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Inhibition of *in vitro* mineralization in osteoblastic cells and in mouse tooth germ by phosphatidylinositol-specific phospholipase C

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Abstract—We have examined the effect of phosphatidylinositol-specific phospholipase C (PIPLC) on the *in vitro* mineralization during cultivation of both osteoblast-like MC3T3-E1 cells and the lower molar tooth germ from mouse embryo. PIPLC not only caused the release of alkaline phosphatase (ALP) from cell membranes into the culture medium but also produced a disturbance of *in vitro* mineralization in MC3T3-E1 cells and in mouse embryonic tooth germ. These findings strongly suggest that ALP, functioning as an ectoenzyme, is involved in the physiological mineralization process of both bone and teeth.

Tissue non-specific alkaline phosphatase (ALP*) plays a key role in the formation and calcification of hard tissues [1]. Although its precise function is still not well understood, several possible actions of ALP in mineralization have been proposed: increasing local concentrations of inorganic phosphate (Pi), local destruction of mineral crystal growth inhibitors, acting as a Pi-transporter, acting as a Ca^{2+}-binding protein, acting as a Ca^{2+}-ATPase, and acting as a regulator of cellular division or differentiation [2].

It has been shown recently that ALP is attached indirectly to the external surfaces of plasma membranes by phosphoethanolamine, which is bound to an oligosaccharide, which is, in turn, covalently linked to the polar head group of phosphatidylinositol [3, 4]. In fact, as early as 1980 it was found that ALP was released from plasma membranes by phosphatidylinositol-specific phospholipase C (PIPLC) [5], which finding led to the important realization that a variety of proteins were anchored in this way to cell surfaces by glycosylphosphatidylinositol [6].

MC3T3-E1 cells, a clonal line of osteoblastic cells derived from mouse calvaria [7], have the phenotypic characteristics of osteoblasts; they synthesize type I collagen, have high ALP activity, high responsiveness to both parathyroid hormone and 1,25-dihydroxyvitamin D_3 [8–10], and in prolonged culture they form bone *in vitro* [11]. Similarly, cultivation of tooth germ dissected from mouse embryo is a useful method for the *in vitro* study of the formation and mineralization of dentin and enamel [12, 13].

It was the aim of the present study to investigate whether ALP attached covalently to the external surface of plasma membranes is involved in the mineralization of hard tissues. For this purpose, we analysed *in vitro* mineralization during the cultivation of both osteoblast-like MC3T3-E1 cells and the lower molar tooth germ from mouse embryo after treatment with PIPLC; direct measurement of the calcium content of these tissues was carried out.

Materials and Methods

Culture of MC3T3-E1 cells. Cloned MC3T3-E1 cells were cultured in plastic dishes in α-minimum essential medium (α-MEM) containing 10% fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37° in air containing 6% CO_2. The culture medium also contained 5 mM β-glycerophosphate, added to facilitate calcification. From day 15, cells were cultured further for 5, 10 or 15 days in the absence or presence of 8.0 mU/mL PIPLC (*Bacillus thuringiensis*) obtained from Funakoshi (Tokyo, Japan). The medium was changed every second day.

Culture of tooth germ. The lower first molar tooth germ was dissected aseptically from 17-day-old embryonic mice. The tooth germ was explanted on a piece of membrane filter on a stainless grid and cultured in Fitton-Jackson modified BGJb medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. The explants were cultured for 7 days in the absence or presence of PIPLC (8.0 or 40 mU/mL) at 37° in air containing 6% CO_2.

Analytical procedure. At the end of the culture period, MC3T3-E1 cell layers (cells and cell matrix) and tooth germ were either dissolved in 6 N HCl or homogenized in 0.9% NaCl solution with 0.1% Triton X-100. The samples dissolved in HCl solution were then used for the determination of calcium content. The sample homogenates were used for the assay of ALP activity and for the determination of protein content. Fractions of the culture medium of MC3T3-E1 cells were collected for the assay of ALP activity in the medium. The calcium content of the cells and the cell matrix was determined by atomic

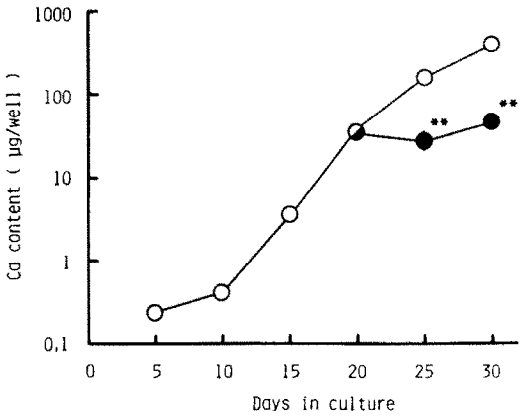

Fig. 1. Effects of PIPLC on the calcium content of the MC3T3-E1 cell layer. MC3T3-E1 cells were cultivated in α-MEM containing 5 mM β-glycerophosphate for 30 days. From day 15, cells were cultivated in the presence (●) or absence (○) of PIPLC (8.0 mU/mL) for 15 days. Abscissa shows calcium content on logarithmic scale. Each point represents the mean value, and the bar indicates the SEM (N = 6). **P < 0.001: statistical difference from the control group by Student's *t*-test or Cochran–Cox test.

* Abbreviations: PIPLC, phosphatidylinositol-specific phospholipase C; ALP, alkaline phosphatase; α-MEM, α-minimum essential medium.

Table 1. Effects of PIPLC on the calcium and protein content of, and ALP activity in a MC3T3-E1 cell layer, and ALP activity in the culture medium

	Control	PIPLC (8.0 mU/mL)
Calcium (μg/well)	334.6 ± 26.6	155.8 ± 22.8†
Protein (μg/well)	970.2 ± 70.6	998.9 ± 126.8
ALP (nmol/min/well)	154.8 ± 33.1	12.7 ± 1.2*
ALP (nmol/min/medium)	6.1 ± 2.5	1311.0 ± 38.5†

MC3T3-E1 cells were cultivated in α-MEM for 25 days. From day 15, cells were cultivated in the presence or absence of PIPLC (8.0 mU/mL). The culture media of cells cultured for 10 days were collected for assay of ALP activity in the medium.

Each value represents the mean ± SEM (N = 6).

* $P < 0.01$, † $P < 0.001$: statistical difference from the control group by Student's *t*-test or Cochran–Cox test.

absorption spectrophotometry [14]. ALP activity was measured by determining the hydrolysis of *p*-nitrophenyl phosphate [15]. Protein content was estimated by the method of Bradford [16], using bovine serum albumin as a standard. To measure the calcium content of the tooth germ, HPLC with electroconductive detection was used. After the decalcification of tooth germ was accomplished with 6 N HCl, the HCl was concentrated by centrifugation under a vacuum (Concentrator CC 180; Tomy, Tokyo, Japan). The resultant sample was then diluted with solution containing 0.4 mM ethylenediamine and 1 mM tartaric acid and an aliquot was then analysed by HPLC. The HPLC system consisted of a Tosoh model CCPD pump, a Tosoh model CM-8000 electroconductive detector, a Reodyne model 7125 sample injector, a Tosoh model CO-8000 column oven and a TSK-IC-cation analytical column (Tosoh Co. Ltd, Tokyo, Japan). The mobile phase was a mixture of 0.4 mM ethylenediamine and 1 mM tartaric acid. The flow rate was 1.2 mL/min and the column was maintained at 40°.

Statistical analyses. The results were analysed for statistical significance by Student's *t*-test or Cochran–Cox test.

Results and Discussion

The mineralization of MC3T3-E1 cells in α-MEM containing 5% β-glycerophosphate was estimated by measuring the calcium content of the cell layer. As shown in Fig. 1, an increased calcium content of the cell layer was observed 15 days after seeding, the increase continuing for

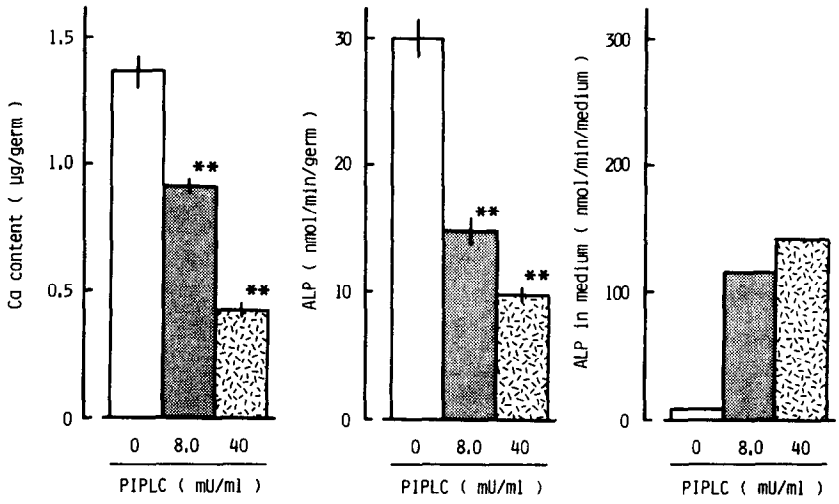

Fig. 2. Effects of PIPLC on the calcium content of and ALP activity in tooth germ, and on ALP activity in culture medium. Eight lower first molar tooth germ were cultivated for 7 days in the presence or absence of PIPLC (8.0 or 40 mU/mL). The mean values of the calcium content of and ALP activity in tooth germ are shown and the bar indicates the SEM (N = 8). To measure the ALP activity in culture medium, all media used for cultivation of eight tooth germs for 7 days were combined and used as the enzyme. **$P < 0.001$: statistical difference from the control group by Student's *t*-test or Cochran–Cox test.

up to 30 days. In the cells treated with PIPLC for 15 days after cultivation under control conditions for 15 days, calcium accumulation was inhibited. The inhibitory effect was sufficient to stop continued mineralization at 25 or 30 days, with a 5-day time lag. The effect of PIPLC on mineralization that was stimulated by β-glycerophosphate was also observed in cells not treated with β-glycerophosphate. Calcium content, protein content and ALP activity in the cell layer, as well as ALP activity in the culture medium of cells treated with PIPLC for 15 days, and those not so treated, are shown in Table 1. PIPLC reduced the calcium content but not protein content in the cell layer. ALP activity decreased in cells treated with PIPLC and increased in the culture medium. Thus, the removal of ALP from the cell surface interfered with mineralization in MC3T3-E1 cells. This suggests that ALP attaching covalently to the external surface of the plasma membrane plays an important role in mineralization in these cells.

Tooth germ dissected from 17-day-old embryonic mice and cultivated for 10 days showed clear mineralization in the dentin and enamel by histological observation. ALP activity and calcium content in the tooth germ increased developmentally during 10-day cultivation. The effects of PIPLC on the ALP activity in and calcium content of tooth germ, and on the ALP activity in the culture medium, are shown in Fig. 2. PIPLC reduced the calcium content of and ALP activity in tooth germ, and increased ALP activity in the culture medium, both effects being dose dependent. Thus, an inhibitory effect of PIPLC on mineralization was observed in teeth, as well as in bone. This suggests that the ALP bioactivity is located outside the cell membrane, but the enzyme has to be covalently linked to the cell membrane. Furthermore, it appears that the enzyme plays a common role in the mineralization of both bone and teeth. The relationship between the natural substrate, ALP, functioning as an ectoenzyme, and mineralization remains to be elucidated.

In conclusion, the findings presented here strongly suggest that ALP, functioning as an ectoenzyme, is involved in the physiological mineralization of bone and teeth. To our knowledge, the physiological role of ALP covalently interacting with membrane phosphatidylinositol in the mineralization process in hard tissues is described here for the first time.

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