Measurement of intracellular Ca^{2+} in single aequorin-injected and suspensions of fura-2-loaded ROS 17/2.8 cells and normal human osteoblasts

Effect of parathyroid hormone

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It is known that parathyroid hormone (PTH) activates the cyclic AMP (cAMP) signalling pathway in osteoblasts. In recent years it has been suggested that an elevation of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) may also be involved in the regulation of osteoblast function by PTH. However, this remains controversial. Here we investigated the effect of PTH on the $[\text{Ca}^{2+}]_i$ of ROS 17/2.8 cells and normal human osteoblasts. The $[\text{Ca}^{2+}]_i$ was measured in single aequorin-injected cells and in suspensions of cells loaded with fura-2. Human PTH-(1–38)-peptide (1–300 nM) had no effect on the $[\text{Ca}^{2+}]_i$ in single aequorin-injected ROS 17/2.8 cells ($n = 17$) measured at various times after injection (1–20 h), or in suspensions of fura-2-loaded ROS 17/2.8 cells ($n = 9$). Ionomycin (1 μM) increased the $[\text{Ca}^{2+}]_i$ in fura-2-loaded and single aequorin-injected ROS 17/2.8 cells by 285 ± 60 nM ($n = 9$) and 312 ± 99 nM ($n = 6$) respectively, indicating that both methods detect changes in $[\text{Ca}^{2+}]_i$ with equal sensitivity. In contrast, human PTH-(1–38) (10–100 nM) markedly stimulated cAMP accumulation in ROS 17/2.8 cells. In single aequorin-injected normal human osteoblasts there was no change in the $[\text{Ca}^{2+}]_i$ in response to 100 nM human PTH-(1–38) or 100 nM bovine PTH-(1–84) ($n = 18$). In contrast, in suspensions of normal human osteoblasts loaded with fura-2, an increase in $[\text{Ca}^{2+}]_i$ in response to human PTH-(1–38) (100 nM) was found (60 ± 28 nM; $n = 6$). Considerable variation in the magnitude of the response was observed between individual preparations and donors. These data indicate that PTH activates cAMP accumulation without affecting $[\text{Ca}^{2+}]_i$ in ROS 17/2.8 cells and that PTH causes a rise in $[\text{Ca}^{2+}]_i$ only in a small subset of normal human osteoblasts. We suggest that the Ca^{2+} response to PTH in osteoblasts is limited by the state of differentiation of the cells, and may be due either to the presence of a distinct Ca^{2+} -mobilizing receptor or to a cAMP-mediated Ca^{2+} response.

INTRODUCTION

Parathyroid hormone (PTH) is one of the main hormones which regulates osteoblast function. Binding of PTH to its cell surface receptor on osteoblasts stimulates adenylate cyclase activity and accumulation of cyclic AMP (cAMP), indicating that cAMP is a major signalling pathway activated by PTH in osteoblasts [1,2]. There is, however, evidence that the cAMP signalling pathway may not account for all of the effects of PTH on osteoblast function [3]. In the past few years it has been suggested that an increase in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is an additional signalling pathway by which PTH regulates osteoblast function [4–9]. However, reports on the effects of PTH on $[\text{Ca}^{2+}]_i$ are conflicting [4–9]. Some workers have found an increase in $[\text{Ca}^{2+}]_i$ in response to PTH [4–8], whereas others could not demonstrate any PTH-induced changes in $[\text{Ca}^{2+}]_i$ [9]. All of the measurements were made in populations of various normal and transformed osteoblast-like cells which were either loaded with fluorescent dyes such as quin2, indo-1 and fura-2, or scrape-loaded with the photoprotein aequorin. Failure to find any $[\text{Ca}^{2+}]_i$ response to PTH has been suggested to be due to the quin2 method used [8,9]. However, other explanations, such as the existence of a second, Ca^{2+} -mobilizing

PTH receptor which is not present on all cell types, could also account for the different results [4]. Here we address the involvement of $[\text{Ca}^{2+}]_i$ in the regulation of osteoblast function by PTH using ROS 17/2.8 cells, which are known to possess a large number of PTH receptors [10], and for the first time using normal human osteoblasts. $[\text{Ca}^{2+}]_i$ was measured in single cells and in cell suspensions. Single cells were injected with the photoprotein aequorin as previously reported for a number of other cells [11–13], and suspensions of cells were loaded with the improved fluorescent dye fura-2.

MATERIALS AND METHODS

Cell culture

ROS 17/2.8 cells were grown in 25 cm² plastic flasks at 37 °C in a humidified atmosphere of air/CO₂ (19:1) in Ham's F12/Dulbecco's modified Eagle's medium (DMEM) (1:1, v/v) supplemented with 5% (v/v) fetal calf serum (FCS), 38 mM-NaHCO₃, 50 μg of streptomycin/ml and 50 units of penicillin/ml. The medium was changed every other day. The cells reached confluence within 8–10 days and were used for experiments between days 7 and 10.

Abbreviations used: PTH, parathyroid hormone (the prefixes b, r and h refer to bovine, rat and human respectively); cAMP, cyclic AMP; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; DMEM, Dulbecco's modification of Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine; fura-2/AM, fura-2-acetoxymethyl ester; FCS, fetal calf serum.

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Bone culture

A detailed description of the methods used in the isolation and culture of the human bone cell populations has been reported previously [14]. Briefly, specimens of human trabecular bone, obtained at surgery or biopsy, were extensively washed in phosphate-buffered saline (pH 7.4) and then dissected into fragments of 0.3–0.5 cm in diameter. Trabecular fragments were seeded as explants into 9 cm diameter plastic culture dishes and cultured at 37 °C in an humidified atmosphere (air/CO₂, 19:1). The culture medium was DMEM containing 10% (v/v) heat-inactivated FCS, supplemented with 50 units of penicillin/ml, 150 µg of streptomycin/ml and 2 mM-L-glutamine. The medium was changed after 24 h and thereafter at 5-day intervals. Cell outgrowths reproducibly occurred within 7 days; confluent monolayers were obtained within 21–28 days. At confluence, cells were subcultured into 9 cm dishes after brief exposure (< 5 min at 37 °C) to trypsin/EDTA (0.05% and 0.02% respectively). All experiments were performed on cultures at first passage. The human bone cell populations obtained by these methods have previously been shown to express osteoblast-like characteristics, including responsiveness to PTH and to 1,25-dihydroxyvitamin D₃, and synthesis of type I collagen and the bone-specific protein osteocalcin [14–17]. Patient details, where known, are given in Figure legends.

Measurement of [Ca²⁺]_i in single aequorin-injected cells

Cells were detached from culture flasks or dishes by incubating them in a Ca²⁺-free EDTA (0.02%) solution for 20–30 min followed by the addition of trypsin to reach a final concentration of 0.01%. Cells were then incubated for another 1–2 min at 37 °C before Ham's F12/DMEM (10% fetal calf serum) was added and cells were washed twice by centrifugation at 500 g for 2 min at 20 °C. Cells were transferred to dishes containing 2% (w/v) agarose type IX prepared in Ham's F12/DMEM containing BSA (0.1%). The cells were incubated in this medium under a layer of liquid paraffin in a humidified incubator (air/CO₂, 19:1) for at least 60 min. Single viable cells (no membrane blebs, absence of vacuolation, distinct nuclei) were then transferred to microslides containing 0.5% agarose type VII prepared in medium as described above and microinjected with aequorin using the protocol described previously [13,18,19]. After the injection cells were incubated for various times ranging from 30 min to 20 h before data were collected as described in detail elsewhere [13,18,19]. The perfusion medium during the experiment was Ham's F12/DMEM (1:1, v/v) containing BSA (0.1%). All substances used were added to the perfusion medium. Data were analysed as described [18,19].

Measurement of [Ca²⁺]_i by fura-2 fluorescence

Cells were detached as described above and were preincubated at a concentration of 0.8×10^6 cells/ml in Ham's F12/DMEM (1:1, v/v) containing BSA (0.1%) at 37 °C for 120 min. Cells were then loaded with fura-2/AM (1 or 2 µM) for 30 min. The cells were centrifuged twice at 500 g for 5 min, washed and resuspended in a Hepes-buffered salt solution (saturated with O₂; pH 7.4, 37 °C), which was composed as follows (mM): NaCl, 150; KCl, 5; CaCl₂, 1.3; MgCl₂, 1.2; Hepes, 10; glucose, 11; pyruvic acid sodium salt, 5; and ascorbic acid, 0.11, plus 0.1% BSA. After about 2 min, fluorescence measurements were made using a protocol described previously [20,21] with a Perkin-Elmer LS-5 spectrophotofluorimeter: excitation wavelength, 342 nm; emission wavelength, 492 nm; slit widths, 5 nm. None of the substances used had any effect on the autofluorescence of unloaded cells or on the maximum or minimum fluorescence of fura-2. Comparing the excitation spectrum of fura-2 free acid

with excitation spectra obtained from lysed cells loaded with fura-2/AM and subsequently corrected for autofluorescence showed no differences. The intracellular concentration of fura-2 was calculated to be about 5 µM. The leakage of fura-2 into the medium was about 1.5% of total fura-2 in the cuvette/min at 37 °C and was not affected by PTH. Measurements were therefore limited to 3–5 min.

cAMP measurement

Cells were detached as described above and incubated as for fura-2 experiments. 3-Isobutyl-1-methylxanthine (IBMX; 200 µM) was added to 500 µl portions of cell suspension [(3–5) × 10⁵ cells] 5 min before the addition of agonist. The cells were then incubated for a further 15 or 30 min in the presence of the agonist. Cells were centrifuged, and the pellet was resuspended in 100 µl of distilled water and then boiled for 5 min. The samples were kept at –20 °C until cAMP levels were determined by the method of Brown *et al.* [22].

Statistics

Unless representative tracings are shown, values are means ± S.E.M. Statistical significance was calculated by Student's *t* test.

Materials

DMEM, Ham's F12, FCS and trypsin (0.05%) were from Flow Laboratories, Rickmansworth, Herts., U.K.; Ca²⁺ ionophore A23187, EDTA (0.02% solution), agarose types VII and IX, penicillin, streptomycin, sodium bicarbonate, ascorbic acid, pyruvic acid, and Hepes from Sigma, Poole, Dorset, U.K.; ionomycin from Novabiochem, Nottingham, U.K.; fura-2 free acid and fura-2/AM from Molecular Probes, Eugene, OR, U.S.A.; BSA from APP, Brockmoor, U.K.; and bovine (b) PTH-(1–84) from the National Institute of Biological Standards and Control, London, U.K. Human (h) PTH-(1–38) was generously provided by Arthost, Hamburg, Germany. Aequorin was obtained from Dr. J. R. Blinks, Department of Pharmacology, Mayo Foundation, Rochester, NY, U.S.A., and was stored at –70 °C and dialysed before use, as described [18].

RESULTS

[Ca²⁺]_i in single aequorin-injected ROS 17/2.8 cells

After the injection, the cells were kept for at least 30 min in an incubator. Before measurement of [Ca²⁺]_i the cells were checked again for their healthy appearance by light microscopy (no membrane blebs, absence of vacuolation, distinct nuclei). Stable resting signals could be recorded from individual ROS 17/2.8 cells for several hours. In 17 such cells, the resting [Ca²⁺]_i within the first 6 h after injection was 178 ± 13 nM. The resting [Ca²⁺]_i in cells which had been incubated for approx. 20 h and allowed to attach and to spread at the bottom of the microslide was not significantly different (194 ± 13 nM; *n* = 10). Adding hPTH-(1–38) (1–300 nM) to the perfusion medium had no effect on the [Ca²⁺]_i of ROS 17/2.8 cells measured at various times between 30 min and 24 h (cells were attached and spread at the bottom of the microslide) after the injection of aequorin (Fig. 1; *n* = 17). In contrast, the ionophore ionomycin (1 µM) caused a rapid increase in the [Ca²⁺]_i of single aequorin-injected ROS 17/2.8 cells (Fig. 1b). The signal peaked sharply and then declined to form a new elevated plateau (Fig. 1b). The peak values in [Ca²⁺]_i reached in response to ionomycin (1 µM) showed great heterogeneity, ranging from 50 nM to 800 nM above prestimulation values. The average ionomycin (1 µM)-induced increase in [Ca²⁺]_i in six single ROS 17/2.8 cells was 312 ± 99 nM.

Fig. 1. Effects of hPTH-(1-38) and the ionophore ionomycin on the $[\text{Ca}^{2+}]_i$ of a single aequorin-injected ROS 17/2.8 cell

Representative tracings with the aequorin signal plotted as $[\text{Ca}^{2+}]_i$ (nM) are shown. Duration of exposure to hPTH-(1-38) (PTH) or ionomycin (Iono) was as indicated by the horizontal bars. The time constant was 10 s.

$[\text{Ca}^{2+}]_i$ in single aequorin-injected normal human osteoblasts

Normal human osteoblasts were treated in the same way as described above. The resting $[\text{Ca}^{2+}]_i$ amount to 185 ± 17 nM ($n = 18$), which was not significantly different from the resting $[\text{Ca}^{2+}]_i$ in ROS 17/2.8 cells. Neither hPTH-(1-38) (1–100 nM) nor bPTH-(1-84) (100 nM) caused any change in the $[\text{Ca}^{2+}]_i$ in 18 cells from different preparations and donors, including those which showed a Ca^{2+} response in fura-2 experiments (Fig. 2a). The ionophore A23187 (2 μM) caused similar changes in $[\text{Ca}^{2+}]_i$ as did ionomycin (1 μM) in ROS 17/2.8 cells (Fig. 2b).

$[\text{Ca}^{2+}]_i$ in fura-2-loaded ROS 17/2.8 cells in suspension

Stable resting signals of fura-2-loaded ROS 17/2.8 cells could be recorded for several minutes. In cells incubated in medium containing 1.3 mM- Ca^{2+} the mean resting $[\text{Ca}^{2+}]_i$ was 232 ± 14 nM ($n = 9$). This is of similar magnitude to that in single aequorin-injected ROS 17/2.8 cells. As shown in Fig. 3, hPTH-(1-38) (1–300 nM) did not cause an immediate change in $[\text{Ca}^{2+}]_i$ ($n = 9$). However, several minutes after the addition of hPTH-(1-38) (100 nM), the fluorescence signal began to rise (Fig. 3b) and reached a fluorescence corresponding to a $[\text{Ca}^{2+}]_i$ of 146 ± 17 nM ($n = 6$) above the resting value after 10 min. A similar rise was observed when medium was added (169 ± 93 nM; $n = 4$; Fig. 3c). To further confirm that this increase was due to dye leaking from the cells, leakage of fura-2 at 37 °C was determined and found to be approx. 15% of total fura-2 in the cuvette after 10 min. Leakage of this order can fully account for the observed increase

Fig. 2. Effects of hPTH-(1-38), bPTH-(1-84) and the ionophore A23187 on the $[\text{Ca}^{2+}]_i$ of a single aequorin-injected normal human osteoblast

Representative tracings are shown with the aequorin signal plotted as $[\text{Ca}^{2+}]_i$ (nM). Duration of exposure to agonist was as indicated by horizontal bars. The time constants were 10 s (a) and 15 s (b). (a) The donor was an elderly lady whose femoral head was obtained during hip replacement because of osteoarthritis; (b) the details of the donor are unknown.

in the fluorescence signal. The addition of ionomycin (1 μM) induced a rapid rise in the $[\text{Ca}^{2+}]_i$ (Fig. 3a). This increase was transient and decayed to a new elevated steady-state value (Fig. 3a). The ionomycin (1 μM)-induced peak increase in $[\text{Ca}^{2+}]_i$ was 292 ± 53 nM ($n = 10$). The shape as well as the amount of the ionomycin-induced increase in $[\text{Ca}^{2+}]_i$ observed in the cell suspension was similar to that found in single aequorin-injected cells (cf. Figs. 1b and 3a). This indicates that the intracellular Ca^{2+} buffering capacity in cells loaded with fura-2 was very low and could not account for the lack of effect of PTH on $[\text{Ca}^{2+}]_i$ in fura-2-loaded ROS 17/2.8 cells.

$[\text{Ca}^{2+}]_i$ in fura-2-loaded normal human osteoblasts in suspension

The resting $[\text{Ca}^{2+}]_i$ in normal human osteoblasts was 246 ± 10 nM ($n = 9$). hPTH-(1-38) caused a transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 4). The average increase in $[\text{Ca}^{2+}]_i$ induced by hPTH-(1-38) in preparations from three different donors was 60 ± 28 nM ($n = 6$). The value, however, is misleading, since there was great variation in magnitude between preparations and donors, as shown in Figs. 4(a)–4(e). Ionomycin (1 or 3 μM) induced similar changes in $[\text{Ca}^{2+}]_i$ to those observed in ROS 17/2.8 cells (Fig. 4).

Fig. 3. Effects of 100 nM-hPTH-(1-38) (PTH) and ionomycin (Iono) on the $[Ca^{2+}]_i$ in ROS 17/2.8 cells loaded with fura-2

(a) Representative tracing; for mean values, see the text. (b) Increase in the fluorescence signal of fura-2-loaded ROS 17/2.8 cells after 5–7 min of measurement. (c) Medium without hPTH-(1-38) (Co) was added.

Fig. 4. Effects of 100 nM-hPTH-(1-38) (PTH) and ionomycin (Iono) on the $[Ca^{2+}]_i$ in normal human osteoblasts obtained from different preparations and donors, loaded with fura-2

The donors were: A and B the same as in Fig. 2(A) (a and b); and two children who had undergone corrective surgery where parts of their ribs had been removed (c,d) and (e,f). The hPTH-(1-38)-induced increases in $[Ca^{2+}]_i$ were 190 nM (a), 76 nM (b), 55 nM (c) and 40 nM (d); there was no response in (e) and (f).

Table 1. hPTH-(1-38)-induced cAMP accumulation in ROS 17/2.8 cells

cAMP was determined in cells under the same conditions as in the fura-2 experiments. Cells were incubated with 200 μ M-IBMX for 5 min before the indicated dose of hPTH-(1-38) was added, and were then incubated for a further 15 min in the presence of agonist. Results are expressed as percentages of the control and were done in triplicate. The control was 84.4 ± 4.4 fmol of cAMP/ 10^6 cells.

Addition		cAMP (% of control)
None (control)		100 $\pm$ 5
hPTH-(1-38)	(10 nM)	290 $\pm$ 80
	(100 nM)	1550 $\pm$ 175
	(500 nM)	1150 $\pm$ 150
Forskolin	(200 μ M)	1650 $\pm$ 325

cAMP measurements

To test whether the ROS 17/2.8 cells used were responsive to hPTH-(1-38), the effect of hPTH-(1-38) on cAMP accumulation was determined. hPTH-(1-38) markedly stimulated cAMP formation in ROS 17/2.8 cells in suspension (Table 1). Fura-2 loading did not affect hPTH-(1-38)-induced cAMP accumulation (results not shown).

DISCUSSION

There have been several previous reports investigating the role of $[Ca^{2+}]_i$ in the regulation of osteoblast function by PTH. The $[Ca^{2+}]_i$ was measured in populations of osteoblast-like cells in suspension or attached to coverslips [4–9]. Small increases in $[Ca^{2+}]_i$ were recorded in UMR-106 cells [4–6] and mouse osteoblasts [7] loaded with fluorescent dyes and treated with PTH. In ROS 17/2.8 cells the results are conflicting. Boland *et al.* could not demonstrate any change in $[Ca^{2+}]_i$ in response to PTH using quin2-loaded cells [9], whereas Donahue *et al.* reported a large increase in $[Ca^{2+}]_i$ on PTH stimulation using cells scrape-loaded with aequorin [8]. It was suggested that the lack of response to PTH in quin2-loaded ROS 17/2.8 cells might be due to a high intracellular Ca^{2+} -buffering capacity introduced by quin2 loading [8,9]. However, this appears unlikely, since in other systems of homogeneous cell suspensions loaded with quin2 large increases in $[Ca^{2+}]_i$ have been reported in response to agonist stimulation [23]. In this study we detected no response of $[Ca^{2+}]_i$ in PTH-treated ROS 17/2.8 cells, either in single cells microinjected with aequorin or in suspensions loaded with fura-2. The ionophore ionomycin produced increases in $[Ca^{2+}]_i$ of similar magnitude and time course in ROS 17/2.8 cells loaded with fura-2 and microinjected with aequorin. This indicates that both methods detect changes in $[Ca^{2+}]_i$ with equal sensitivity and that Ca^{2+} buffering by fura-2 does not appear to account for the lack of effect of PTH on $[Ca^{2+}]_i$ in fura-2-loaded cells. Furthermore, the concentrations of fura-2/AM used were the same as or lower than those used by others who found an increase in $[Ca^{2+}]_i$ in response to PTH stimulation in other osteoblast-like cells [5,6]. The late increase in the fluorescence signal that we observed several minutes after the addition of PTH was most likely due to fura-2 leaking out of the cells. A similar late increase in signal was also observed in controls, and the leakage of fura-2 measured under experimental conditions could fully account for this rise. Single ROS 17/2.8 cells were microinjected with aequorin using protocols which have been applied successfully in a number of other cell types [11–13,19]. Microinjection damage is unlikely to account for the absence of a PTH-induced Ca^{2+} response, since in cells which had been incubated in full growth medium for

10–20 h after the injection PTH did not elicit any change in $[\text{Ca}^{2+}]_i$. Under these circumstances cells were attached and had spread at the bottom of the microslide and some had divided, indicating that the cells were in a healthy condition. In a recent study measuring $^{45}\text{Ca}^{2+}$ uptake in various osteoblast-like cells neither hPTH-(1–34) nor rPTH-(1–34) caused any increase in $^{45}\text{Ca}^{2+}$ uptake in ROS 17/2.8 cells [24], indicating that differences in the sequence of the human and rat PTH fragments may not account for the lack of effect of hPTH in this study. In contrast with the Ca^{2+} measurements, PTH markedly stimulated cAMP accumulation in ROS 17/2.8 cells in suspension, indicating that PTH receptors were present and functioning under the experimental conditions. Therefore a rise in $[\text{Ca}^{2+}]_i$ does not appear to be a signalling pathway activated by PTH in the ROS 17/2.8 cells used in this study. This demonstrates that the cAMP and the Ca^{2+} signalling pathways can be dissociated. This is in line with the findings of Boland *et al.* [9] but in contrast with those of Donahue *et al.* [8]. This observed variability of results could be due either to a 'phenotypic drift' of cell cultures or to the variation in the culture conditions used in the different studies [8,9].

For the first time $[\text{Ca}^{2+}]_i$ was measured in cells derived from normal human bone. These cells have been shown in the past to express many osteoblast characteristics, including the production of cAMP in response to PTH [14–17]. In fura-2-loaded cells an increase in $[\text{Ca}^{2+}]_i$ in response to PTH could be detected. The increase in $[\text{Ca}^{2+}]_i$ was transient and of similar shape to the ones found in other osteoblast-like cells [4–8]. However, the magnitude of the Ca^{2+} rise was highly variable, depending on the preparation and donor, and in some preparations there was no change in $[\text{Ca}^{2+}]_i$ in response to PTH. This indicates that only a subset of normal human osteoblasts show a rise in $[\text{Ca}^{2+}]_i$ in response to stimulation by PTH. The lack of a Ca^{2+} response to PTH in single aequorin-injected cells is consistent with a response restricted to a subset of cells. There is evidence for heterogeneity of the osteoblast-like phenotype [25,26]. Recently, Guenther *et al.* [26] provided further evidence for great heterogeneity of the osteoblastic phenotype using clones of osteoblast-like cells derived from mouse calvaria. In some clones, for example, there was no cAMP accumulation in response to PTH, but other osteoblastic characteristics were expressed [26]. Heterogeneity has also been demonstrated in the response of clonal osteoblast-like cells to prostaglandins [27]. In view of such heterogeneity of the osteoblastic phenotype, a Ca^{2+} response to PTH restricted to a subset of osteoblasts by their state of differentiation appears to be conceivable. A Ca^{2+} response limited by the degree of differentiation has been reported from other blast-like cells [28].

An increase in $[\text{Ca}^{2+}]_i$ in response to PTH in only some osteoblast-like cells could be due to a distinct Ca^{2+} -mobilizing PTH receptor with restricted expression, or to one PTH receptor and a restricted cAMP-mediated Ca^{2+} response, or to a combination of both. A cAMP-mediated increase in $[\text{Ca}^{2+}]_i$ via voltage-dependent Ca^{2+} channels has been reported in other cell types [29], and Ca^{2+} channels have been found on osteoblast-like cells [30]. The characteristics of the Ca^{2+} channels described on osteoblast-like cells were different from those reported for other cell types [31,32]. It remains to be shown whether the Ca^{2+} channels present on osteoblast-like cells can be modulated by cAMP-dependent mechanisms. Further support for a cAMP-mediated increase in $[\text{Ca}^{2+}]_i$ via voltage-dependent Ca^{2+} channels comes from the findings that extracellular Ca^{2+} was required for a PTH-induced increase in $[\text{Ca}^{2+}]_i$ and that Ca^{2+} -channel blockers such as verapamil could block the effect of PTH on $[\text{Ca}^{2+}]_i$ [4,6,7].

Evidence for a distinct Ca^{2+} -mobilizing receptor on osteoblast-like cells coupled to the phosphatidylinositol/ Ca^{2+} signalling pathway also exists. Various PTH fragments have different

effects on the accumulation of cAMP and on $[\text{Ca}^{2+}]_i$ [4,8]. PTH caused an increase in $\text{Ins}(1,4,5)\text{P}_3$ in populations of neonatal mouse osteoblasts [33], and the PTH-induced increase in $[\text{Ca}^{2+}]_i$ reported by one group was not affected by removal of extracellular Ca^{2+} [5]. This is consistent with an $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} response. Interestingly, this was reported from UMR-106 cells, the same cell line which have been used by others who have demonstrated that the PTH-induced Ca^{2+} response was dependent on extracellular Ca^{2+} and could be blocked by Ca^{2+} -channel blockers [4,6]. It appears that not only in ROS 17/2.8 cells, but also in UMR-106 cells, the Ca^{2+} response to PTH exhibits certain variability. Since there is evidence for two distinct PTH receptors and for a cAMP-mediated Ca^{2+} response that is not present on all osteoblast-like cells, it might be that both could occur depending on the degree of differentiation.

If transformed osteoblast-like cells represent distinct phenotypes of normal osteoblastic cells, they might prove useful tools for investigating the role of each signalling pathway in osteoblast function. The use of clones of normal osteoblast-like cells as described [26] and of imaging techniques such as video or charge-coupled device (CCD) camera systems, which allow large numbers of single cells to be monitored at the same time, will further help to clarify this complex issue.

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