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Cell-Mediated Mineralization in Culture at Low Temperature Associated With Subtle Thermogenic Response

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Abstract In both the growth plate and in marrow stromal cell cultures cell-mediated mineralization is preceded by characteristics of anaerobic and low efficiency energy metabolism. Reagents that increase mineralization like malonate and dexamethasone (DEX) also increase the mitochondrial membrane potential (MtMP) especially 1 week after DEX stimulation. Contrarily, levamisole, which decreases mineralization, also decreases MtMP. Modulation of MtMP and energy metabolism could be linked to regulation of mineralization by the uncoupling of oxidative phosphorylation. This uncoupling should be associated with thermogenesis in cells that induce mineralization. We examined whether cold temperature affects mineralization, and whether cellular thermogenesis takes place at cold temperature in parallel to changes in MtMP. Osteoprogenitor cells (OPC) induced, in DEX stimulated rat marrow stroma, higher mineralization at 33°C than at 37°C. Increased mineralization by cold temperature required long incubation since incubation in the cold during short intervals, 3–4 days, did not increase mineralization relative to (37°C) controls. Marrow stromal cells in the presence of valinomycin responded to incubation at 33°C by retaining all the vital dye after 4 h, unlike the cells at 37°C; however, after 24 h the level of dye retention at 33°C was the same as at 37°C. The delayed response of the temperature-dependent (> 37°C) K^+ ionophor to incubation in the cold indicated that certain cells may respond to low temperature by local intracellular heating, and by heat conduction to the plasma membrane. DEX-stimulated stromal cells, unlike unstimulated cells, showed increased mitochondrial rhodamine 123 retention in the presence of valinomycin after 24 h in the cold, which corresponds to day 4 of OPC induction. This is consistent with the concept that valinomycin-induced cell damage is mediated by (cold-induced) local heating. The mechanism of this cell damage should selectively prefer non-thermogenic (rhodamine retaining) over thermogenic (rhodamine leaking) cells such as OPC. At cold temperature DEX-stimulated stromal cells showed the best anti-OPC selection under exposure to valinomycine between days 3–7, concurrent with the period of rhodamine leakage from the mitochondria. These results indicate that thermogenesis is enhanced during the period of low MtMP in mineralizing cells, and prolonged exposure to cold increases mineralization also due to induction of subtle thermogenesis.

Key words: thermogenesis, osteoprogenitor cells, valinomycin, mitochondria, inner membrane, rhodamine 123, uncoupling, oxidative phosphorylation

Mineralization depends on the accumulation of calcium and phosphate ions in the extracellular matrix of bone, teeth, and cartilage. This, in turn, is dependent on cellular Ca [Wuthier, 1993; Zimmermann et al., 1994], and phosphate [Montessuit et al., 1994] accumulation. The mitochondria (in addition to extracellular matrix) was implicated long ago as a potential regulator of mineralization based on changes in energy metabolism associated with calcium fluxes [Lehninger, 1970; Shapiro and Lee, 1975; Shapiro et al., 1982; Brighton and Hunt, 1978; Posner, 1978]. In the growth plate, these changes are associated with a local change in oxygen tension [Haselgrove et al., 1993] and a switch from aerobic to anaerobic metabolism in which the mitochondria plays a central role. In soft tissues, under aerobic conditions, mitochondrial accumulation and release of calcium are respiration dependent. However, the release of calcium occurs via channels different than those of calcium uptake [Lehninger et al., 1978a]. Calcium release can occur by Na^+-dependent or Na^+-independent mechanisms and also by a nonspecific way [Richter, 1992]. It is not known how such calcium cycling contributes to mineralization in calcified tissues. In several soft tissues mitochondrial calcium retention is associated with a high NADH/NAD ratio, whereas calcium release in

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exchange for protons is associated with a low NADH/NAD ratio [Lehninger et al., 1978b]. This ratio should depend on the respiration level which is reflected by the membrane potential. Thus, the high NADH/NAD ratio found in hypertrophic chondrocytes [Shapiro et al., 1982] agrees with a low oxygen tension, and with calcium retention by the mitochondria in this zone of the growth plate. Profiles of membrane potential in the growth plate are not known. It is possible that after a sustained low membrane potential at the hypertrophic zone, membrane potential should increase prior to mineralization in front of the zone of provisional calcification. This should be expected on the basis of studies with a different osteogenic differentiation system (cultured rat marrow stromal cells) in which a drop in mitochondrial membrane potential (MtMP) has been shown to occur from days 3–4 of subconfluency, and maintained up to day 10 [Klein et al., 1993c]. When cells were stimulated to mineralize by dexamethasone (DEX) medium, the MtMP had a similar pattern but with a twofold increase in MtMP and an eightfold burst in MtMP on day 7–8 [Klein et al., 1993c]. In this system, in which mineralization becomes detectable on day 13–14, such an MtMP surge could be a prelude to mineralization. Inhibition of succinate dehydrogenase (SDH, complex II) by malonate results in osteoprogenitor cell (OPC) selection and a 20% increase in mineralization. The maximal efficiency of OPC selection is concurrent with the period of lower MtMP [Klein et al., 1993a]. Levamisole, as opposed to malonate, was shown to inhibit mineralization by a manner which was unrelated to its inhibitory effect on alkaline phosphatase [Klein et al., 1993b]. The opposing effects of malonate and levamisole on mineralization are accompanied by opposing effects on the profile of the MtMP during OPC differentiation (day 3–7) [Klein et al., 1996]. The period of low MtMP seems important in initiation of mineralization. Its growth plate version is characterized by low ATP reserves and by calcium efflux which is consistent with uncoupling of oxidative phosphorylation. Therefore it is possible that uncoupling is involved in exchanging protons for mitochondrial calcium. Reshuffling protons through the inner membrane via channels alternative to the ATP synthase causes thermogenesis [Nedergraad and Cannon, 1992]. It is therefore relevant to ask whether induction of thermogenesis in cell culture would affect mineralization. Furthermore, is it possible to induce thermogenesis in cultured cells other than brown fat, such as stromal OPC? In the present work we examined the ability of OPC to increase mineralization in association with thermogenesis in response to cold temperature.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate, valinomycin, neutral-red (NR) and rhodamine 123 were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY).

Stromal Cell Culture

Bone marrow cell suspensions obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25 cm^2 flasks, 10^8 cells/flask [Maniatopoulos et al., 1988]. Stromal cells were obtained by removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium that consisted of DMEM (Dulbecco modified Eagle's medium) suplemented with 15% fetal calf serum and antibiotics. The cells were incubated at 37°C in a humid 9% CO_2 atmosphere, as in all our previous studies, since increased CO_2 is associated with a decreased redox level in differentiating bone cells. For experiments involving mineralization, stromal cells were removed 2 weeks later by trypsinization and were plated in 96 well microtiter plates, 5,000 cells/well, and grown in ordinary medium or osteoprogenitor cell (OPC) stimulation medium. This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate, and 10 mM β glycerophosphate (DEX medium). Media were changed periodically as indicated in the text or the figure legends. For cells grown in the cold the temperature was either 30 ± 1 or 33 ± 1°C, compared with 37°C controls. For experiments in which mineralization was not examined the culture conditions are described in the text.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day-11 of dexamethasone stimulation was set for ALP assay and cell count [Klein et al., 1993a]. Growth

medium was removed and the cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TBS, 1.33 mg/ml was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min and optical density of the hydrolysed pNPP was measured in a multichannel optical densitometer at 405 nm wavelength. This protocol enables long incubation times as hydrolysis is 30 times slower than the usual protocol [Klein et al., 1993b]. ALP specific activity was calculated as nMol/90 min/50,000 cells.

Quantitative Cell Staining

After the ALP or rhodamine retention assays, cells at the lower half of the plates were counted using the methylene blue (MB) staining method. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O and air dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water and air dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. OD of the eluted MB was measured at 620 nm by a multichannel optical densitometer. Cells seeded at different seeding levels on day 0 (250, 500, 1,000, 2,000, and 4,000/well). On days 10 and 13, close to the usual time for cell counting in our experiments, the cells were divided in 2 groups. Four wells for each were trypsinized and counted and in parallel 8 wells were MB stained. Linear regressions for counts and MB staining on both days 10 and 13 were practically coalescent and indicate that linearity of the OD reading at least up to 73,000 cells/well, 1.0 U, is equivalent to 5×10^4 stromal cells.

Cell Viability Test

The neutral red (NR) cell viability test described by Lowik et al. [1993] was used with a modification. NR, 0.2 ml of 0.01% in phosphate buffered NaCl (PBS), was added to each well after removal of the growth medium, and incubated for 40 min at 30°C. Excess NR dye was washed once with PBS, retained NR was eluted from intracellular microsomes by incubation in 1% $NaHPO_4$ in 50% ethanol for 10 min with gentle shaking. The yellow eluate (in PBS) was read on a plate reader of an Anthos absorption photometer using a 450 nm filter.

Rhodamine Retention Measurement

Rhodamine 123 (Rho) is a cationic dye trapped by the mitochondria depending on the content of its negative charges, and directly related to the inner membrane potential [Chen, 1989].

Rho stock solution × 20 in distilled water was added to the cultures, 10 μl/well. After an incubation period as indicated, the Rho containing medium was removed. The cells were washed twice with TBS (50 mM Tris pH 7.6, 150 mM NaCl) and continued their cultivation after the short incubation period untill Rho retention measurement on the following day.

After removal of the growth medium and washing with TBS the cells were incubated for 30 min at 37°C with 150 μl of 0.2% Triton-X 100 to dissolve membranes and to release intracellular Rho. The disolved cell sap (of 5 pools of 4 wells, 20 wells/sample) was transferred to 1 ml cuvettes (cells). Rho content was measured in a Perkin Elmer (Oak Brook, IL) LS-5B luminescence spectrometer by exitation at 505 nm and emission at 540 nm.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, OPC stimulation medium as opposed to ordinary medium induces cell mediated calcifications in uninhibited cultures [Klein et al., 1993b]. To measure the precipitates plates were washed twice with TBS and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by the arsenazo-III metallochromic method adapted to microplates [Klein et al., 1995] against samples of known calcium concentrations. Mineralization is expressed as μg calcium/well. Under these conditions calcium phosphate is precipitating at a ratio of 1.6:1 and detectible either as amorphous or after transition to apatite [Maniatopoulos et al., 1988]. Results are best comparable with controls from the same plate since controls are affected by the experimental well perhaps by a volatile effect [Klein et al., 1994].

RESULTS

Cell Mediated Mineralization at Low Temperature

Figure 1 shows a quantitative comparison of cell proliferation and activity of two differentiation markers, between DEX-stimulated stromal

cells cultured at 37° and 33°C. To exclude a putative effect of alkaline pH on mineralization, the medium was buffered by 20 mM HEPES. Figure 1A shows that day 12 specific ALP activity increased in DEX medium at 37°C more than at 33°C. Reciprocally, cell number increased in ordinary medium at 33°C more than at 37°C (Fig. 1B). Mineralization at 33°C was higher than at 37°C. When HEPES buffer was excluded from the medium the pattern of specific ALP activity and cell counts remained similar to that obtained with HEPES (not shown), although in the buffered medium the absolute level of cell counts has diminished. Without HEPES, in the cold temperature, mineralization was higher both in ordinary and in DEX medium than in the presence of HEPES probably due to the increased pH. These results indicated that cell-mediated mineralization increases in response to lower temperature beyond its response to pH changes.

Temporal Dissection of Cold Temperature Effect on Mineralization

Cultures stimulated with (DEX) and without dexamethasone (bGP + Asc) were exposed to 33°C for different time intervals and during the remaining time the cells were cultured at 37°C. Figure 2 shows the mineralization measured on day 21 in all cultures including the same media incubated without cells. The pattern of the 37°C control was similar to that seen in the respective Figure 1C; however, none of the relations of partial exposures to cold vs. control was comparable to the relation between mineralization in cold and normal temperature seen in Figure 1C. This indicates that in order to obtain increased mineralization at cold temperature, a prolonged and continuous exposure to cold is required; otherwise, at short intervals, cold temperature is rather inhibitory to mineralization. In most of the samples the presence of cells significantly contributed to mineralization under these conditions. In the controls (see control panel in Fig. 2) there was no mineralization of DEX medium without cells, clearly requiring the presence of cells. Results in Figures 1C and 2 also indicate that the presence of HEPES abrogated the effect of DEX medium on mineralization perhaps in addition to its pH buffering capacity, since also at 37°C with whole DEX medium the mineralization was lower than with bGP + Asc without dexamethasone.

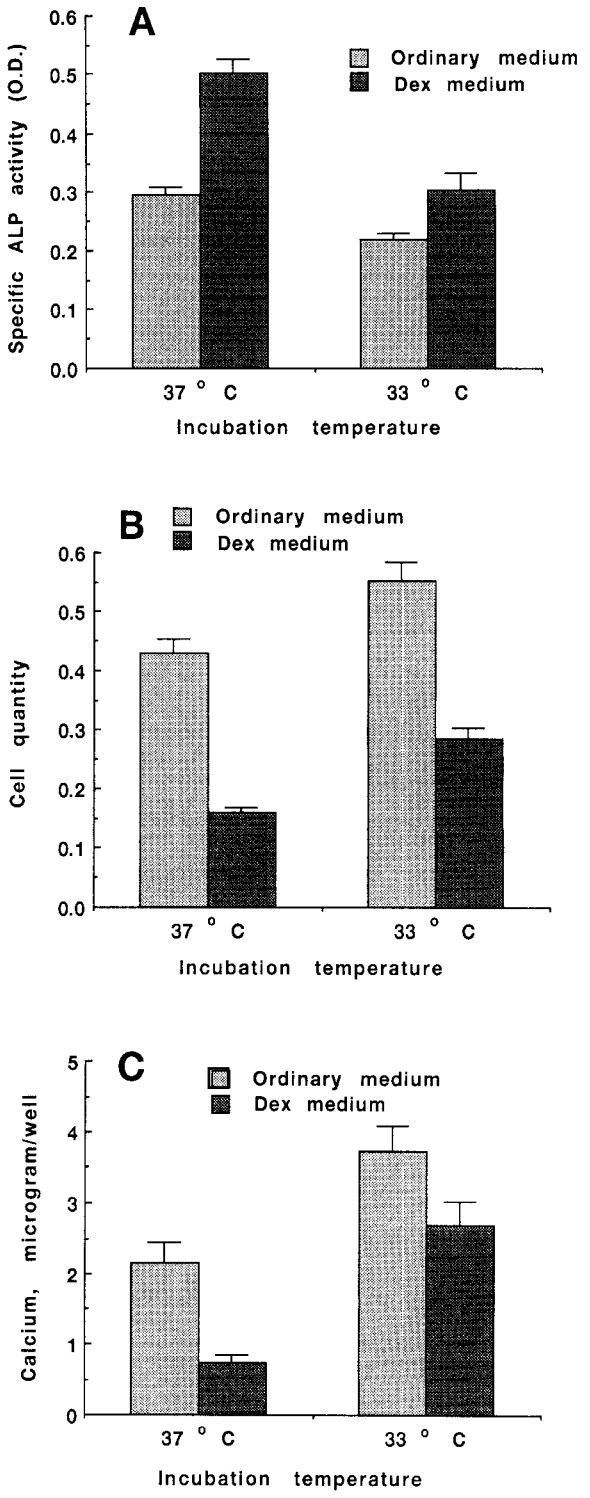

Fig. 1. Osteogenic stimulation of marrow stromal cells in the cold. Cultures were stimulated with whole DEX medium from day 0 to 21. Media were changed on days 0, 7, 12, and 16. **A:** Day 12 specific ALP activity. **B:** Day 12 cell proliferation. **C:** Day 21 cell-mediated mineralization. The differences between calcium/well at 33° and 37°C are significant under both ordinary and DEX-medium. The media contained 20 mM HEPES buffer and the cold incubator was set at 33°C. Each sample represents the mean ± SEM, n = 20.

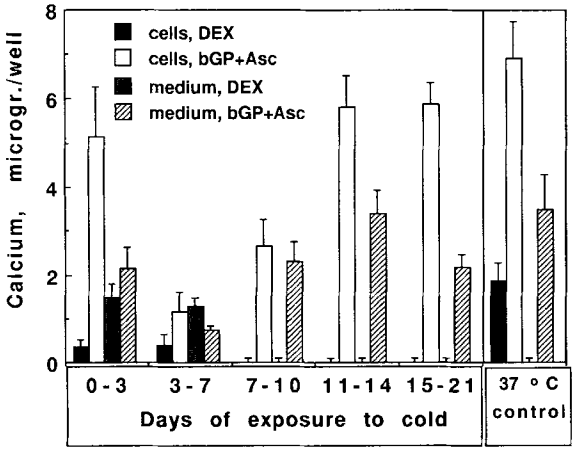

Fig. 2. Dissection of exposure to the cold of differentiating stromal cells and its effect on mineralization. DEX-stimulated cultures were transferred from the warm to the cold incubator on indicated days. Day 21 calcium deposits were measured after removal of the media and after washing soluble calcium from the wells with TBS. Mineralization is compared with controls which were cultured at 37°C during the entire period. Media were changed on days 0, 7, and 14. DEX = whole DEX medium; bGP + Asc = medium without DEX; cells = stromal cell culture; medium = medium incubated without cells. Each bar represents the mean ± SEM, n = 20.

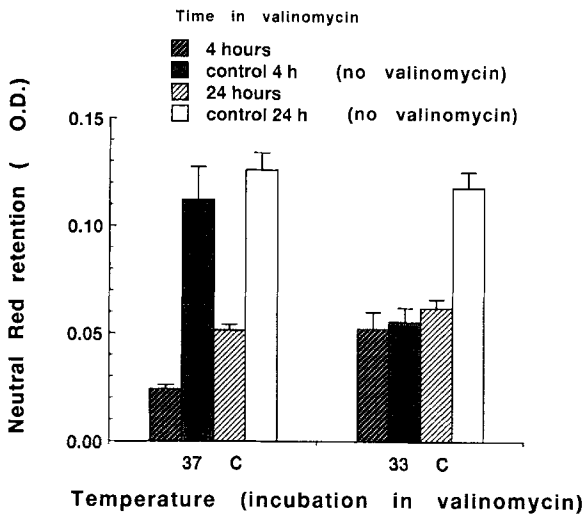

Fig. 3. Temperature effect on vital-dye retention by unstimulated stromal cells in the presence of valinomycin. Cells seeded on day (−)3 and cultured in ordinary medium at 37°C were removed to room temperature on day 0 for 10 min to cool. Valinomycin (10^{-8} M) was added to one half of each plate and the plates were quickly placed in either 37° or 33°C incubator. At indicated time points the respective plates were removed to room temperature and neutral red (NR) was added and, after washing of excess NR, cell-retained NR was eluted and counted (n = 40 per sample).

Temperature-Dependent Sensitivity of Stromal Cells to Valinomycin

Stromal cells seeded in microtiter plates on day (−)3 and cultured at 37°C up to day 0, were then transferred to room temperature for 10 min. Valinomycin was added in one half of each plate to a final concentration of 10^{-8} M and the bare solvent of the corresponding stock solution was added to the second half of each plate. The plates were than divided between two incubators, the temperature of one set at 33°C and the other at 37°C, both at 9% CO_2. Cells were not DEX-stimulated for this analysis. At the end of the specified temperature and valinomycin incubations, neutral red (NR) was added to all samples, which were then shortly incubated with the dye for an additional 60 min, both groups at 33°. Figure 3 shows the vital dye (NR) retention in cultured cells exposed to valinomycin for 4 and 24 h. The NR retention by controls, which were not exposed to valinomycin, shows that after 4 h control, cultures at 37°C contained twice as much NR than the controls at 33°C. Therefore the temperature effect on the uptake of the indicator dye was normalized. After 4 h of incubation at 37°C less then 20% of NR resisted the exposure to valinomycin, whereas at 33°C practically all the NR was retained. However, after 24 h the level of NR retention at 33°C was about the same as at 37°C. Namely, after 24 h the valinomycin effect on cell vitality at 33°C has closed the gap with its effect on vitality at higher temperature. At 33°C the lipid bilayer membrane structure does not permit valinomycin movement [Krasne et al., 1971; Stryer, 1988]. Therefore these results indicate local heating of the membrane as a response to cold temperature. Figure 4 shows, in a similar experiment, that such a thermogenic response can occur already after 18 h. In this experiment cell counts, obtained by methylene blue (MB) staining, were normalized (Fig. 4B). The MB staining was not parallel to the NR vital staining but the results were indicative of a similar effect on the cells.

Mitochondrial Rhodamine Retention in Valinomycin-Loaded Plasma Membranes in the Cold

We tested the fidelity of the rhodamine retention assay in valinomycin-loaded plasma membranes after incubation in the cold. Stromal cells seeded on day (−)3 were grown during the entire experiment in ordinary medium, and only up to day 0 at 37°C. On day 0 the mitochondria was loaded with 1 μg/ml of rhodamine for 1 h at 37°C and then transferred to a 30°C incubator. On day 1 the valinomycin was added to the left

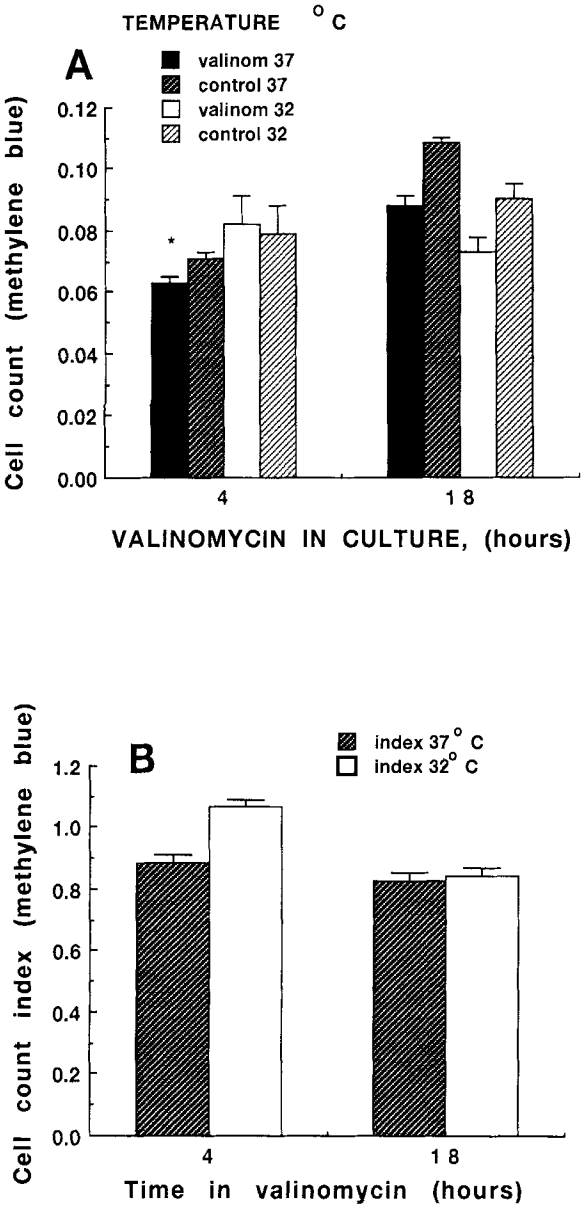

Fig. 4. Temperature effect on methylene blue cell counts of stromal cells cultured in the presence of valinomycin. Unstimulated stromal cells. **A:** Cell counts in the presence and absence of valinomycin. * (asterisk) indicates that after 4 h the difference between cell counts with valinomycin and controls was significant at 37° but not at 32°C. **B:** Cell index, valinomycin treated/control cell counts. Cultures were treated as described in Figure 3 but instead of loading with NR the cells were fixed with glutaraldehyde at indicated time points for methylene blue staining. n = 40.

half of the plates. Figure 5A shows that after 4 h valinomycin does not affect the cell count (as in Fig. 3, 33°C). However, later the control cell counts gradually increase relative to valinomycin treated cultures. Figure 5B shows a slight but insignificant increase in rhodamine retention per cell in the presence of valinomycine at 4 and 25 h in the cold. This indicates that the rhodamine retention assay is not significantly affected at 4 and 24 h by the presence of valinomycin, and after 49 and 73 h (equivalent to days 3 and 4 of DEX-stimulated cultures) the values in treated cultures were almost equal to controls. Additionally, the pattern of rhodamine retention in the presence of valinomycin on days 1–4 is preserved relative to unstimulated controls as previously shown by Klein et al. [1993c]. Figure 5A also shows that not all the stromal cells persist under valinomycin. This is consistent with the presence of 2 cell populations, one that is resistant and a second smaller population that is sensitive to valinomycin.

Rhodamine Retention in the Cold Under DEX Stimulation of Osteoprogenitor Cells

Stromal cells seeded on day (−)3 and stimulated on day 0 with DEX medium were subjected on day 3, for 1 h, to mitochondrial loading with rhodamine using a medium that contained 1 μg/ml of rhodamine 123. On day 4 plates were transferred to cold incubation (30°C) for 1 h. Subsequently, before the incubation with DEX medium was continued, the plasma membranes of cells at the left half of each plate were loaded with valinomycin by growing the cultures continuously in 10^{-7} M of this ionophor. The cell counts in Figure 6A confirm the cytolytic response to valinomycin in the cold, which is used here as a marker for thermogenic response to cold temperature. Figure 6B shows that rhodamine retention per cell, 4 h after addition of valinomycin, was 50% lower than in the controls and it has doubled 24 h later to reach and slightly pass the controls. Most of this is related to the increased cell counts after 4 h in the valinomycin treated samples. Notice that 4 and 24 h of valinomycin presence corresponds to days 4 and 5 of DEX stimulation, respectively, unlike the presentation in Figure 5 where 4 and 24 h of valinomycin presence correspond to day 1 and 2 of the (DEX) unstimulated controls. There was a lower cell count after 25 h associated with valinomycin, relative to controls, at 30°C (Fig. 6A). This was in face of a significant but relatively small increase in specific Rho retention (Fig. 6B). This may indicate that the mitochondria of valinomycin resistant cells show almost no change in Rho retention. Therefore, it is possible that the valinomycin sensitive cells have an increased Rho retention capacity, since

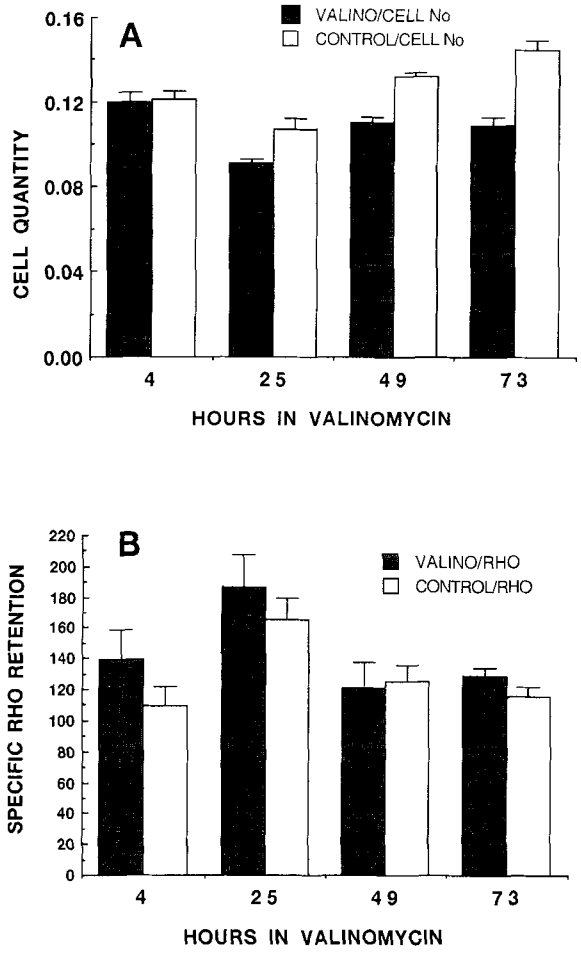

Fig. 5. Mitochondrial rhodamine retention at cold temperature in unstimulated stromal cells in the presence of valinomycin. Cells seeded on day (−)3 were grown at 37°C and on day 0 the ordinary medium was replaced by fresh ordinary medium. On day +1 the cells were loaded with rhodamine 123 (1 μg/ml for 1 h), and after restoring the growth medium the cells were transferred to the cold incubator, after adding valinomycin to a half side of each plate. **A:** At indicated time points retained rhodamine was extracted and fluorescence units for pools of 5 wells each (8/sample, n = 40) were divided by the methylene blue (MB) OD. The MB OD was derived from cells counted in respective pooled wells of appropriate separate plates. **B:** Cell counts/well. n = 40.

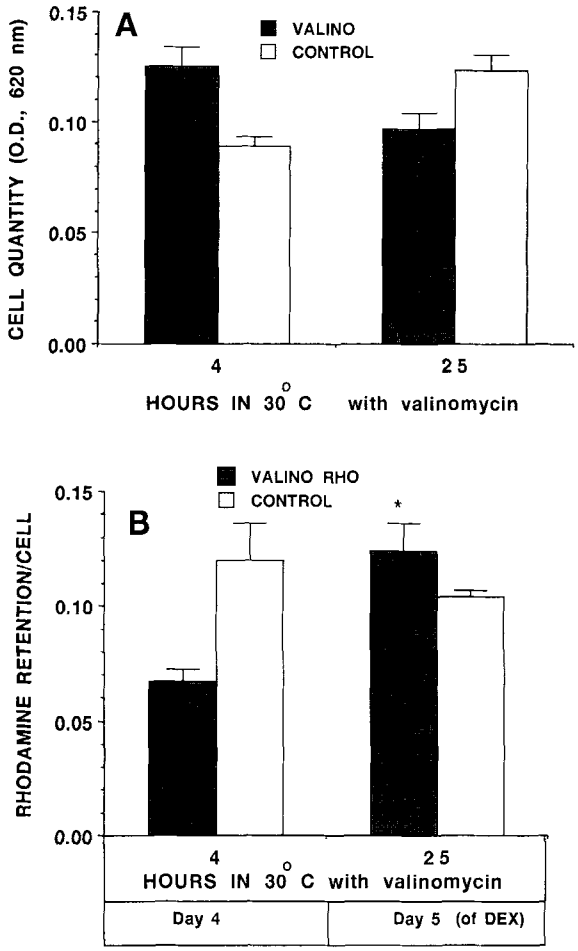

Fig. 6. Mitochondrial rhodamine retention at 30°C in DEX-stimulated stromal cells in the presence of valinomycine. Cell treatment was similar to that in Figure 5. However on day 0 the ordinary medium was changed to DEX medium. **A:** Cell count/well. **B:** Specific rhodamine retention. n = 40. *Slight but significant increase in Rho retention.

in a previous work DEX stimulation showed a twofold increased Rho retention in overall unselected stromal cell population [Klein et al., 1993c].

Selection of Non-Mineralizing Cells by Valinomycin

Figure 7 shows a temporal dissection of exposure to valinomycin of DEX-stimulated stromal cells incubated in the cold (30°C). The combined effect of temperature and valinomycin on mineralization and on cell count was tested. Valinomycin increased the cell counts mainly when applied during the first 7 days, but it decreased mineralization concomitantly. This indicates that valinomycin has either selected non-mineralizing cells or inhibited their mineralization at cold temperature. The decreased mineralization in Figure 7 is parallel to the minute change in Rho retentin at 25 h in Figure 6B, in spite of a decreased cell count at 25 h (Fig. 6A). This favors the possibility of negative selection against those mineralizing cells responsible for the fraction of calcium deposition which is associated with thermogenesis.

DISCUSSION

The increased mineralization below physiological temperatures was quantitatively unrelated

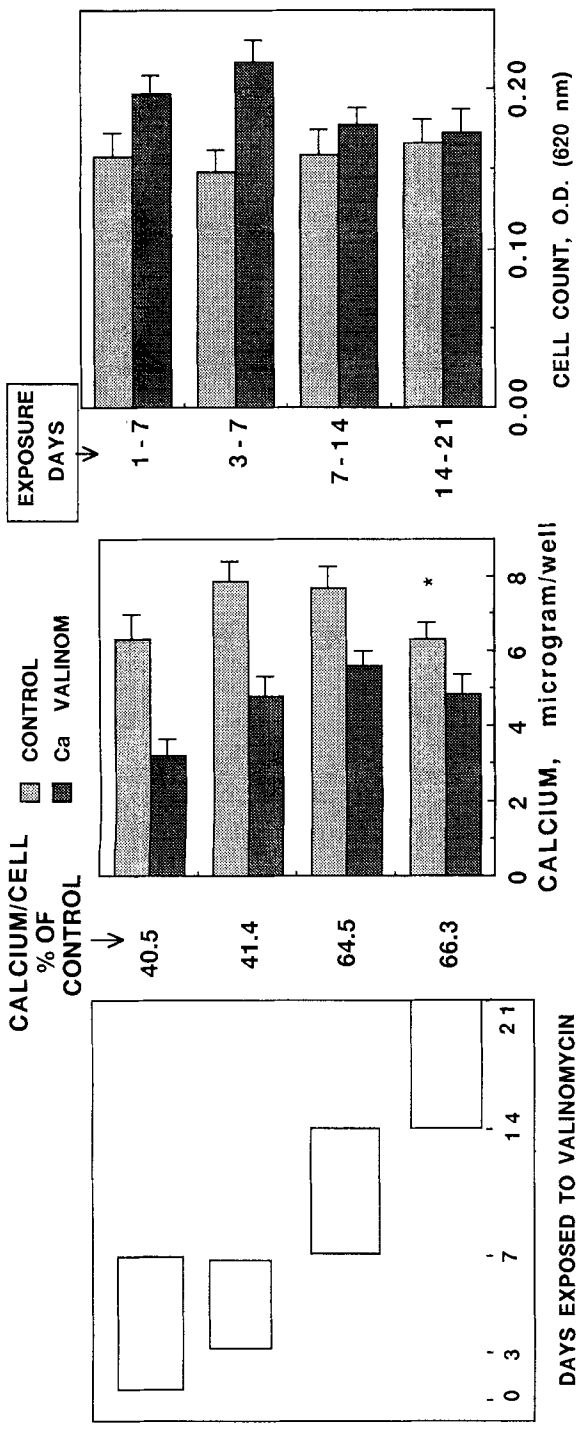

Fig. 7. Dissection of the exposure-time of DEX-stimulated stromal cells to valinomycin and its effect on mineralization. Stromal cells in separate pairs of plates were analysed on day 21 of DEX-stimulation for mineralization and cell counts. All plates were transferred on the day of DEX-stimulation (day 0) to a 30°C incubator and one half side of each pair of plates was exposed to valinomycin for the period indicated on the **left.** Media were changed on days 0, 7, and 14. **Middle:** Calcium/well on day 21; *small but significant. **Right:** Cell counts/well on day 21. Notice the negative selection of mineralizing cells induced by valinomycin mainly during the first 7 days (especially days 3–7).

to the activity of ALP. Such dissociation between ALP and mineralization, where the later is believed to depend on the former [Whyte, 1989], has been exhibited as decreased mineralization in the face of increased ALP activity, by the use of tetramisole [Klein et al., 1993b]. In the present study, dissociation between these functions is shown also to occur in an inverted manner, i.e., increased mineralization in the cold in the face of a decreased ALP activity. In this previous work we presented evidence that part of the inhibitory effect of tetramisole on mineralization is conducted through the energy metabolism. Like tetramisole, the low temperature effect, which has dissociated the mineralization from ALP, could also have acted through the energy metabolism. Nevertheless we cannot exclude the possibility that the cold-induced low ALP activity is to some extent a result of an earlier onset of mineralization, which may inhibit ALP activity earlier than usual.

Since mineralization can occur in solution in the absence of cells by rising the pH and by lowering the temperature [Thermine and Posner, 1970], we used HEPES to buffer the pH in the growth medium. This way much of the chemical effect of alkaline pH on mineralization, in the presence of cells, was excluded. In HEPES buffered medium, β-GP in the absence of dexamethasone caused higher mineralization than in its presence at both temperatures. Incubation of buffered DEX medium resulted in mineralization at the physiological temperature only in the presence of cells, suggesting that the chemical precipitation of mineral may be inhibited by dexamethasone contrary to osteoprogenitors which are clearly able to use DEX for cell-mediated mineralization. It is also clear from the dissected time interval analysis, of exposure to cold temperature, that a continuous exposure to cold is required to induce increased mineralization. For cold temperature to successfully increase mineralization, it is probably required to act uninterruptedly from the very begining of the mineralization initiation process. Thus exposure to cold should include the biochemical level of the mitochondrial energy metabolism, and continue throughout the chemical effect on extracellular amorphous calcium deposition and later transition into apatite crystal growth. It should be noted that differential quantities of mineral phases deposited by cell-mediated mineralization at 33 vis-a-vis 37°C are not known yet.

How does continuous exposure to cold temperature increase cell-mediated mineralization?

Is it associated with energy conservation or rather energy dissipation? The inner mitochondrial membrane can respond to cold temperature by expression of thermogenin [Nedergraad and Cannon, 1991], an uncoupling protein which serves as a channel for proton influx. This channel, being similar to the ATP synthase channel, detours the protons away from the site of ATP synthase, such as to waste its released energy on dissipation of heat instead of ATP synthesis. This thermogenic mechanism is specific to brown fat cells and is activated in vivo under the control of the sympathetic nervous system, which is responding immediately to cold exposure [Mory et al., 1984]. The physiological interaction between systemic hormone effects and brown fat, which results in thermogenesis, occurs in the context of the entire intact organism, whereas our experiments show that individual cells in culture can also respond to cold. The increased cell-mediated mineralization in response to cold could result from association of calcium efflux from mitochondria in exchange for short-circuiting protons influx. If, as a result of cold temperature, protons enter the mitochondria via inner membrane channels other than the F_0F_1 ATPase channel, failure of protons to drive ATP synthesis may translate into dissipated heat. Concomitantly the unused phosphate may become available to leave the cells with calcium and initiate apatite crystal nucleation. The endoplasmic reticulum (ER) has a larger storage of calcium than the mitochondria [Pozzan et al., 1994]. However, the ER is not a site of oxidative phosphorylation-uncoupling, and therefore it is less likely to contribute to the fraction of mineralization induced by thermogenesis. Since the volume of the growth medium is 10^8 times larger than the volume of the cells, changes in temperature as a result of intracellular heating should be undetectable. However, heat could perhaps be transmitted to the plasma membrane before and against temperature equilibration with the cold medium. If such heating would raise membrane temperature to 39–40°C [Stryer, 1988] its fluidity could enable valinomycin movement back and forth across the lipid bilayer to cause cellular potassium depletion. In the present study we counted on this special characteristic of valinomycin to mark indirectly local cellular heating by changing measurable cell functions or viability. Cell counts increased at the first several hours in the cold under valinomycin but diminished after 18–24 h. We expected the action of valinomycin to select those cells which do not respond to cold by thermogenesis and against those which are thermogenic in the cold. Accordingly, increased rhodamine retention/cell was observed after 24 h surpassing the controls. This is consistent with elimination of thermogenic cells with decreased rhodamine retention and enrichment of the cultures with cells of increased rhodamine retention. If indeed influx of protons into the mitochondria can generate heat, then their ability to neutralize inner negative charges (and thereby displace some of the rhodamine from the mitochondria) should agree with our results. The present work focused on the question whether thermogenic response to cold increases mineralization by associating the proton influx with mineralization. The experiment summarized in Figure 7 suggested that this may be the case. DEX-stimulated stromal cells growing at different time intervals with valinomycin in the cold, showed the greatest decrease in day 21 mineralization upon exposure to the ionophor from day 3 to 7. This time interval is consistent with the drop in rhodamine retention which was previously shown in DEX-stimulated stromal cells [Klein et al., 1993c]. This agrees with the above methodological concept that the cold induced thermogenesis increases mineralization in the controls but thermogenically activates valinomycin which, in turn, decreases mineralization by selecting nonthermogenic cells. Thermogenesis by brown fat is probably not related to the ability of mitochondria in bone or other tissues to produce heat as these lack thermogenin. Possibly in the present case other (non-ATP synthase) channels for proton reflux are generated, perhaps permeability transition pores (PTP). It is not known whether mitochondrial PTP in osteoblasts could be related to mineralization. However, their opening is inhibited [Petronilli et al., 1994] by cyclosporin A (CsA) a drug which is also implicated in osteopenia [Movsowitz et al., 1988]. We have recently found that a nonimmunosuppressive analogue of CsA, which can block PTP, can indeed inhibit mineralization in the same system of cultured stromal cells.

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