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Ecto-Alkaline Phosphatase Considered as Levamisole-Sensitive Phosphohydrolase at Physiological pH Range During Mineralization in Cultured Fetal Calvaria Cells

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Abstract Alkaline phosphatase (ALP) activity expressed on the external surface of cultured fetal rat calvaria cells and its relationship with mineral deposition were investigated under pH physiological conditions. After replacement of culture medium by assay buffer and addition of *p*-nitrophenyl phosphate (*p*NPP), the rate of substrate hydrolysis catalyzed by whole cells remained constant for up to seven successive incubations of 10 min and was optimal over the pH range 7.6–8.2. It was decreased by levamisole by a 90% inhibition at 1 mM which was reversible within 10 min, dexamisole having no effect. Values of apparent Km for *p*NPP were close to 0.1 mM, and inhibition of *p*NPP hydrolysis by levamisole was uncompetitive (K_i = 45 μM). Phosphatidylinositol-specific phospholipase C (PI-PLC) produced the release into the medium of a *p*-nitrophenyl phosphatase (*p*NPPase) sensitive to levamisole at pH 7.8. The released activity whose rate was constant up to 75 min represented after 15 min 60% of the value of ecto-*p*NPPase activity. After 75 min of PI-PLC treatment the ecto-*p*NPPase activity remained unchanged despite the 30% decrease in Nonidet P-40–extractable ALP activity. High levels of ^{45}Ca incorporation into cell layers used as index of mineral deposition were decreased by levamisole in a stereospecific manner after 4 h, an effect which was reversed within 4 h after inhibitor removal, in accordance with ecto-*p*NPPase activity variations. These results evidenced the levamisole-sensitive activity of a glycosylphosphatidylinositol-anchored *p*NPPase consistent with ALP acting as an ecto-enzyme whose functioning under physiological conditions was correlated to ^{45}Ca incorporation and permit the prediction of the physiological importance of the enzyme dynamic equilibrium at the cell surface in cultured fetal calvaria cells.

Key words: *ecto-enzyme, ALP inhibitor, Ca incorporation, glycosylphosphatidylinositol-anchored proteins, PI-PLC,* bone differentiation

The bone/liver/kidney ALP isoenzyme is present in the outer plasma membrane of osteoblasts and matrix vesicles of calcifying tissues [Whyte, 1989; Morris et al., 1992]. ALP belongs to the class of proteins that are anchored to plasma membrane via covalent linkage to glycosylphosphatidylinositol, and the fact that ALP can be released by membrane impermeable glycosylphosphatidylinositol-specific phospholipases C or D has provided evidence of its ecto-orientation [Low, 1987]. Enzymes, such as ALP, which can act on appropriate substrates of the extracellular medium are referred to as ecto-enzymes [DePierre and Karnovsky, 1974]. ALP has long been associated with the mineralization process, and the strongest inference about its function comes from the association between enzyme presence and bone mineralization. The transfection into negative cells of ALP cDNA gives them a mineralization capacity [Yoon et al., 1989]. Conversely, lack of ALP activity causes defective mineralization in hypophosphatasia [for review see Caswell et al., 1991]. Furthermore, observations concerning abnormal extracellular metabolism of several phosphorylated compounds as phosphoethanolamine, pyridoxal 5′-phosphate, and pyrophosphate support that ALP functions as an ecto-enzyme [Whyte, 1989]. Despite the generally admitted necessity of ALP for mineral-

Abbreviations used: ALP, alkaline phosphatase; *p*NPPase, *p*-nitrophenyl phosphatase; *p*NPP, *p*-nitrophenyl phosphate; PI-PLC, phosphatidylinositol-specific phospholipase C.

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ization, its precise biochemical function as an ecto-enzyme which may act on extracellular matrix proteins or soluble substrates remains controversial. The enzyme has been thought to hydrolyze phosphate esters leading to an increase in local Pi concentration, to hydrolyze an inhibitor of hydroxyapatite crystal growth, to function as a tyrosine-specific phosphoprotein phosphatase, to act as a Ca^{2+}-binding protein or a Pi-binding protein, and to be involved in the Pi transport system [for review see Wuthier and Register, 1985; Whyte, 1989].

The most striking feature of phosphohydrolase ALP activity, when measured using a variety of organic phosphate esters as substrates, is its optimum alkaline pH [Bowers and McComb, 1966]. Under these pH conditions, ALP activity levels in the serum are currently used as a screening test for bone and liver diseases. On the other hand, ALP activity levels of cell extracts supplied by in vitro studies as marker of the osteoblastic phenotype at strongly alkaline pH give little insight into the enzyme functions in in vivo circumstances. In addition, it has been emphasized that the glycosylphosphatidylinositol-anchoring of the enzyme [Hsu et al., 1993; Harrison et al., 1995], as well as its interactions with other proteins of the extracellular matrix such as collagen type I and II able to modulate catalytic parameters of ALP, contributes to the mineralization process [Wu et al., 1991; Bossi et al., 1993]. Thus, it was of interest to have information about the kinetics of the phosphohydrolase activity of the enzyme at physiological pH range in its integrated membrane state.

Primary cultures of fetal rodent calvaria cells express osteoblast phenotype markers, such as ALP, osteocalcin, and type I collagen, and form a mineralizing matrix following a time sequence resembling that observed in vivo [Nefussi et al., 1985, 1989; Bellows et al., 1986; Ecarot-Charrier et al., 1988; Aronow et al., 1990]. The expression of high levels of ALP activity in cultured fetal rat calvaria cells [Collin et al., 1992] provides the opportunity to investigate the enzyme under physiological pH conditions concomitantly with the onset of mineralization. In the present study an approach was developed to measure ALP activity at 37°C on intact fetal calvaria cells at pH 7.8 with p-nitrophenyl phosphate (*p*NPP) as substrate in order to define the functioning of ALP at the external surface in its native environment. Levamisole, which inhibits phosphohydrolase activity of bone/liver/kidney ALP isoenzyme at physiological pH in a stereospecific manner [Cyboron et al., 1982], was a useful tool to distinguish ecto-ALP activity from other *p*-nitrophenyl phosphatase (*p*NPPase) ecto-activities and to study the functioning of ecto-ALP in relation with the rate of ^{45}Ca incorporation into cell layers. Our data showed that cultured fetal calvaria cells expressed ALP characterized by an ecto-oriented active site which was functional at a physiological pH range. They evidenced the ALP dynamics at the cell surface after release of the ecto-enzyme by phosphatidylinositol-specific phospholipase C (PI-PLC) and revealed a correlation between ecto-ALP activity and ^{45}Ca incorporation in cultured fetal calvaria cells.

MATERIALS AND METHODS

Materials

Dulbecco Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY) and fetal calf serum (FCS) (batch 158350) was from Biosys (Compiègne, France). Antibiotics and PI-PLC from Bacillus cereus were from Boehringer (Mannheim, Germany). Tricine, Hepes and Pipes were from Fluka (Buchs, Switzerland). Dexamisole, (+)-R-2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b)thiazole monohydrochloride, was from Janssen Research Products (Olen, Belgium). Levamisole, L(-)-2,3,5,6 tetrahydro-6-phenylimidazole (2,1-b)thiazole hydrochloride, and *p*-hydroxymercuriphenylsulfonic acid as well as all other chemical reagents were from Sigma (St Louis, MO). $^{45}CaCl2$ (10-40 mCi /mg calcium was obtained from Amersham (Buckinghamshire, England).

Culture Procedure

Calvaria bone cells were isolated as previously described [Nefussi et al., 1985]. Briefly, calvaria bones from 18-day-old fetal rats (Sprague Dawley) were dissected and incubated with 2.5 mg/ml (type I) collagenase in PBS for 1 h at 37°C. Cells were dissociated from bone fragments by repeated pipetting and washed several times in PBS. They were then counted and seeded at a final density of 2×10^4 cells/cm^2 in culture medium. This culture medium consisted of DMEM supplemented with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 50 UI/ml penicillin, and 50 μg/ml streptomycin with or without 10% selected fetal calf serum. The nonadhering hematopoietic cells were removed 4 h

after plating. Cells were cultured in a humidified atmosphere containing 95% ambiant air and 5% CO_2 at 37°C. The medium was changed every 48 h. All experiments were performed after 8 days of culture.

Assays of Phosphohydrolase Activity of ALP

ALP phosphohydrolase activity was assessed by measuring p-nitrophenol release from *p*NPP by absorbance spectrophotometry at 410 nm. Ecto-*p*NPPase assays were realized on the cell layers in the culture dish in a humidified incubator *in the* absence of CO_2 at 37°C. The culture medium was removed, and cell layers were washed twice with buffer A (136 mM NaCl, 2.6 mM KCl, 20 mM Hepes, 10 mM Tricine, 5.5 mM glucose, pH 7.8). The reaction was carried out in 0.9 ml of buffer A supplemented with 1 mM $MgCl_2$ (assay buffer) and containing various *p*NPP final concentrations (reaction mixture). After 10 min intervals, the supernatant solution was transferred into hemolyse tubes containing 0.1 M NaOH in order to stop the reaction, and *the p-nitrophenol* released was measured. Most measurements were carried out at pH 7.8. In some experiments, assay buffer supplementation with organic buffers was modified to obtain various pH over the range 6.8–8.2.

The results are expressed as units (U) of ecto-*p*NPPase activity per milligram of cell protein, proteins being measured according to the bicinchoninic acid method using BSA as standard [Smith et al., 1985]. One unit is the enzyme activity which hydrolyses 1 nmol *p*NPP per minute at pH 7.8 and at 37°C. With *p*NPP as a substrate at the physiological pH range, levamisole is a stereospecific uncompetitive enzyme inhibitor of ALP [Cyboron et al., 1982]. To distinguish the ecto-ALP activity from other ecto-*p*NPPase activities, the activity not inhibited by maximal concentrations of levamisole was subtracted, levamisole-sensitive ecto-*p*NPPase activity being referred to as ecto-ALP activity.

ALP activities were also determined at pH 7.8 in cell extracts by measuring the *p*NPPase activity in the presence and absence of 1 mM levamisole. Cells were harvested using a rubber policeman and solubilized in the assay buffer containing 0.2% Nonidet P-40. Then they were sonicated with 0.6 s/35 W pulses at 4°C, and the levamisole-sensitive *p*NPPase activity was determined on aliquots.

Calcium Incorporation Into Cell Layers

Experiments were performed in the presence of $^{45}CaCl_2$ (0.5 to 2.5 μCi/μmol), the total calcium concentration of the medium being 2 mM. Cells were incubated for 1–4 h with $^{45}CaCl_2$ (5 μCi/ml) in serum-free medium supplemented with levamisole, dexamisole, or their solvent. At the end of incubation, cells were rapidly washed 4 times with serum-free medium and incubated at 37°C for five successive periods of 4 min. This chase step allowed the release of rapidly exchangeable ^{45}Ca as seen under Results. Cells were then washed once with PBS at 4°C, and the radioactive material of cell layers was extracted in 10% formic acid for 24 h at room temperature as previously described [Bellows et al., 1991]. Aliquots were used to determine ^{45}Ca counts by liquid scintillation spectrometry. The results are expressed as micromole of calcium per milligram of cell protein.

PI-PLC Cell Treatment

PI-PLC of bacterial origin known to release glycosylphosphatidylinositol-anchored proteins was tested for its ability to release ecto-ALP activity into the incubation medium [Low, 1987]. Cells were rapidly washed twice with NaCl-free buffer A, pH 7.8, and were then incubated at 37°C for successive periods of 15 min in the same buffer with or without 0.1 U/ml PI-PLC, whose activity is inhibited by NaCl [Sundler et al., 1978]. At the end of each incubation, buffer was collected and supplemented with 136 mM NaCl and 1 mM $MgCl_2$ to measure *p*NPPase activity released into incubation buffer. For ecto-*p*NPPase activity measurements, after discarding PI-PLC–containing buffer, cells were washed with buffer A supplemented with 4.6 mM *p*-hydroxymercuriphenylsulfonic acid, a thiol-blocking agent which inhibited the anchor-degrading activity of the PI-PLC used, and the assay was performed as described above, except that the inhibitor was present at 2 mM, a concentration which did not interfere with the reaction.

RESULTS

Evidence of a Levamisole-Sensitive *p*NPPase Activity on Intact Cells

In order to investigate the phosphohydrolase activity of ALP expressed at the cell surface, substrate hydrolysis was measured directly on cultured fetal calvaria cells under physiological conditions (i.e., at pH 7.8 at 37°C). A commonly

used in vitro substrate, *p*NPP, was chosen because it does not penetrate the cellular membrane [DePierre and Karnovsky, 1974]. At day 8 of culture, which corresponded to early bone nodule formation with high level of ALP expression [Nefussi et al., 1989], culture medium was replaced by the assay buffer containing the saturating 1 mM concentration of *p*NPP. Then cells were submitted to seven successive incubations of 10 min at 37°C with *p*NPP in assay buffer. The rate of hydrolysis measured at the end of each 10 min incubation was close to 7 U/mg cell protein and remained constant for up to 70 min (Fig. 1). The assays were also performed in the presence of levamisole, a known specific inhibitor of ALP activity, or dexamisole, its inactive *d*-isomer [Van Belle, 1976; Cyboron et al., 1982]. When 1 mM levamisole was present during the first four successive incubations, ecto-*p*NPPase activity was inhibited by more than 90%, the inhibition extent being identical during the 40 min period. Then levamisole was removed, and the ecto-*p*NPPase activity was restored within 10–20 min to the value of untreated cultures. By contrast, dexamisole at 1 mM was without any effect on ecto-*p*NPPase activity (6.5 ± 0.4 vs. 6.7 ± 0.3 U/mg cell protein representing the mean value ± SD for the seven successive 10 min incubations in the presence and absence of dexamisole, respectively; $P > 0.1$).

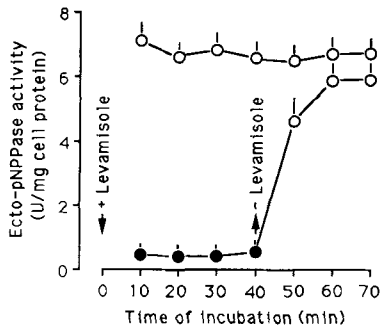

Fig. 1. Ecto-*p*NPPase activity measured in intact cells in the presence and absence of levamisole. At day 9, cells were incubated with assay buffer containing 1 mM *p*NPP at pH 7.8 at 37°C for seven successive incubations of 10 min. The reaction mixture was supplemented or not with 1 mM levamisole for the first four successive incubations of 10 min. Then levamisole was suppressed, and cells were further incubated during three successive 10 min periods. At the end of each 10 min incubation, the supernatant solution was analyzed for the release of *p*-nitrophenol. Cultures incubated in the presence of 1 mM levamisole (●) or with solvent alone (○) are shown. Results are presented as means ± SD for measurements of triplicate cultures from the representative experiment shown.

PI-PLC–Induced Release of *p*NPPase Activity

Incubation of cultured cells with bacterial PI-PLC, known to selectively remove glycosylphosphatidylinositol-membrane anchored proteins [Low, 1987], was used to determine the contribution of glycosylphosphatidylinositol-anchored ALP to ecto-*p*NPPase activity. In the absence of PI-PLC, *p*NPPase activity was not detected in the incubation buffer during two successive 15 min incubations (Fig. 2). PI-PLC addition to cultured cells at a concentration of 0.1 U/ml caused a release into the incubation buffer of *p*NPPase activity whose rate increased progressively during 30 min and remained constant for the last four 15 min incubations (4.4 ± 0.2 U/mg cell protein, n = 4). In the presence of 1 mM levamisole, the *p*NPPase activity released into the incubation buffer was totally inhibited. When ecto-*p*NPPase activity was measured at the end of each 15 min incubation before and following

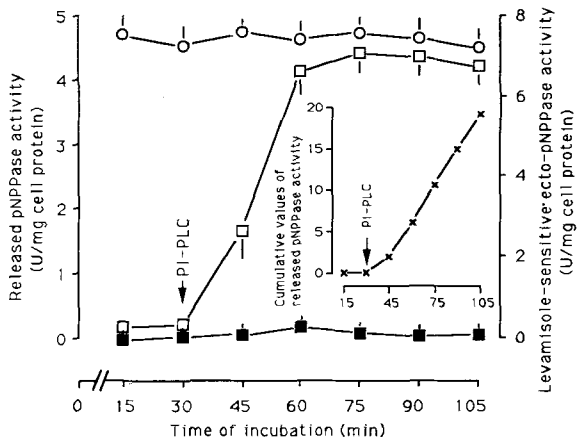

Fig. 2. Time-dependent effect of PI-PLC on the release of *p*NPPase activity and on levamisole-sensitive ecto-*p*NPPase activity. At day 8, cells were first incubated for two successive periods of 15 min in NaCl-free buffer A. Then 0.1 U/ml PI-PLC was added to the buffer, and cells were further incubated for five successive 15 min periods in the presence of PI-PLC. To measure the *p*NPPase activity released, buffer was collected at the end of each incubation period and supplemented with 136 mM NaCl and 1 mM $MgCl_2$ before being incubated with 1 mM *p*NPP. In a set of parallel cultures, to measure the ecto-*p*NPPase activity, PI-PLC–containing buffer was removed, cells were washed rapidly twice with 4.6 mM p-hydroxymercuriphenylsulfonic acid, and ecto-*p*NPPase activity was measured as described in Fig. 1, except that 2.2 mM p-hydroxymercuriphenylsulfonic acid was present. Both activities were measured in the absence and presence of 1 mM levamisole. Released *p*NPPase activity assayed in the absence (□) and presence of levamisole (■) and levamisole-sensitive ecto-*p*NPPase activity (○) are shown. **Inset:** Cumulative values of released *p*NPPase activity (×). Results are presented as means ± SD for measurements of triplicate cultures from the representative experiment shown.

the addition 0.1 U/ml PI-PLC, its amount remained unchanged throughout the experiment and close to 7.2 U/mg cell protein. When cumulative values of levamisole-sensitive *p*NPPase activity released during the five successive 15 min incubations in the presence of PI-PLC were considered (Fig. 2, inset), their total equal to 20 U/mg cell protein corresponded to about three times the constant value of ecto-*p*NPPase activity. On the other hand, the levamisole-sensitive Nonidet P-40–extractable *p*NPPase activity remaining associated with cells after PI-PLC treatment for 75 min, when measured at pH 7.8, was decreased by about 30% as compared to untreated cells (19.8 ± 1.8 vs. 28.2 ± 3.7 U/mg cell protein in three independent experiments). In addition, the protein content was not modified, and no morphological change or cell detachment was observed during PI-PLC treatment.

pH Dependence of Levamisole-Sensitive Ecto-*p*NPPase

To define optimal pH for ecto-*p*NPPase activity, *p*NPP hydrolysis catalyzed by cultured cells was measured with a saturating substrate concentration as a function of pH, which was stabilized with appropriate combinations of organic buffers permitting the pH to vary over the range of 6.8–8.2 [Eagle, 1977]. Ecto-*p*NPPase activity progressively augmented as the pH increased from 6.8 to 7.6. Then it plateaued between pH 7.6 and 8.2 when measured with *p*NPP at 1 mM, a saturating substrate concentration for the ecto-enzyme activity within this pH range (Fig. 3A). When compared to the value obtained at pH 6.8, the activity measured at pH 7.4 and 7.8 was increased by 1.7- and twofold, respectively. The inhibition pattern of ecto-*p*NPPase activity by 1 mM levamisole was also studied as a function of pH. The proportion of levamisole-sensitive ecto-*p*NPPase activity, which was 66% at pH 6.8, increased progressively between pH 7.1 and 7.6 to represent 90% and remained unchanged as far as pH 8.2. Ecto-*p*NPPase activity considered at the given pH 7.1, 7.4, or 7.8, as well as effectiveness of levamisole on ecto-enzyme activity, appeared to be independent of specific organic buffer additives (Pipes, Hepes, and Tricine) when used alone or in combination. The rate of *p*NPP hydrolysis catalyzed by ecto-*p*NPPase activity of cultured cells was also measured with increasing substrate concentrations as a function of pH. For all pH considered, the apparent Vmax was reached with concentrations equal or superior to 0.8 mM, its value being augmented by about 35% as the pH increased from 7.4 to 8.2 (Fig. 3B).

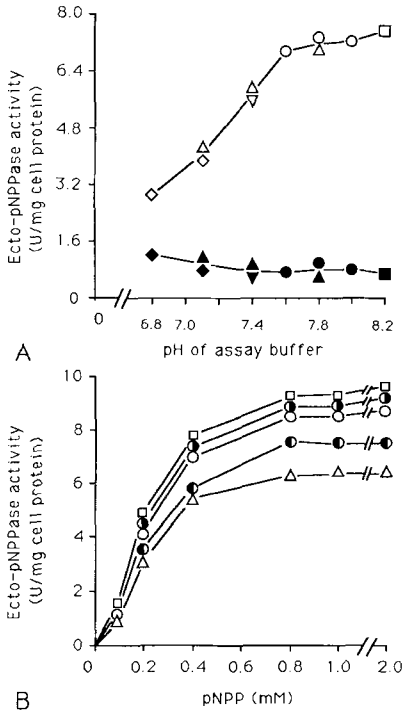

Fig. 3. Influence of buffer pH on ecto-*p*NPPase activity in intact cells. At day 10, culture medium was replaced by assay buffer whose supplementation with organic buffers varied in order to obtain various pH over the range 6.8–8.2. Then cells were incubated for three successive 10 min periods with 1 mM *p*NPP in the absence or presence of 1 mM levamisole (**A**) or with increasing concentrations of *p*NPP in the absence of levamisole (**B**). At the end of each 10 min incubation, the supernatant solution was analyzed for the release of *p*-nitrophenol, and the mean of the values obtained with the three successive incubations was determined. In A, cultures incubated with 1 mM levamisole (*solid symbols*) or its solvent (*open symbols*) at pH 6.8 with 10 mM Pipes (◇, ◆), 7.1 either with 10 mM Pipes (◇, ◆) or 10 mM Hepes (△, ▲), 7.4 either with 10 mM Hepes/6.7 mM Pipes (▽, ▼) or 15 mM Hepes (△, ▲), 7.6 with 15 mM Hepes/2.5 mM Tricine (○, ●), 7.8 with either 15 μM Hepes (△, ▲) or 20 mM Hepes/10 mM Tricine (○, ●), 8.0 with 15 mM Hepes/15 mM Tricine (○, ●), and 8.2 with 15 mM Tricine (□, ■) and, in B, cultures incubated with Hepes at pH 7.4 (△), with Hepes/Tricine at 7.6 (◐), 7.8 (○), 8.0 (◑), and with Tricine at 8.2 (□) are represented. A representative experiment is shown where the SD values for measurements of triplicate cultures were in all cases less than 10%.

Kinetic and Inhibition Parameters of Ecto-*p*NPPase

To determine the nature of inhibition of ecto-*p*NPPase activity by levamisole, assays were performed on cultured cells in the presence of

various concentrations of *p*NPP (0.03–1 mM) and increasing doses of levamisole (0.1–1 mM). Under these conditions the value of apparent Km for *p*NPP was close to 100 μM and that of Vmax equal to 7.4 U/mg cell protein (Fig. 4A). Levamisole inhibited ecto-*p*NPPase activity in a dose-dependent manner. When measured at the saturating concentration of 1 mM *p*NPP, maximal inhibition of enzyme activity (90%) was observed with levamisole concentrations superior to 0.5 mM and half-maximal inhibition with a dose of 0.07 mM. The results shown as a plot of S/V vs. S indicated that levamisole at concentrations from 0.1–0.5 mM was an uncompetitive inhibitor of *p*NPP hydrolysis (Fig. 4B). The intersection of Vo/Vapp vs. levamisole concentration indicated an apparent Ki of 45 μM (Fig. 4B, inset).

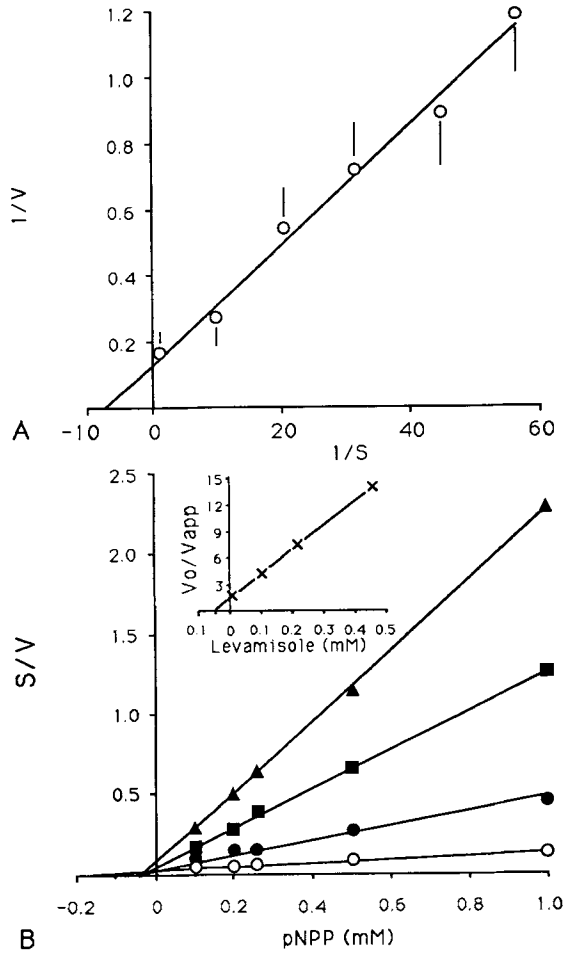

Fig. 4. Influence of *p*NPP concentration on ecto-*p*NPPase activity in the absence and presence of various concentrations of levamisole. At day 8, cells were incubated with the assay buffer containing various concentrations of *p*NPP for three successive 10 min periods, parallel cultures being incubated with the reaction mixture supplemented with increasing levamisole concentrations. At the end of each 10 min incubation, the supernatant solution was analyzed for the release of *p*-nitrophenol. The plot 1/V vs. 1/S **(A)** in the absence of levamisole and the Hanes plots of substrate concentration (S) divided by initial velocity (V) vs. (S) **(B)** for cultures incubated in the absence of levamisole (○) or in its presence at 0.1 mM (●), 0.22 mM (■), and 0.46 mM (▲) are represented. The replot of Vo/Vapp (Vmax in the absence of inhibitor/Vmax in the presence of inhibitor) vs. levamisole concentration is also shown (**inset**, ×).

The kinetic parameters of Nonidet P-40–extractable *p*NPPase activity were then compared to those obtained for ecto-*p*NPPase activity, both being measured at pH 7.8. Similar values were found for cell extracts and whole cells except for Vmax which was fourfold superior for extractable activity (Table I). This suggests that ALP was partly present as an ectoenzyme, approximately 75% of the total levamisole-sensitive ALP activity being intracellular or not accessible to exogenous *p*NPP.

Relationship Between Levamisole-Sensitive Ecto-*p*NPPase Activity and ^{45}Ca Incorporation Into Cell Layers

In order to define the role of ecto-*p*NPPase activity in the mineral deposition, ^{45}Ca incorporation into cell layers, previously used as an index of mineralization [Kato and Iwamoto, 1990; Bellows et al., 1991], was investigated. In the present study a chase step was applied after cell incubation in the presence of ^{45}Ca allowing

TABLE I. Kinetic and Inhibition Parameters of *p*NPP Hydrolysis by Intact Cells and Cell Extracts*

	Hydrolysis by *p*NPP at pH 7.8	
	By intact cells	By cell extracts
Km (μM)	105	125
Vmax app (nmol/min/mg cell protein)	7.4	33
Maximal effect of lev (% of control)	90	82
Ki app (μM)	45	66

*Data concerning ecto-*p*NPPase activity were issued from Fig. 4. *p*NPPase activity of cell extracts was measured by hydrolysis of various concentrations of *p*NPP at pH 7.8 in the assay buffer in the absence and presence of levamisole. Kinetic and inhibition parameters were determined by graphic analysis, and values of apparent Km, apparent Vmax for *p*NPP, maximum effect of 1 mM levamisole, and the inhibition constant Ki are represented.

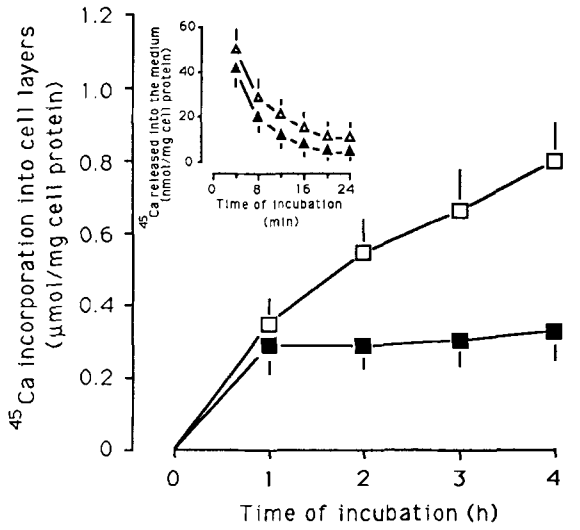

Fig. 5. ^{45}Ca incorporation into cell layers in the presence and absence of levamisole. At day 8, medium was replaced by a serum-free medium containing ^{45}Ca (5 μCi/ml) and supplemented with 1 mM levamisole or its solvent. After incubation times up to 4 h, ^{45}Ca incorporation into cell layers was measured as follows. Cells were first rapidly washed four times with serum-free medium and incubated in the same medium at 37°C for six successive periods of 4 min (chase step), ^{45}Ca released into the medium being measured at the end of each incubation. Then the remaining radioactivity incorporated into cell layers after the different incubation times with ^{45}Ca was determined. The radioactivity incorporated into cell layers after incubation in the presence of ^{45}Ca with levamisole (■) or its solvent (□) are represented. The amount of ^{45}Ca released into the medium during the chase step after incubation in the presence of ^{45}Ca for 4 h with levamisole (▲) or its solvent (△) is also shown **(inset)**. Corrections were made for the nonspecific release and cell incorporation of ^{45}Ca by incubating parallel cultures with ^{45}Ca for 1 min at 37°C. Results are presented as means ± SD for three experiments performed with different cell preparations.

the rapidly exchangeable radioactivity to be released into the culture medium. The high rate of the early ^{45}Ca release decreased progressively to lead to a constant low residual rate, which might correspond to the slow turnover rate of ^{45}Ca deposits (Fig. 5, inset). The radioactivity remaining incorporated into cell layers was then measured. Under these conditions at day 8 of culture ^{45}Ca incorporation rate was high during the first hour (0.73 nmol/min/mg cell protein—i.e., about fortyfold that observed at day 2, stage of cell confluence) and decreased gradually from 1 to 4 h (Fig. 5). The corresponding rate in the presence of levamisole represented after 1 h 85% of the value obtained in its absence and was strongly decreased thereafter, so that after 4 h it corresponded to 40% of the control values. Levamisole-sensitive ecto-*p*NPPase activity measured in parallel cultures remained unchanged during the 4 h incubation period and was close to 7.4 U/mg cell protein.

The amount of radioactivity incorporated from ^{45}Ca was also determined after two successive 4 h incubation periods in labeled culture medium (0–4 h and 4–8 h) in the presence or absence of 1 mM levamisole or dexamisole. In the absence of levamisole (control cultures), as well as in the presence of dexamisole, the rate of ^{45}Ca incorporation appeared to be important (0.75 nmol/mg cell protein) and similar during the two successive 4 h incubation periods (Table II). A single addition of levamisole performed at time zero or after 4 h was followed within the next 4 h by a 50% inhibition of ^{45}Ca incorporation. In parallel cultures, after a first addition of levamisole at time zero, the drug was maintained in or removed from the fresh medium after 4 h. When levamisole was maintained, the ^{45}Ca incorporation was again lowered. The inhibitory effect of levamisole was reversed after its removal within 4 h, the ^{45}Ca incorporation rate returning to 90% of the control value. The values of ecto-*p*NPPase activity in control cultures remained unchanged when assayed at time zero and after either of the two successive 4 h incubations, and dexamisole was without effect. Ecto-*p*NPPase activity was inhibited by levamisole by more than 90% to be restored 4 h after inhibitor removal to the value of control cultures. Actually, this reversal effect of levamisole occurred as early as 10 min, as shown in Figure 1.

DISCUSSION

The present study showed that cultured fetal rat calvaria cells were able to hydrolyze *p*NPP in the external medium under physiological pH conditions, suggesting the functioning of a *p*NPPase whose active site is ecto-oriented. Among criteria commonly considered to identify an ecto-enzyme is included the demonstration that the enzyme is capable of acting on a substrate in the external medium and that the enzyme is not leaked out of the cell; neither is its activity due to broken cells. The substrate we used, *p*NPP, is known not to penetrate the plasma membrane [DePierre and Karnovsky, 1974]. The *p*NPPase activity was not detected by direct measurement in the assay buffer, which ruled out the possible implication of soluble contaminants or the leakage of intracellular enzymes. The *p*NPP hydrolysis was linear with time, showing that cell break-

TABLE II. Reversal Effect of Levamisole on the Rate of ^{45}Ca Incorporation and Ecto-*p*NPPase Activity*

Addition		Period of labeling (h)	^{45}Ca incorporation into cell layers (μmol/mg cell protein)	Ecto-*p*NPPase activity U/mg cell protein	
At zero time	At 4 h			At 4 h	At 8 h
None	—	0–4 h	0.72 ± 0.09	7.42 ± 1.03	—
1 mM dexamisole	—	0–4 h	0.76 ± 0.09	6.97 ± 0.84	—
1 mM levamisole	—	0–4 h	0.37 ± 0.04	0.76 ± 0.08	—
None	None	4–8 h	0.87 ± 0.07	—	7.73 ± 1.08
None	1 mM dexamisole	4–8 h	0.81 ± 0.09	—	6.97 ± 1.07
None	1 mM levamisole	4–8 h	0.39 ± 0.04	—	0.61 ± 0.08
1 mM levamisole	1 mM levamisole	4–8 h	0.27 ± 0.03	—	0.75 ± 0.11
1 mM levamisole	None	4–8 h	0.72 ± 0.07	—	7.88 ± 1.10

*At day 8, medium was replaced by a serum-free culture medium with or without 1 mM levamisole or dexamisole. Cells were then incubated for two successive periods of 4 h, ^{45}Ca (5 μCi/ml) being present either from 0–4 h or from 4–8 h. In a set of cultures which received 1 mM levamisole at time zero, levamisole was removed from or maintained in labeled medium added after 4 h. Incorporation of ^{45}Ca into cell layers was measured at the end of each incubation. In parallel cultures, ecto-*p*NPPase activity was determined at the end of each 4 h period in the assay buffer containing 1 mM *p*NPP, using three successive incubations of 10 min in the presence or absence of 1 mM levamisole or dexamisole according to culture conditions of the preceding period. Results are presented as mean ± SD for three experiments performed on different cell preparations.

age which would have enhanced appreciably the activity did not occur during the incubation. The important ecto-*p*NPPase activity was evidenced at a stage of osteoblastic differentiation characterized by a high ALP expression [Nefussi et al., 1989; Collin et al., 1992].

Under the above conditions, the ecto-*p*NPPase activity was inhibited by levamisole in a stereospecific and reversible manner. The drug inhibition increased gradually with substrate concentrations, a situation typical for uncompetitive inhibition. This is in agreement with levamisole inhibition of the phosphohydrolase activity of purified ALP isoenzyme from bone/liver/kidney at alkaline pH, whereas intestinal and placental isoenzymes are barely sensitive to the drug [Van Belle, 1976]. At pH 7.4, the *p*NPPase activity of purified or membrane-bound ALP from chick cartilage and bone and rat liver is also inhibited by levamisole [Cyboron et al., 1982; Farley et al., 1982; Sarrouilhe et al., 1993] as well as the transphosphorylase activity of skeletal ALP [Müller et al., 1991]. The insensitivity of the phosphohydrolase activity of other membrane enzymes reveals the specificity of the inhibition of ALP by levamisole [LeBel et al., 1980; el Kouni and Cha, 1982]. Levamisole is an aromatic analog whose transport across plasma membrane cannot be excluded, yet the fact that the assay was performed with a membrane nonpermeable substrate prevented the detection of a levamisole-sensitive intracellular *p*NPPase activity. Because of its stereospecific levamisole sensitivity, the ecto-*p*NPPase expressed on cultured fetal calvaria cells represented ecto-ALP.

Ecto-*p*NPPase activity increased with pH and was optimal over the pH range from 7.6–8.2 in which the apparent Km values did not vary significantly. As to levamisole inhibition, it concerned more than 90% of ecto-*p*NPPase activity for pH equal or superior to 7.4. Of interest was the fact that the optimum pH was not a peak but a plateau which appeared as from pH 7.8. A similar plateau has been described for phosphotyrosine phosphohydrolase activity of ALP measured directly on cultured bone cells [Puzas and Brand, 1985]. The pH of the extracellular microenvironment of bone is not known; however, a shift to an alkaline pH has been described in the hypertrophic zone of growth plate cartilage and during bone formation [Cuervo et al., 1971]. Alkalinization of medium pH from 7.6 to 8.0 during 2 days of culture in fetal chick calvaria cells resulted in an increase in both mineralization and ALP content [Ramp et al., 1994]. Our study revealed an ecto-ALP functional at physiological pH and whose increased activity at mild alkaline pH might mimic some regulated process occurring in fetal bone formation.

The *p*NPPase activity released into the incubation buffer after addition of PI-PLC of bacterial

origin corresponded to a soluble form of ALP, as indicated by its sensitivity to levamisole. The constant rate of released ALP activity every 15 min represented 60% of the value of ecto-ALP activity, the latter being unchanged after 75 min when a 30% decrease in the Nonidet P-40–extractable activity was observed. A spontaneous release of ALP activity was not observed under our experimental conditions, contrary to what has been found in calvaria cells and osteosarcoma cell lines [Farley and Baylink, 1986; Noda et al., 1987; Nakamura et al., 1988; Fukayama and Tashjian, 1990]. The ALP activity released by PI-PLC was thus likely issued from ecto-ALP that would be rapidly restored, as shown by the constant rate of release with no modification of ALP activity at the cell surface. The fact that at pH 7.8 Nonidet P-40–extractable ALP activity, which represented four times the value of ecto-*p*NPPase activity, was decreased by PI-PLC treatment argued for the presence of an intracellular pool. The reappearance of the enzyme activity could be due to the translocation of the stored intracellular protein to the cell surface rather than to an upregulation of ALP synthesis. This may be compared with results obtained in neutrophils, where the majority of ALP activity is intracellular and can rapidly associate with the cell surface upon cell stimulation by a chemoattractant or an active phorbol ester [Kobayashi and Robinson, 1991]. Like in SKG3a cells, where the newly synthesized placental ALP is sensitive to PI-PLC as soon as it is integrated into the membrane [Wong and Low, 1994], the ecto-ALP of cultured fetal calvaria cells was probably sensitive to PI-PLC as soon as it appeared at the cell surface. These results let us think that the release of a PI-PLC–sensitive ecto-ALP activity triggered a new association of the intracellular enzyme with the cell membrane to compensate for the released activity.

^{45}Ca incorporation into cell layers represented at day 8 of culture more than fortyfold the value obtained at day 2, the stage of cell confluence. As previously described [Nefussi et al., 1985], the cell population was osteoblastic in character with typical nodules in formation, the slight contamination by chondroblasts at day 2 of culture being no longer detectable. Nodule formation has been found to be associated with expression of type I collagen, ALP, and low levels of osteocalcin [Nefussi et al., 1985; Collin et al., 1992]. The sequential expression of these proteins is characteristic of the matrix maturation whose mineralization is a process essential to complete expression of the mature osteoblast phenotype [Stein and Lian, 1993]. From this culture stage cells progressed to full mineralization in less than 2 weeks. Autoradiographic analysis of ^{45}Ca after pulse-labeling shows a distribution of silver grains occurring in the mineralized osteoid matrix and in the osteoblasts of nodules with very few grains in the neighboring cell layers [Nefussi et al., 1989]. In addition to positive Von Kossa staining, calcium phosphate crystals in association with matrix vesicles or collagen fibers has been revealed in nodules by electron microscopic examination [Nefussi et al., 1985]. At day 8 of culture, which represented the preparation phase for complete osteoblastic differentiation, ^{45}Ca incorporation reflecting active mineral deposition concomitantly to high ecto-ALP activity allowed the study of levamisole effect on both parameters.

Levamisole decreased ^{45}Ca incorporation into cell layers in a reversible and stereospecific manner in accordance with ecto-*p*NPPase activity variations without affecting the enzyme level. The short exposure effect of levamisole during the early phase of mineralization of cultured fetal calvaria cells, because of the unmodified ALP level and the full recovery of ^{45}Ca incorporation after removal of the drug, seemed unrelated to the long-term drug effect reported on cell differentiation in stromal cells [Klein et al., 1993]. It has been reported that levamisole can inhibit mineralization by interfering with Pi-binding protein function of ALP, a situation in which dexamisole and levamisole produced similar inhibition of ion uptake by isolated matrix vesicles [Register et al., 1984]. The discrepancy with our results may be explained by the nonsimilar surrounding environment of the enzyme in the two experimental systems which could influence ^{45}Ca incorporation. In the present study, the differences observed in the time dependence of the levamisole inhibition on both parameters could be related to the development stage of culture where nascent crystals were already present, as ALP activity inhibition does not modify crystal growth per se while it alters calcium and phosphate uptake [Register and Wuthier, 1984]. Ecto-ALP catalytic activity degrades phosphoethanolamine, pyridoxal-5-phosphate, and PPi which are supposed to be ALP substrates in vivo [Fedde et al., 1988]. In cultured fetal calvaria cells, the increase in ^{45}Ca incorporation in the presence of organic phos-

phate linked to the ecto-ALP–dependent enhancing of Pi levels was inhibited by levamisole, whereas the similar increase produced by corresponding levels of Pi was unaffected by the drug [F. Anagnostou, C. Plas, J.R. Nefussi, and N. Forest, in preparation]. These findings suggest that the levamisole effect observed is due to the specific effect of the drug on the catalytic activity of ecto-ALP and indicates the enzyme involvement in the mineralization process.

In conclusion, the functioning of ALP as a phosphohydrolytic ecto-enzyme under physiological conditions was evidenced in cultured fetal calvaria cells which expressed high levels of ALP at the early phase of mineralization because of the optimum of levamisole-sensitive ecto-*p*NPPase activity obtained at physiological pH, the low background of other non-levamisole-sensitive ecto-*p*NPPases at pH inferior to 8.0, and the release by PI-PLC of the levamisole-sensitive ecto-*p*NPPase activity followed by rapid restoration of the ecto-enzyme. The results obtained allowed us to establish a correlation between levamisole-induced variations of the ecto-ALP functioning and modifications of the mineral deposition. They also revealed the dynamics of the enzyme after PI-PLC treatment which may lead to study of the regulation of intracellular ALP shift to the cell surface.

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