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Role of β-GP-Derived Pi in Mineralization via Ecto-Alkaline Phosphatase in Cultured Fetal Calvaria Cells

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Abstract The permissive effect of β-GP on mineralization in cultured rat fetal calvaria cells was investigated in relationship with phosphohydrolase activity of ecto-ALP at physiological pH range. β-GP present in the culture medium for 8 days exerted a stimulatory effect on ^{45}Ca incorporation into matrix cell layers while the ecto-ALP activity level measured on intact cells with a saturating concentration of *p*NPP was similar for cells grown either in the presence or absence of β-GP. In both types of cultures, β-GP addition inhibited *p*NPP hydrolysis in a competitive and reversible manner and increased Pi concentration in the medium. The dose dependency of the effect of β-GP on ^{45}Ca incorporation and generation of Pi was similar ($k\phi$ = 3 mM). Levamisole, but not dexamisole, inhibited both *p*NPP and β-GP hydrolyses, which were likely catalyzed by the same ecto-enzyme. The rate of ^{45}Ca incorporation into matrix cell layers, which was high (0.90 μmol/4h/mg cell protein) in cells grown in the absence of β-GP, was inhibited by 50% by levamisole. In cells grown in the absence of β-GP, the ^{45}Ca incorporation rate increased progressively after β-GP addition, reaching after 12 h the value of cultures grown in the presence of β-GP, the increase being totally inhibited by levamisole. In both types of cells, addition of exogenous Pi at concentrations corresponding to medium levels of β-GP-derived Pi rapidly led to high ^{45}Ca incorporation rate which was unaffected by levamisole. β-GP removal from cultures grown in its presence reduced by 50% the ^{45}Ca incorporation rate which recovered the initial value after exogenous Pi addition independently of levamisole presence. Thus, mineral deposition did not affect the level and catalytic efficiency of ecto-ALP to hydrolyze β-GP in cultured fetal calvaria cells, yet it influenced the β-GP-stimulatory effect on mineralization so as to render this process not sensitive to high medium Pi levels.

Key words: ecto-ALP, β-GP, Pi, mineralization

Skeletal tissue formation involves the synthesis of an organic extracellular matrix and proteins characteristic of the bone phenotype which occurs during osteoblastic differentiation in a stepwise fashion [Rodan and Noda, 1991]. The subsequent matrix mineralization is regulated by a combination of cellular events including matrix vesicle formation, synthesis of matrix molecules and ionic milieu regulation, such process being sensitive to changes in extracellular Ca^{2+}, Pi, and organic phosphates, such as AMP, ATP, and β-GP [Anderson and Morris, 1993]. In many culture cell systems, induction of mineralization requires the addition of β-GP to the incubation medium [Ecarot-Charrier et al., 1983; Nefussi et al., 1985; Bellows et al., 1986; Aronow et al., 1990; Zimmermann et al., 1992]. Even if, in the absence of β-GP, the mineralization is self-sustained when medium Pi concentration is high [Aronow et al., 1990; Whitson et al., 1992] or when mineralized bone is already present [Gronowicz et al., 1989], β-GP accelerates the onset and extent of extracellular matrix mineralization [Gerstenfeld et al., 1987; Stein and Lian, 1993].

β-GP is a common in vitro substrate of ALP and its effect on bone metabolism is closely linked to the high levels of ALP but the mechanism leading to differentiation and/or mineral deposition is not completely understood. β-GP

Abbreviations used: ALP, alkaline phosphatase; β-GP, β-glycerophosphate; Pi, inorganic phosphate; pNNPase, *p*-nitrophenyl phosphatase *p*NPP; *p*-nitrophenyl phosphate.

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as well as exogenous Pi has been shown to modulate the amount of cellular ALP activity [Lee et al., 1992; Aronow et al., 1990; Farley et al., 1994] whose increase expresses a shift to a more differentiated osteoblast state [Stein and Lian, 1993]. On the other hand, ALP is known to be essential for mineralization [Whyte, 1989], yet its catalytic activity on organic phosphate is linked to initiating mineralization, but not to its continuation [Tenenbaum, 1987; Bellows et al., 1991; Anderson and Morris, 1993]. ALP is able to function as an ectoenzyme [Low, 1989; Whyte, 1989] and its catalytic parameters contributing to the mineralization process may be modulated by the ALP glycosylphosphatidyl-inositol anchoring to the cell membrane [Hsu et al., 1993; Harisson et al., 1995] and or the enzyme interaction with developing mineral phase or with proteins of extracellular matrix [Genge et al., 1988; Bossi et al., 1993]. However, there is a lack of data on the functioning of the phosphohydrolase activity of the ALP in its integrated state in the presence or absence of mineral deposition. For example, it is not clear whether the effects of β-GP on mineralization reflect changes in the level or kinetic properties of ALP or its subcellular distribution.

Cultured fetal rat calvaria cells grown in the presence of β-GP express osteoblast phenotype markers such as ALP, osteocalcin, and collagen type I and form a mineralizing matrix following a time sequence similar to that observed in vivo [Nefussi et al., 1985, 1989a; Bellows et al., 1986; Aronow et al., 1990; Collin et al., 1992]. We have previously shown that ALP anchored at the surface of cultured osteoblast cells is able to function at physiological pH as a *p*-nitrophenylphosphatase (pNPPase) whose activity variations are correlated with modifications of ^{45}Ca incorporation into cell layers [Anagnostou et al., in press]. In the present study the ALP-mediated effect of β-GP on mineralization was investigated in relation with the kinetic properties of the enzyme on intact cells in its integrated state at physiological pH and in the presence or absence of mineralized matrix. Our data provided direct evidence that the main ectoenzyme implicated in the production of β-GP-derived Pi is ecto-ALP, whose level as well as kinetic properties were not affected by β-GP-derived Pi or β-GP-induced mineralization. High local levels of Pi produced via ecto-ALP-mediated β-GP hydrolysis triggered the initiation of mineral formation. Pi was able to mimic the effect of β-GP-derived Pi on ^{45}Ca incorporation into cell layers, yet ultrastructural analysis showed differences in the foci of mineralization.

MATERIALS AND METHODS

Materials

Culture medium Dulbecco Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY) and fetal calf serum was from Biosys (Compiègne, France). Antibiotics were from Boehringer (Mannheim, Germany). *N*-(Tris(hydroxymethyl)methyl)glycin (Tricine) and 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (Hepes) 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 as well as all other chemical reagents were from Sigma (St Louis, MO). $^{45}CaCl_2$ (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 without removal of suture area and incubated with 2.5 mg/ml (type I) collagenase in phosphate-buffered saline (PBS) for 1 h at 37°C. Cells were then dissociated from partially digested bone fragments by repeated pipetting, total cell dissociation being rapidly obtained. After three washes in PBS, cells were counted and seeded at a final density of 2×10^4 cells/cm^2 in DMEM culture medium supplemented with 10% selected fetal calf serum, 50 UI/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml ascorbic acid. β-GP was added at final increasing concentrations from 1 to 20 mM as indicated for each experiment. The non-adhering cells were removed 4 h after plating. Cells were cultured in a humidified atmosphere containing 95% ambient air and 5% CO_2 at 37°C. The medium was replaced every 48 h and all experiments were performed after 8 days of culture, corresponding to the initiation of matrix nodule mineralization [Collin et al., 1992]. β-GP or Pi (consisting of NaH_2PO_4 and Na_2HPO_4

0.2 M mixed in proportion of 10 parts Na_2HPO_4 to 1 part NaH_2PO_4, pH 7.8) were added to give final concentrations from 1 to 10 mM.

Assays of Phosphohydrolase Activity of ALP

Ecto-ALP phosphohydrolase activity was assessed on intact cells by measuring *p*-nitrophenol release from *p*-nitrophenyl phosphate (*p*NPP). As previously described [Anagnostou et al., 1996], ecto-*p*NPPase assays were realized at 37°C on the cell layers in the culture dish. 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 allowed to occur in buffer A supplemented with 1 mM $MgCl_2$ (assay buffer) and containing various *pNPP final* concentrations. 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. The data presented for each culture corresponded to the mean of values obtained with three successive 10 min-incubation periods. The results are expressed as units (U) of ecto-*p*NPPase activity per mg cell protein. The protein content was measured according to the bicinchoninic acid method using bovine serum albumin (BSA) as a standard (Kit Pierce, Rockford, IL). One unit is the enzyme activity which hydrolyzes 1 nmol *p*NPP per min at pH 7.8 and at 37°C. With *p*NPP as a substrate at physiological pH range, levamisole is a stereospecific uncompetitive inhibitor of ALP [Cyboron et al., 1982]. To distinguish the activity of ecto-ALP from those of other ecto-*p*NPPases, the activity not inhibited by maximal concentrations of levamisole was subtracted, and the levamisole-sensitive ecto-*p*NPPase activity was 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 30 times with 0.6-sec/35-W pulses at 4°C and the levamisole-sensitive *p*NPPase activity was determined on aliquots. In some cases, for comparison with the standard assay at pH 10.2 [Bowers and McComb, 1966], 2-amino 2-methyl-1-propanol buffer was substituted to buffer A.

Calcium Incorporation into Cell Layers

Two sets of labeling experiments in the presence of $^{45}CaCl_2$ were performed, the total calcium concentration of the medium being 2 mM. First, in continuous-labeling experiments, medium was replaced by culture medium whose serum ALP activity was heat inactivated. After standing at 37°C for 10 min, the medium was again replaced by the same one containing ^{45}Ca (1 μCi/ml) and various doses of β-GP, and cells were incubated for 36 h. Second, in short-labeling experiments, cells were incubated for 1–4 h with $^{45}CaCl_2$ (5 μCi/ml) in serum-free medium supplemented with agents to be tested. At the end of incubation cells were rapidly washed 4 times with serum-free medium and incubated at 37°C for four successive periods of 10 min (continuous-labeling experiments) or five successive periods of 4 min (short-labeling experiments). This chase step allowed the release of rapidly exchangeable ^{45}Ca [Anagnostou et al., 1996]. Cells were then washed once with PBS at 4°C and the total cellular material 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. Corrections were made for the nonspecific ^{45}Ca cell labeling by incubating parallel cultures with ^{45}Ca for 1 min at 37°C.

Phosphate Determination

After addition of the agents to be tested, cells were incubated in 1 ml of medium for various times and the phosphate levels in the medium were determined on aliquots after precipitation of proteins and lipid-bound phosphates with trichloroacetic acid. The supernatant was reacted with ammonium molybdate and reduced with the Fiske and Subbarow reagent to form a blue phosphomolybdenum complex measured by absorbance spectrophotometry at 595 nm (kit 670 Sigma).

Light and Transmission Electron Microscopy

Observations of cell cultures were performed with an inverted phase contrast microscope at the end of the culture period (Leitz Diavert). Then medium was removed and cultures were washed three times with serum-free medium, fixed in situ with 5% formaldehyde for 5 min and stained for calcium phosphate salt forma-

tion by Von Kossa method. For electron microscopic observation, after removal of medium, cultures were washed three times with serum-free medium and fixed with Karnovsky fixative solution for 1 h. After several rinses in 0.2 M sodium cacodylate buffer pH 7.2, cells were post-fixed in 1% osmium tetroxide for 1 h, dehydratated in a graded series of ethanols, and embeded in situ in Epon + araldite. Ultrathin sections were obtained with a diamond knife, perpendicularly through bone nodules, and were stained with uranyl acetate for 4 min and with lead citrate for 2 min. They were examined in a Philips CM 12 electron microscope.

RESULTS

Effect of β-GP on Characteristics of Ecto-ALP Activity

Fetal calvaria cells grown in the presence of β-GP express high levels of ecto-ALP activity on which levamisole exerts inhibitory effects correlated with modifications of the mineral deposition [Anagnostou et al., 1996]. Experiments were performed to see how ecto-ALP behaved as a function of the availability of β-GP whose beneficial effects on mineralization are well-known [Bellows et al., 1992]. Mineralization was measured after cells were grown in the absence or presence of various doses of β-GP for 8 days using a 36-h labeling period with $^{45}CaCl_2$ during which the same doses of β-GP were maintained. A clear dose-dependent stimulatory effect of β-GP on ^{45}Ca incorporation into cell layers was observed (Fig. 1A). When compared to cells grown in the absence of β-GP, the stimulation of ^{45}Ca incorporation was maximal (20-fold) for concentrations of β-GP equal to 10 mM with a half-maximal response at 3 mM. In parallel cultures, the activity of levamisole-sensitive ecto-*p*NPPase referred to as ecto-ALP was determined on intact cells using the saturating concentration of 1 mM *p*NPP in the assay buffer. The amount of ecto-ALP activity expressed by cells grown in the presence of increasing doses of β-GP did not differ from that found in cells grown in the absence of β-GP. In addition, Nonidet P-40-extractable ALP activity assayed at pH 7.8 or 10.2 exceeded the value of ecto-ALP activity by 4-fold and 40-fold, respectively, and did not depend on the availability of β-GP during the culture period (Fig. 1B). Furthermore, β-GP at 1–10 mM did not modify significantly protein content or protein synthesis as measured by

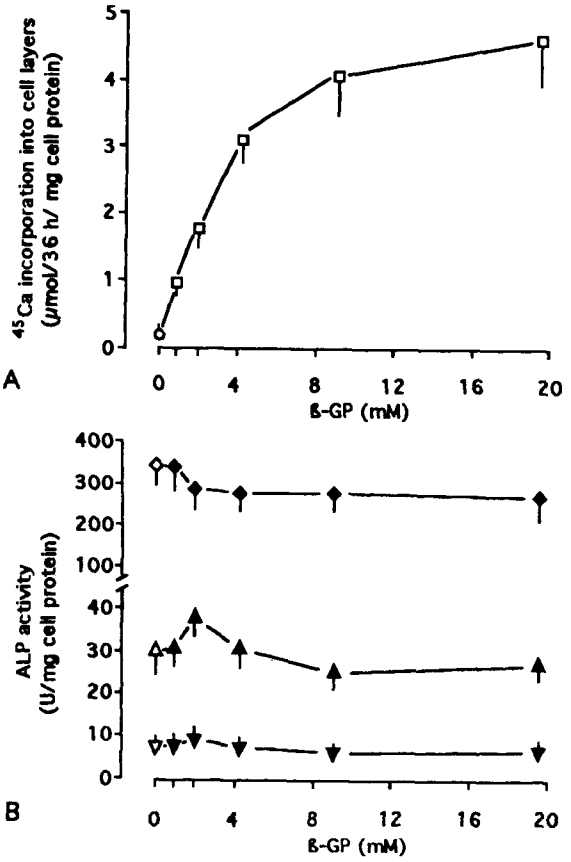

Fig. 1. Dose-dependent effect of β-GP on ^{45}Ca incorporation and ALP activity in cultured fetal calvaria cells. **A:** After cells were grown for 8 days in the presence of 1–20 mM β-GP or in its absence, the medium was replaced by a medium containing serum whose ALP activity was heat-inactivated, supplemented with the same dose of β-GP together with $^{45}CaCl_2$ (1 μCi/ml; specific activity 0.2 μCi/μmol). After a 36-h incubation, ^{45}Ca incorporated into cell layers was determined. **B:** At the same time, ecto-ALP activity was determined in parallel cultures by incubating cells with assay buffer at pH 7.8 containing 1 mM *p*NPP for three successive 10 min periods in the absence and presence of 1 mM levamisole. The ALP activity of NP-40-extractable cellular enzyme was also measured at pH 7.8 and 10.2 with 1 and 7 mM *p*NPP, respectively. **A:** ^{45}Ca incorporation into cell layers (○, □). **B:** Levamisole-sensitive *p*NPPase activities for ecto-ALP at pH 7.8 (▽, ▼) and Nonidet-P-40-extractable ALP at pH 7.8 (△, ▲) and 10.2 (◇, ◆) in cultures grown in the presence of β-GP (□, ▼, ▲, ◆) or in its absence (○, ▽, △, ◇) are represented. Results are presented as means ±SD for measurements of triplicate cultures from the representative experiment shown.

[^{14}C]leucine incorporation during 36 h under conditions described in Figure 1A (data not shown).

Ecto-*p*NPPase activity was also assayed in cells grown with or without 10 mM β-GP in assay buffer supplemented with various concen-

trations of *p*NPP (0.01 to 1 mM) and increasing doses of β-GP (1–10 mM). Values of apparent Km for *p*NPP were close to 0.135 mM, those of Vmax being equal to 7.4 U/mg cell protein, independently of the presence of β-GP in the medium during the culture period (Fig. 2A). Ecto-*p*NPPase activity was inhibited by 10 mM β-GP in a similar way in cells grown either in the presence or absence of β-GP. The results expressed as a plot 1/V versus 1/S indicated that β-GP at concentrations of 1 to 10 mM was a competitive inhibitor which increased apparent Km for *p*NPP without affecting Vmax (Fig. 2B). On the other hand, Nonidet P-40-extractable ALP activity was inhibited by β-GP added to the assay buffer at pH 7.8, but not at pH 10.2 (data not shown). Thus, β-GP proved to be a competitive inhibitor of the phosphohydrolase activity of ecto-ALP at physiological pH range.

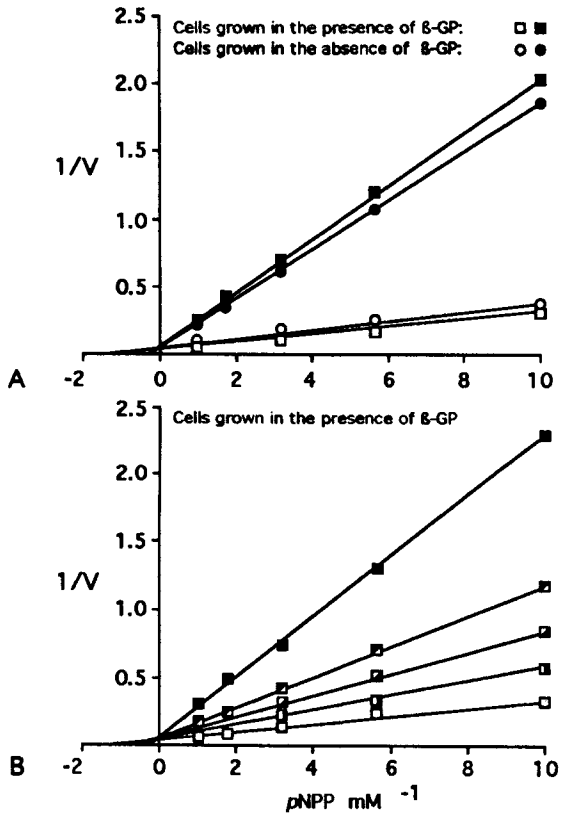

Fig. 2. Effect of β-GP on *p*NPP hydrolysis by ecto-ALP. Parallel cultures were grown either in the presence or absence of 10 mM β-GP. At day 8, cells were incubated at pH 7.8 at 37°C for three successive 10-min periods with the assay buffer containing various concentrations of β-GP and *p*NPP and ecto-ALP activity was determined at the end of each 10-min incubation. The plot 1/V vs. 1/S for cultures grown for 8 days in the presence of 10 mM β-GP (**A,B**: squares) or in its absence (**A**: circles) and incubated for 30 min in the presence of β-GP at 10 mM (■, ●), 4.64 mM (◩), 2.15 mM (◪), and 1 mM (◨) or in its absence (□, ○), are represented.

Ecto-ALP Activity and Generation of Pi in the Medium

β-GP conversion to Pi was studied in relation with the ecto-ALP phosphohydrolase activity to define its role in the β-GP hydrolysis under physiological pH conditions. For this purpose, cells grown with β-GP were incubated in serum-free culture medium containing 0.9 μmol/ml Pi in the presence or absence of 10 mM β-GP together with 1 mM levamisole or dexamisole for six successive 10-min incubations. After the first 10-min period in the presence of 10 mM β-GP, Pi concentration in the medium was increased by 2-fold, the rate of Pi generation (0.6 μmol/10 min/mg protein) remaining constant all the time (Fig. 3A). Levamisole at 1 mM suppressed β-GP-generated Pi without affecting the Pi concentration of β-GP-free medium, while dexamisole was inefficient. Ecto-*p*NPPase activity when measured using successive 10-min incubations in β-GP-free assay buffer did not change during the 60-min experiment (10 U/mg cell protein) and was decreased by 45% when the buffer contained 10 mM β-GP (Fig. 3B). Whether β-GP was present or not, a stereospecific inhibition of the ecto-*p*NPPase activity by levamisole (by 90%) was observed. Exogenous Pi added at 10 mM resulted in a decreased ecto-*p*NPPase activity (by 20%), whose inhibition by β-GP was maintained. When increasing concentrations of β-GP were used, medium Pi concentration increased in a dose-dependent manner, the maximal value (1.8 μmol/ml) being obtained at 10 mM β-GP and the half-maximal one at 3 mM, the stereospecific inhibition of Pi formation by levamisole being observed for all β-GP concentrations (data not shown).

β-GP Conversion to Pi and ^{45}Ca Incorporation into Cell Layers

The time dependence of ^{45}Ca incorporation into cell layers was studied together with β-GP conversion to Pi in cultures grown either in the presence or absence of 10 mM β-GP, which expressed similar ecto-ALP activity level. In cells grown in the absence of β-GP and upon addition of 10 mM β-GP, Pi concentration in the medium increased in a linear manner during the first 30

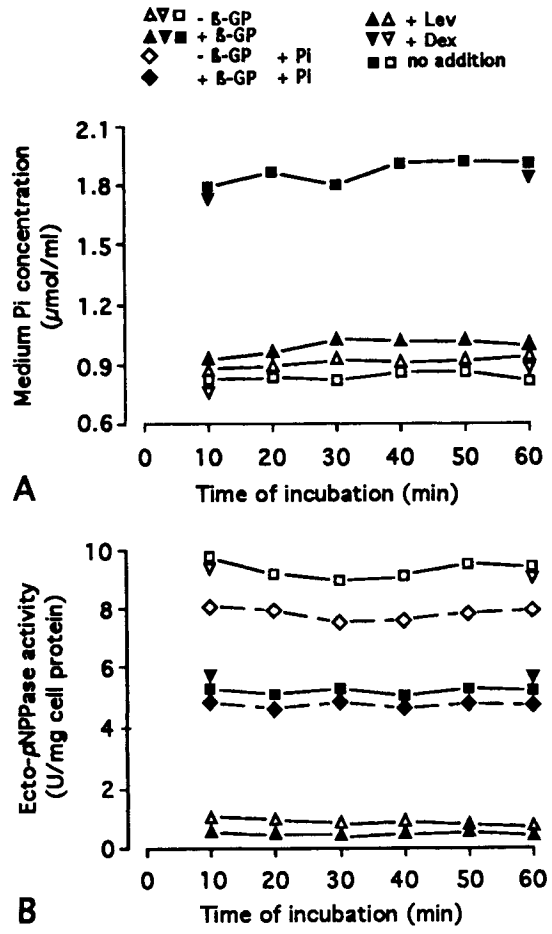

Fig. 3. β-GP conversion to Pi in the medium and ecto-ALP activity. β-GP conversion to Pi (**A**) and ecto-ALP activity (**B**) were measured in parallel cultures grown in the presence of 10 mM β-GP. In (A), medium was replaced by a serum-free medium supplemented with or without 10 mM β-GP and with 1 mM levamisole or dexamisole or vehicle alone. **B:** Medium was replaced by the assay buffer containing 1 mM *p*NPP and supplemented as described in A and also with 10 mM Pi. Cultures were then incubated for successive 10 min incubations, and at the end of each 10 min, Pi levels of the medium (**A**) and ecto-ALP activity (**B**) were determined. Cultures incubated for times up to 60 min with levamisole (▲, △), dexamisole (▼, ▽), Pi (◇, ◆) or vehicle alone (■, □) in the presence of β-GP (solid symbols) or in its absence (open symbols) are represented. A representative experiment is shown where the SD values for measurements of triplicate cultures were in all cases less than 10%.

min at a rate of 90 nmol Pi per min, and then augmented more slowly to reach a plateau after 2 h (Fig. 4A). The time course of Pi generation was identical for cells grown in the presence of β-GP and in both cases, levamisole at 1 mM inhibited Pi generation. When ^{45}Ca incorporation was measured after addition of 10 mM β-GP in cultures previously grown in the absence of β-GP, its rate was low and constant for 4 h (0.05 µmol/h/mg cell protein) and inhibited by levamisole as early as 1 h (Fig. 4B). In cells grown in the absence of β-GP, the rate of ^{45}Ca incorporation was high during the first hour (0.35 µmol/h/mg cell protein) and tended to be lower thereafter, the inhibition of ^{45}Ca incorporation by levamisole being only seen after a lag period of about 1 h. Dexamisole at 1 mM had no

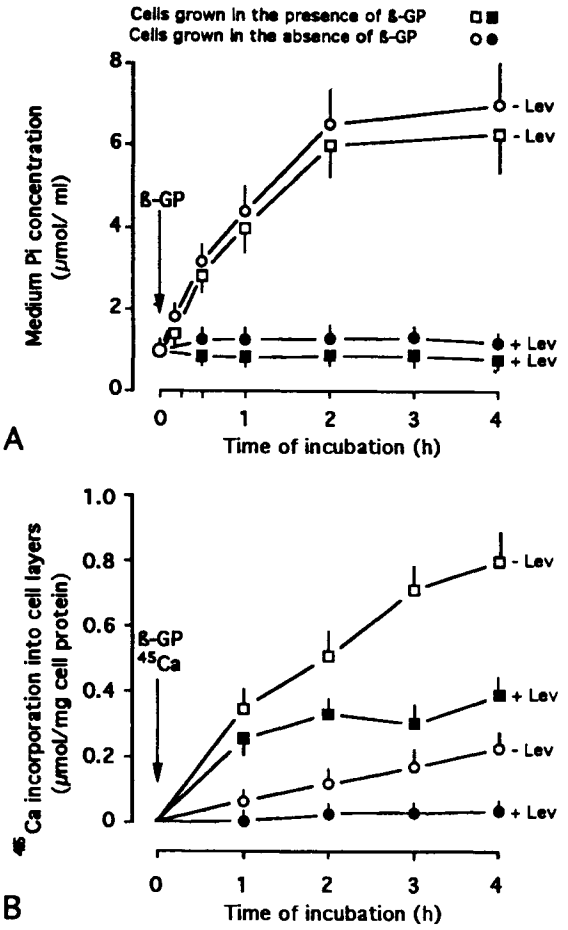

Fig. 4. Time course of β-GP conversion to Pi in the medium and of ^{45}Ca incorporation into cell layers. In parallel cultures grown either in the presence or absence of 10 mM β-GP, medium was replaced at day 8 by a serum-free medium supplemented with 10 mM β-GP and with or without 1 mM levamisole, (time zero of the experiment), one set of cultures receiving at the same time ^{45}Ca (5 µCi/ml; specific activity 1 µCi/µmol). At the times indicated, medium was removed and Pi levels of the medium (**A**) and ^{45}Ca incorporated into cell layers (**B**) were determined. Cultures which had been grown in the presence of β-GP (□, ■) or in its absence (○, ●), and which received at time zero β-GP, together with levamisole (solid symbols) or its vehicle alone (open symbols), are represented. Results are presented as means ±SD for three experiments performed with different cell preparations.

effect on the amount of ^{45}Ca incorporation into cell layers for 4 h (0.76 ± 0.09 vs. 0.84 ± 0.12 μmol/mg cell protein in cells grown in the presence and absence of β-GP, respectively).

Compared Effects of β-GP-Derived Pi and Exogenous Pi on ^{45}Ca Incorporation

In parallel cultures grown in the presence or absence of β-GP, after replacement of medium with one supplemented with either 10 mM β-GP or 9 mM Pi, the rate of ^{45}Ca incorporation was measured as a function of time. ^{45}Ca was added immediately (time zero) or at various times up to 20 h after β-GP or Pi addition and the radioactivity present in cell layers was measured after a 4-h incubation. In cells grown in the absence of β-GP, the initial weak rate of ^{45}Ca incorporation increased progressively from 4 to 12 h following β-GP addition to reach a constant high rate (0.90 μmol/4h/mg cell protein) as from 12 h (Fig. 5A). Such a rate was equal to that obtained in cultures grown continuously with β-GP. Levamisole at 1 mM completely inhibited ^{45}Ca incorporation in cultures which had only received β-GP at time zero in contrast to the partial inhibitory effect (50%) observed in cultures grown continuously in the presence of β-GP. Upon exogenous Pi addition in the medium, the rate of ^{45}Ca incorporation was high as soon as after the first 4-h incubation and identical for cultures grown in the absence or presence of β-GP to remain constant for the five following 4-h incubations. It was unaffected by 1 mM levamisole in both cases (Fig. 5B). The rate of ^{45}Ca incorporation obtained after addition of Pi was similar to that seen with β-GP, except that the latter reached its maximal value only after 12 h in cultures previously grown in the absence of β-GP.

^{45}Ca incorporation was further studied in cells grown in the presence of β-GP after β-GP removal in order to examine to what extent it was dependent on the availability of Pi in the medium. ^{45}Ca incorporation rate showed a decrease during the first 4-h incubation after β-GP withdrawal (by 50%) to remain at the same level up to 24 h despite the levels of medium Pi lesser than 1 mM (Fig. 6A). It must be pointed out that the rate of ^{45}Ca incorporation then obtained (0.4 μmol/4h/mg cell protein) was close to that observed in the presence of levamisole in cultures grown continuously in the presence of β-GP, as seen in Figure 5A. The addition of various doses of exogenous Pi either immediately or 24 h after

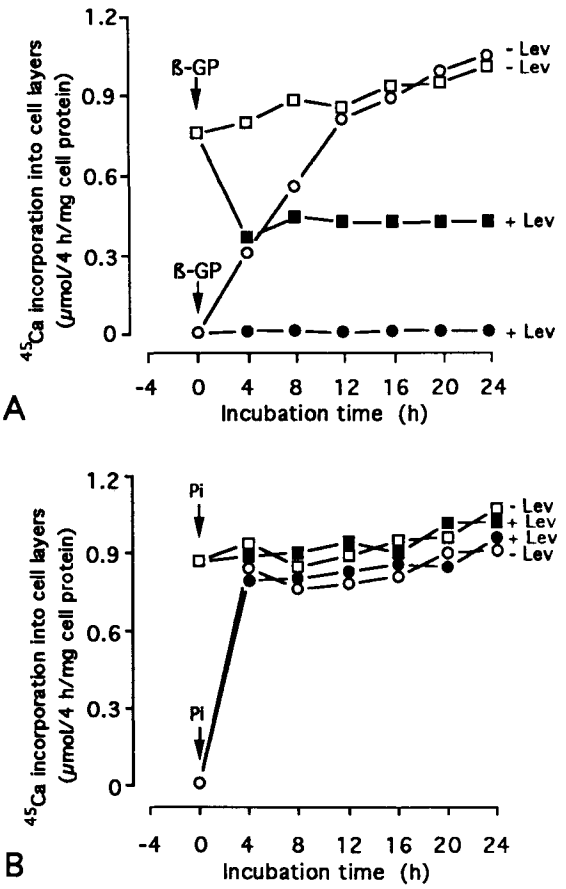

Fig. 5. Time-dependent variations of ^{45}Ca incorporation rate induced after β-GP or Pi addition. In parallel cultures grown in the presence or absence of 10 mM β-GP the medium was replaced at day 8 by a β-GP-free medium supplemented with either 10 mM β-GP (**A**) or 9 mM Pi (**B**) together with or without 1 mM levamisole (time zero of the experiment). At the times indicated, medium was replaced by a serum-free medium containing ^{45}Ca (5 μCi/ml; specific activity 1 μCi/μmol) and supplemented as indicated above. The radioactivity incorporated into cell layers was determined 4 h later. Cultures grown in the presence of β-GP (■, □) or in its absence (●, ○), and that received at time zero β-GP (**A**) or Pi (**B**) together with levamisole (solid symbols) or its vehicle alone (open symbols), are represented. Results are presented as means ±SD for three experiments performed with different cell preparations.

β-GP removal stimulated significantly the ^{45}Ca incorporation rate with a similar dose dependency (Fig. 6B), the values corresponding to 9 mM Pi being close to those obtained in the presence of 10 mM β-GP.

Light and electron microscopic observations were performed to compare β-GP-induced mineralization to the one obtained after a 24 h exposure to Pi in cultures grown in the absence of β-GP for 8 days. Cells grown and maintained in the absence of β-GP developed nodules which did not show any Von Kossa-positive staining

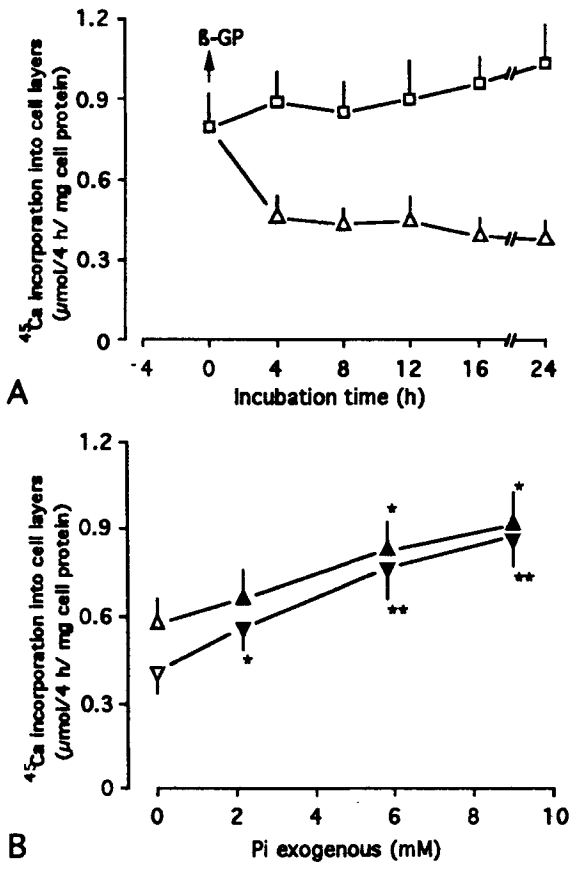

Fig. 6. Effect of removal of β-GP and supplementation with Pi on ^{45}Ca incorporation rate. In parallel cultures grown in the presence of β-GP, the medium was replaced at day 8 by a medium containing or deprived of β-GP (time zero of the experiment). **A:** Medium was further replaced at the times indicated by the respective serum-free medium supplemented or not with 10 mM β-GP, and containing ^{45}Ca (5 μCi/ml; specific activity 1 μCi/μmol). **B:** After β-GP removal, the medium was replaced immediately or after 20 h by a serum-free medium supplemented with the doses of Pi indicated and containing ^{45}Ca (5 μCi/ml). The radioactivity incorporated into cell layers was determined 4 h after ^{45}Ca addition. **A:** Cultures for which β-GP was maintained (□) or removed (△). **B:** Cultures that immediately (▲) or 20 h (▼) after β-GP removal received Pi (solid symbols) or not (open symbols), are represented. Results are presented as means ±SD for three experiments performed with different cell preparations. **B:** $^{*}P < 0.01$, $^{**}P < 0.001$ compared to the corresponding control in the absence of Pi.

(Fig. 7A,B, culture 3). After exposure to either β-GP or Pi for 24 h, a similar positive Von Kossa staining was observed which was confined to nodules (Fig. 7C,D, culture 4; Fig. 7E,F, culture 5). Nodules consisted of a few flat surface cell layers which lay above a row of osteoblast cells under which was observed a fiber matrix with an irregular mineralization front formed of small round areas of mineralization as shown by ultrastructural analysis (Fig. 8). Differences between the mineralization obtained after β-GP and Pi addition were noted. In addition to the mineralization foci present in collagen fibers in both cases (Fig. 8C and D), in cultures incubated with Pi crystallite formation next to intracellular material was revealed (Fig. 8E). The somewhat different mineralization process in the presence of Pi could reflect some cellular damage. These results indicate that the mode of phosphate supply may be an important factor in the mineralization process.

DISCUSSION

Considering that in cultured rat fetal calvaria cells a close correlation exists between the β-GP availability in the culture medium and the increase in ^{45}Ca incorporation, it was of interest to define the level and the functioning of ALP. The presence of β-GP in the culture medium during cell proliferation and matrix maturation did not cause any alteration in the ecto-ALP activity level. Neither was the level of total cellular enzyme activity and protein content modified by β-GP. In contrast to our findings, β-GP decreases the level of ALP activity which was measured per cell in cultured embryonic chick calvaria cells [Tenenbaum et al., 1989] or assayed at pH 10.2 in total cellular extracts from calvaria cells and various cell lines [Tenenbaum, 1987; Chung et al., 1992]. The addition of β-GP in post-confluent cultured calvaria cells or in sub-confluent SaOS-2 cells increases total cellular ALP activity and ALP mRNA levels [Gerstenfeld et al., 1987; Aronow et al., 1990; Lee et al., 1992; Farley et al., 1994], suggesting a direct effect of β-GP-derived Pi or an indirect effect of β-GP-induced mineralization on enzyme synthesis. The discrepancies between the above studies might be explained by the dependence of β-GP-regulated ALP expression on culture cell system and the stage of culture when β-GP is added. Under our experimental conditions, the fact that β-GP did not regulate the total cellular activity of ALP or its expression at the cell surface provided the possibility to compare the catalytic potency of the ecto-enzyme in non-mineralizing and β-GP-induced mineralizing cultures.

Stereospecific levamisole inhibition of β-GP conversion to Pi and competition of β-GP with *p*NPP for the same enzyme binding site evidenced that ecto-ALP mediated the hydrolysis of β-GP on intact cells. Km and Vmax for *p*NNP, β-GP competition profiles and the rate of Pi

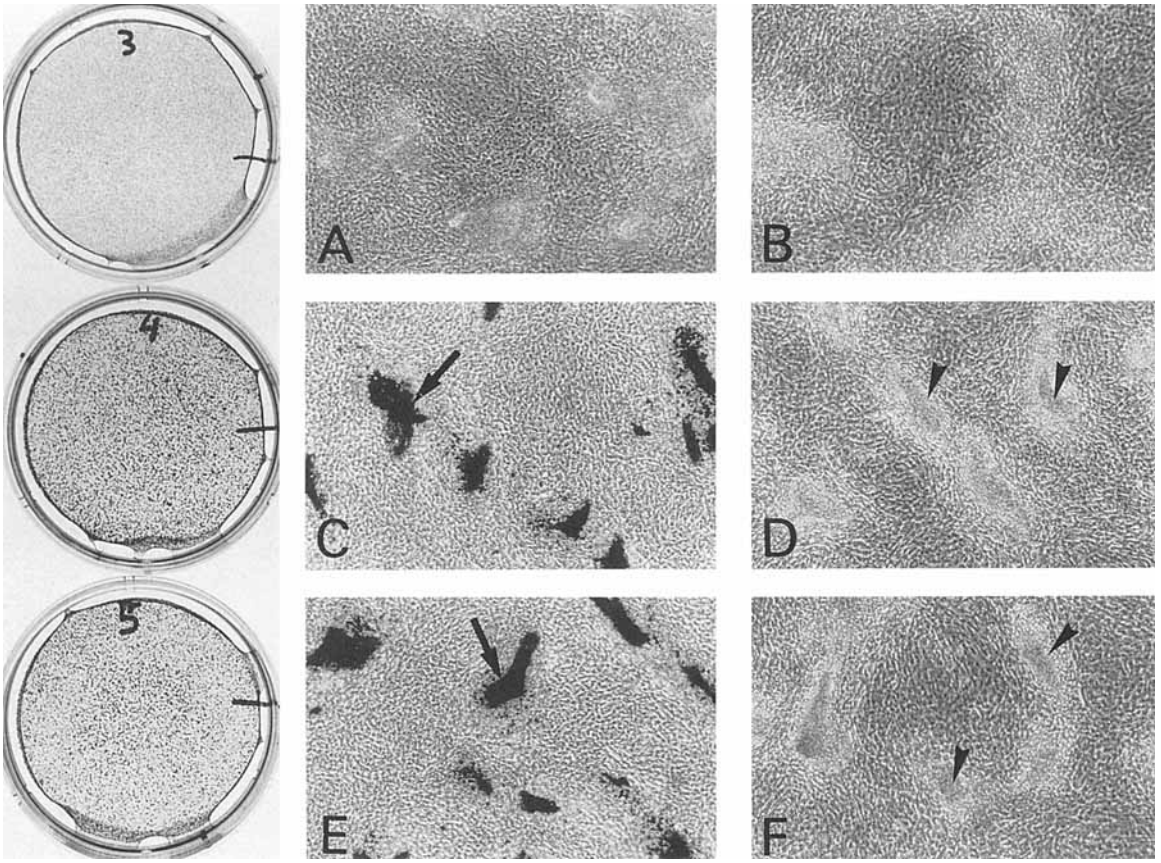

Fig. 7. Compared effect of β-GP and exogenous Pi on Von Kossa-positive nodule formation. In cultures grown in the absence of β-GP, the medium was replaced at day 8 by a β-GP-free medium supplemented or not with 10 mM β-GP or 9 mM Pi. After a 24-h incubation period, phase contrast microscopic observations were performed, and cultures were then fixed with 5% formaldehyde and stained by Von Kossa method. Cultures dishes (3,4,5), and photomicrographs after Von Kossa staining (**A,C,E**) and before Von Kossa staining (**B,D,F**) for cells maintained in the absence of β-GP (culture 3; **A,B**) or which received β-GP (culture 4; **C,D**) or Pi (culture 5; **E,F**) are represented. A similar Von Kossa staining of mineralizing nodules after β-GP or Pi exposure could be noted.

production were similar in cells either grown in the presence or absence of β-GP. Moreover the ecto-ALP activity did not appear to be regulated by Pi generated during the reaction. Pi has been shown to behave as a competitive inhibitor with respect to *p*NPPase activity for both purified and membrane-bound ALP at alkaline and physiological pH range [Farley et al., 1980; Sarrouilhe et al., 1993], the membrane-bound enzyme being less affected at neutral pH [Cyboron et al., 1982]. Our results indicated that ecto-ALP functioning was particular as regards its regulation by Pi. Moreover ecto-ALP was equally efficient for hydrolyzing extracellular β-GP despite striking differences in the mineral deposition. This argues against an interaction of the developing mineral phase with the active site of the enzyme, which has been evoked to explain the loss of the total ALP activity accompanying the accumulation of mineral ions during isolated matrix vesicle mineralization [McLean et al., 1987; Genge et al., 1988]. Our findings showed that the ecto-ALP in its native environment at physiological pH was able to generate Pi from β-GP, its catalytic efficiency being not de-

Fig. 8. Electron micrographs of cross section of mineralizing bone nodules after β-GP or Pi addition. In cultures grown in the absence of β-GP, the medium was replaced at day 8 by a β-GP-free medium supplemented or not with 10 mM β-GP or 9 mM Pi. After a 24-h incubation period, cultures were fixed with Karnovsky solution, and after dehydration they were embedded in Epon + Araldite. The sections were stained with uranyl acetate and with lead citrate. Cultures exposed to β-GP (**A,C,E**) or Pi (**B,D,F**) are represented. (**A,B:** ×5,100) Mineralized matrix (M) is observed in the lower part of the micrographs. A mineralization front (*arrowheads*) could be noted in **A.** (**C,D:** ×16,000) Mineralized matrix (M) in a network of collagen fibers (*small arrow*). (**E,F:** ×35,200) Mineral deposits are clearly associated with collagen fibers in (**E**) while they seem to be associated with intracellular materials in (**F**).

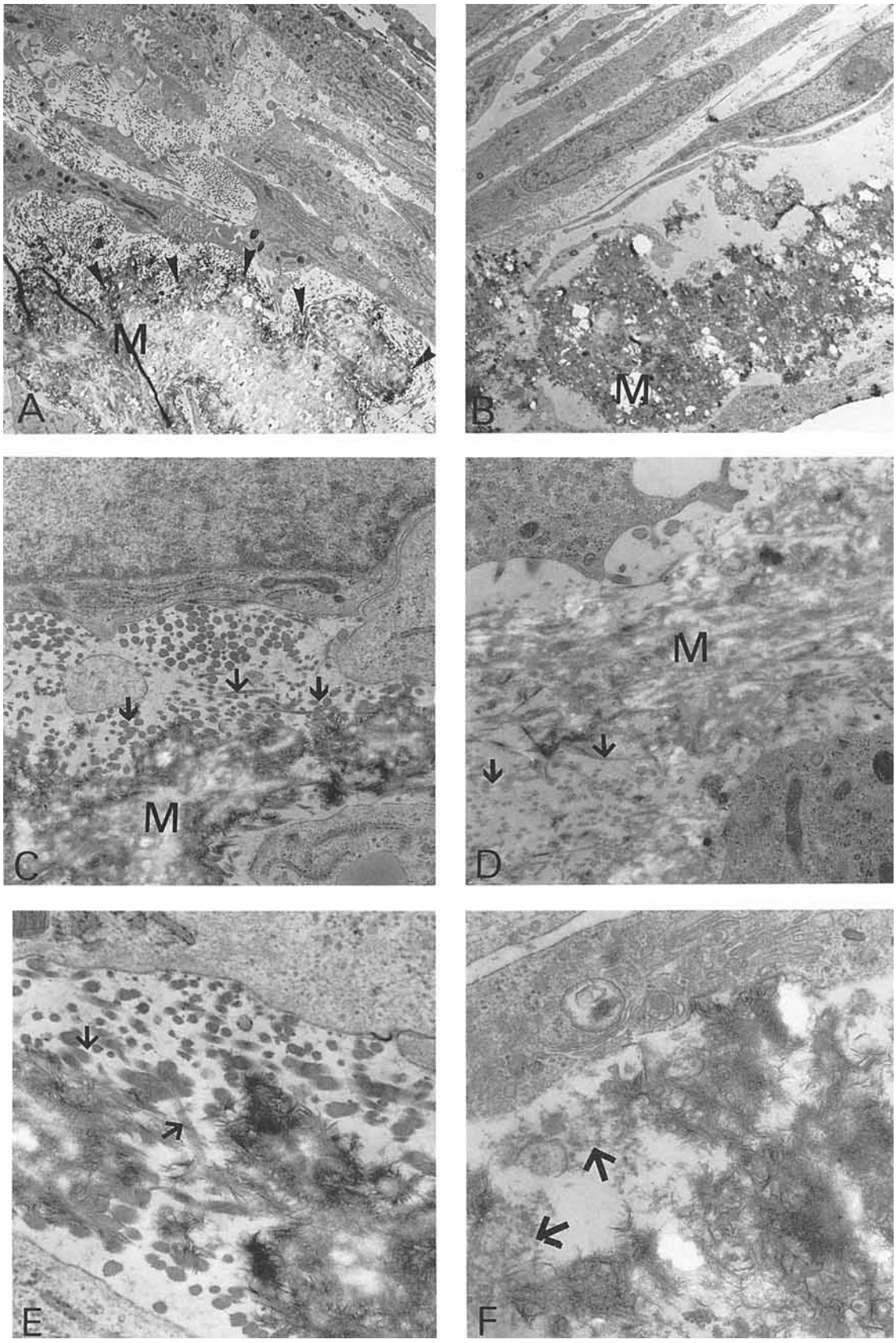

Figure 8.

pendent on the amount of Pi formed and on mineral deposition occurring possibly at some distance from the active site of the enzyme.

Kinetic analysis of the ^{45}Ca incorporation rate after addition of β-GP in cells grown for 8 days in its absence revealed a linear increase up to 12 h. By contrast, after exogenous Pi addition maximal ^{45}Ca incorporation rate was obtained as soon as the first 4-h incubation. This could be due to some time-dependent process induced by β-GP, which was not mimicked by exogenous Pi. At variance with our study, a lag period of 8–12 h has been observed after exposure to β-GP in fetal calvaria cells, which may represent the initial phase of mineralization during which accumulation of Ca and Pi occurs within matrix vesicles [Bellows et al., 1991]. β-GP per se or β-GP-induced mineralization does not modulate the synthesis of osteoblast phenotype components which are implied in the regulation of mineralization, in particular phospholipids, type I collagen, and osteocalcin [Chung et al., 1992; Lee et al., 1992; Kasugai et al., 1991]. It has been suggested that β-GP increases mineralization of the matrix once it is formed, partly by serving as a Pi source and partly by stimulating matrix degradation, facilitating crystal formation and growth (Dean et al., 1994). Such modifications of the matrix dynamics could participate in the progressive development of β-GP-induced mineralization contrasting with the fast effect of Pi expressed in the present work.

The inhibition by levamisole of the stimulatory effect of β-GP on ^{45}Ca incorporation into cell layers appeared more pronounced in cells grown in the absence of β-GP than in cells grown in its presence though the kinetics of β-GP-derived Pi generation in both situations was similar. This indicated a strict dependence of ^{45}Ca incorporation on β-GP hydrolysis by ecto-ALP in nonmineralizing cultures, and a partial one in mineralizing cultures in which ^{45}Ca incorporation was half-decreased after β-GP removal and resumed following addition of exogenous Pi. Other studies have shown that levamisole prevents β-GP hydrolysis in culture medium and ^{45}Ca incorporation during the initiation phase of β-GP-induced mineralization [Tenenbaum, 1987; Bellows et al., 1991, 1992]. ALP activity has been also reported to be implicated in calcium and phosphate uptake but not in crystal growth per se [Register and Wuthier, 1984]. The cell system used in the present work has been characterized by a first synchronized generation of nodules followed by successive nodule generations as revealed by microcinematographic kinetic studies (Nefussi et al., 1989a). Our data indicated that the availability of high local levels of Pi via ecto-ALP-mediated β-GP hydrolysis triggered the initiation of mineral formation in the different generations of nodules, the continuation of mineral deposition being likely able to proceed in the absence of high levels of medium Pi.

Exogenous Pi enhanced mineralization to a similar extent to that seen for medium Pi concentrations corresponding to β-GP-derived values, independent of the presence of β-GP during the culture period. Morphological observations demonstrated the same apparent development of positive Von Kossa staining in nodules from cultures exposed for 24 h to Pi or β-GP, whereas ultrastructural analysis showed differences in the foci of mineralization. In addition to the mineralization of fiber matrix, Pi showed some cell damage with mineral formation in association with intracellular material. During β-GP induced mineralization, deposits of needle-shaped mineral associated with proteoglycans and lipid material have been observed along the collagen fibers and at matrix vesicle surface (Nefussi et al., 1985; 1989b; 1992). It has been shown, using an in vitro mineralizing system that the mineral formed in the presence of β-GP and ALP is more apatitic than the one formed from Pi [Golub et al., 1992] and that the mineral formed is influenced by ALP anchor (Harrison et al., 1995). Our results are in line with these studies suggesting an altered physicochemical state of nascent Pi from organic phosphate and a crystallization pathway dependent on Pi source. This led us to postulate that despite the same extent of Pi and β-GP effect on ^{45}Ca incorporation into cultured fetal calvaria cells, the mode of Pi supply via the action of ecto-ALP on organic phosphate could be more appropriate for mineralization.

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