and acted as a main pathway in the regulation of ALP activity by PTH and PTHrP in osteoblasts.

Parathyroid hormone (PTH) is a major calcium regulating hormone, which binds to recently cloned G protein-linked receptor in osteoblasts (1,2) and modulates various osteoblastic phenotypes. PTH-related peptide (PTHrP), a causative peptide associated with humoral hypercalcemia of malignancy, has been purified from the conditioned medium derived from a human lung cancer cell line and its amino acid sequencing revealed high similarity with PTH at the amino-terminus (3,4). Successful cloning of complementary DNA (cDNA) of PTH/PTHrP receptor demonstrated that a single cDNA clone, derived from either rat bone or opossum

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Abbreviations: cAMPS, adenosine 3',5'-monophosphorothioate; EDTA, ethylene diaminetetraacetic acid.

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kidney PTH receptor, mediates the stimulation by PTH or PTHrP of both adenylate cyclase and phospholipase C, when expressed in COS cells (2). We recently demonstrated that the activation of cAMP-dependent kinase (PKA) was directly linked to the inhibition of proliferation and collagen synthesis by PTH and PTHrP in osteoblastic osteosarcoma cells (UMR-106), using Rp-cAMPS which could directly inhibit PKA and that this cAMP analogue was useful to characterize the cAMP-mediated cellular events (5-7). Alkaline phosphatase (ALP) is a membrane bound enzyme which is abundant in bone, liver, kidney, small intestine and placenta. Although the role of ALP in bone is not fully elucidated, ALP activity is thought to be a marker of osteoblast differentiation and PTH regulates its activity (8-11). Although there are previous reports suggesting the involvement of cAMP in the regulation of ALP activity by PTH (8-11), its precise mechanism remains still unclear. In the present study, we sought to characterize the involvement of PTH-responsive dual signal transduction systems in the regulation of ALP activity by PTH in UMR-106 cells. Furthermore, we also performed the same study using PTHrP and compared this result with that of PTH.

Materials and Methods

Materials

UMR-106 cells were the generous gift from Dr. T. J. Martin (Melbourne, Australia). Human (h) PTHrP-(1-34) and hPTH-(1-34) were purchased from Peptide Institute Inc. (Osaka, Japan). Sp-diastereoisomer of adenosine cyclic 3', 5'-phosphorothioate (Sp-cAMPS) and Rp-diastereoisomer of adenosine cyclic 3', 5'-phosphorothioate (Rp-cAMPS) from Biolog Life Science Institute (Bremen, Germany), phorbol 12-myristate 13-acetate (PMA), N^6, $O^{2'}$-dibutyryl adenosine 3', 5'-cyclic monophosphate (dbcAMP) and ionomycin from Sigma Chemical Co. (St. Louis, MO), and A23187 from Hoechst Japan Co. (Tokyo, Japan). All other chemicals were of analytical grade.

Cell culture

UMR-106 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum and penicillin G (100 IU / ml) in a 5% CO_2-95% air atmosphere at 37°C, as previously described (12). Cells were weekly passed, using 0.05% trypsin-0.02% EDTA solution.

Assay of ALP activity and DNA content

Forty-eight hours after treatment of nearly confluent cells with the indicated concentration of substances, the cells were rinsed three times with phosphate buffered saline and 600ml of distilled water was added to each well. The DNA assay procedure of Labarca and Paigen was employed (13). This method is based upon the enhancement of fluorescence that occurs when bisbenzimidazole binds to DNA. Calf thymus DNA was used as a standard. Our preliminary experiments revealed a linear correlation between DNA contents of the cells and cell numbers. ALP activity was assayed at 37 °C by a method modified from Lowry et al (14). The assay mixtures contained 0.1M 2-amino-2-methyl-1-propanol, 1mM $MgCl_2$, 8mM p-nitrophenyl phosphate disodium, and cell homogenates. After 30 minutes of incubation, the reaction was stopped with 0.1N NaOH and the absorbance was read at 405 nm. Standard curves were prepared with p-nitrophenol. Data were expressed

as the mean ± SEM of six determinations. Statistical analysis was performed, using Student's t test or Duncan's multiple range test.

Results and Discussion

First we examined the effect of PTH and PTHrP on ALP activity in UMR-106 cells. As shown in figure 1, UMR-106 cells had high ALP activity at basal level and hPTH-(1-34) at 10^{-8}M significantly increased it in these cells. Human PTHrP-(1-34) at the same concentration increased it to the similar degree. In osteoblasts, the effect of PTH on ALP activity is conflicting. For example, PTH inhibited ALP activity in ROS 17/2 cells (9), while PTH stimulated it in MC3T3-E1 cells (8). Moreover, PTH stimulated and inhibited ALP activity in primary cultures of fetal mouse and rat osteoblasts, respectively (11). These discrepancies are considered to be partly due to the differences of species, differentiation stages of cells used and study protocols. It has been reported by us and others that PTH as well as PTHrP cause an increase in cAMP production followed by the activation of PKA and that they also act on polyphosphoinositide metabolism, another signal transduction system, resulting in the elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) as well as the activation of protein kinase C (Ca/PKC) in UMR-106 cells (15-19). The next experiment, therefore, was performed to clarify the involvement of these dual signal transduction systems in the regulation of ALP activity by PTH and PTHrP, using second messenger analogues. Dibutyryl cAMP (10^{-5} to 10^{-4}M) caused a concentration-dependent increase in ALP activity and 10^{-4}M Sp-cAMPS, a direct stimulator of PKA, also increased it (figure 1). On the other hand, PMA (10^{-7} to 10^{-6}M), which could substitute for diacylglycerol and stimulate PKC, did not affect ALP activity. Two types of calcium ionophores (A23187 and ionomycin, 10^{-7} to 10^{-6}M), which

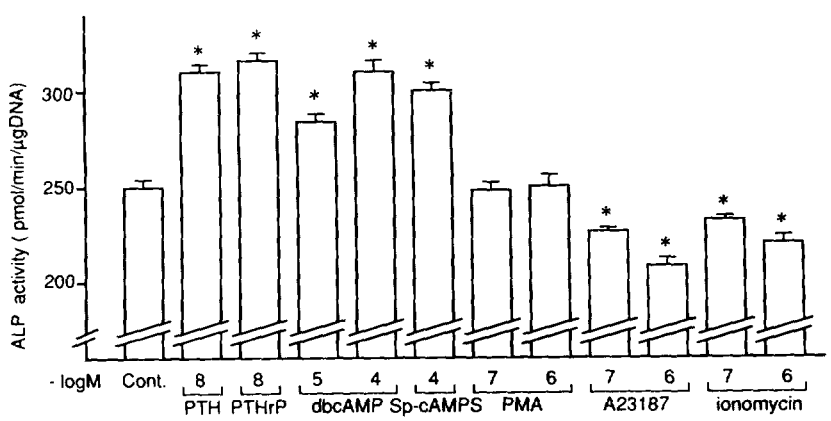

FIG. 1. Effect of PTH, PTHrP, dbcAMP, Sp-cAMPS, PMA and calcium ionophores on ALP activity in UMR-106 cells. After treatment with 10^{-8}M hPTH-(1-34), 10^{-8}M hPTHrP-(1-34), dbcAMP (10^{-5} and 10^{-4}M), 10^{-4}M Sp-cAMPS, PMA (10^{-7} and 10^{-6}M) or calcium ionophores (A23187, ionomycin, 10^{-7} and 10^{-6}M) for 48 hr, ALP activity was measured, as described in Materials and Methods. Each bar represents the mean ± SEM of six determinations. * $P<0.01$, compared to control.

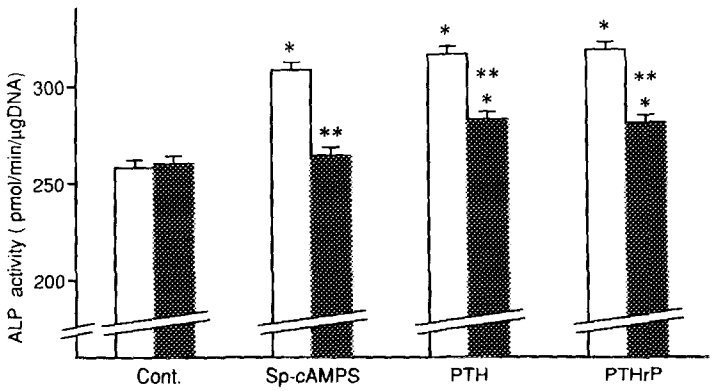

FIG. 2. Effect of Sp-cAMPS, PTH and PTHrP on ALP activity in the presence or absence of Rp-cAMPS in UMR-106 cells. Thirty minutes after pretreatment with 10^{-4}M Rp-cAMPS (closed bar) or vehicle (open bar), cells were treated with 10^{-4}M Sp-cAMPS, 10^{-8}M hPTH-(1-34) or 10^{-8}M hPTHrP-(1-34) for 48 hr and ALP activity was measured, as described in Materials and Methods. Each bar represents the mean ± SEM of six determinations. * $P<0.01$, compared to each control, ** $P<0.01$, compared to each Rp-cAMPS-untreated group.

could elevate $[Ca^{2+}]_i$, caused a concentration-dependent decrease in ALP activity. These results suggested that PKA activation but not Ca/PKC would be involved in the stimulation of ALP activity by PTH as well as PTHrP. Since we recently demonstrated that Rp-cAMPS, a direct inhibitor of PKA, is a useful tool to characterize the involvement of cAMP-dependent pathway in the regulation of osteoblast proliferation and collagen synthesis by PTH and PTHrP in these cells (5-7), this cAMP analogue was employed in the next experiment. As shown in figure 2, Rp-cAMPS at 10^{-4}M completely antagonized Sp-cAMPS-induced increase in ALP activity, although it unaffected ALP activity by itself. Furthermore, Rp-cAMPS also significantly antagonized PTH- and PTHrP-induced increase in ALP activity. These results first indicated that the activation of PKA was directly linked to the regulation of ALP activity by PTH in osteoblasts and that PTHrP-induced regulation of ALP activity was exerted presumably through the same mechanism as PTH-induced one.

It is unlikely that this effect of Rp-cAMPS is toxic one, since our previous studies revealed that it antagonized PTH- and PTHrP-induced inhibition of osteoblast proliferation and collagen synthesis in these cells. In the present study, Rp-cAMPS could not completely antagonize PTH- and PTHrP-induced increase in ALP activity. Three possibilities must be considered. First, Rp-cAMPS even at 10^{-4}M was not potent enough to completely antagonize the activation of PKA. Second, Rp-cAMPS has been reported to inhibit phosphodiesterase activity in cultured Leydig tumor cells (20). It is, therefore, possible that an inhibition of phosphodiesterase activity in turn might allow endogenous cAMP to accumulate, resulting in the partial compensation of PKA inhibition. Third, it might be presumed that PKA is not the only signal transduction system involved in the regulation of ALP activity by PTH and

PTHrP in osteoblasts, as demonstrated by us about the regulation of osteoblast proliferation by PTH (21). However, it is unlikely about the regulation of ALP activity by PTH and PTHrP. Because, among second messenger analogues examined, only PKA activators but not PKC activator and calcium ionophores mimicked the effect of PTH and PTHrP on ALP activity. The present study did not clarify the role of cross-talk of these dual signal transduction systems in the regulation of ALP activity by PTH and PTHrP, although we and other group reported its important role in the regulation of osteoblast proliferation and collagenase production by PTH in these cells, respectively (21,22). Further study is necessary to clarify it.

In conclusion, the present study first indicated that the activation of PKA was directly linked to the regulation of ALP activity by PTH in osteoblasts and that PTHrP had the same effect on ALP activity presumably through the same mechanism as PTH.

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