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Single-Cell Analysis of Cyclic AMP Response to Parathyroid Hormone in Osteoblastic Cells

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ABSTRACT

We previously demonstrated that the $[Ca^{2+}]_i$ response to PTH is heterogeneous in single UMR-106-01 osteogenic sarcoma cells. To verify whether response heterogeneity is a universal feature of PTH signal transduction, cAMP production was monitored in monolayer cultures of UMR-106-01 cells and human trabecular bone osteoblasts (HOB) using the cAMP-sensitive fluorescent indicator FlCRhR. FlCRhR was microinjected into single cells, and the 500–530/>560 nm fluorescence ratio was monitored by confocal laserscanning video imaging as a measure of cAMP concentration ([cAMP]). Virtually all UMR-106-01 cells exposed to bovine PTH(1–34) (10^{-7} M) exhibited an increase in intracellular [cAMP], with an average fluorescence ratio change of 145 ± 17% of baseline (n = 15), corresponding to nearly maximal dissociation of protein kinase A. In the continued presence of the hormone (10^{-7} M), [cAMP] remained elevated for at least 30 minutes. This effect was accompanied by a slow translocation of the fluorescein-labeled catalytic subunit of protein kinase A from the cytoplasm to the nucleus. In contrast, PTH(1–34) caused no detectable increase in [cAMP] in HOB cells, although PGE_2 (3×10^{-6} M) stimulation was able to increase the FlCRhR ratio (154 ± 27%, n = 10). The truncated fragment PTH(2–34) was only 67% as potent at PTH(1–34), but deletion of the first two amino acids at the N terminus abolished the hormone's ability to stimulate cAMP production in UMR-106-01 cells. Brief exposure to 10^{-7} M of either PTH(3–34) or PTH(7–34) did not affect the amplitude of the fluorescence ratio change induced by equimolar doses of PTH(1–34). Thus, in osteoblast-like cells stimulated with PTH, the [cAMP] response is much more homogeneous from cell to cell than the $[Ca^{2+}]_i$ response.

INTRODUCTION

SIGNAL TRANSDUCTION for parathyroid hormone (PTH) is mediated by both the adenylyl cyclase and phospholipase C pathways. While receptor coupling to adenylyl cyclase leads to the production of cAMP and activation of protein kinase A (PKA),[1-3] phospholipase C hydrolysis of phosphatidylinositol-4,5-bisphosphate generates inositol 1,4,5-trisphosphate and diacylglycerol, which in turn produce transient elevations of cytosolic calcium concentration $[Ca^{2+}]_i$ and activation of protein kinase C, respectively.[4-7] The relative roles of these two intracellular signaling systems for the physiologic action of PTH are still uncertain, but they are both necessary to express the full hormonal effect.[8,9] Most of the studies on PTH signal transduction were initially performed on cell populations.[1,3-5] Results obtained with these methods provide useful information on the average effect of an agonist on a colony of cells, but they do not allow us to distinguish and analyze potential intercellular differences within a particular cell population. This issue is very important in a tissue like bone, in particular for the osteoblast lineage, which is constituted by an heterogeneous array of cells, from the osteoblast precursors residing in the marrow space to the differentiated cells lining the bone surfaces. Each of these cell types expresses different patterns of osteoblast phenotypic

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markers, depending on their differentiation stage.[10-12] In particular, PTH receptors have been found to be more concentrated on the osteoblast precursors, whereas they are virtually absent in the more differentiated bone-lining cells.[13] Therefore, the capacity of osteoblastic cells to receive and elaborate external input signals may be different in relation to the phases of their differentiation pathway.

We recently observed that the distribution of PTH receptors and the hormone's ability to generate $[Ca^{2+}]_i$ responses are heterogeneous in monolayer cultures of the osteogenic sarcoma cell line UMR-106-01.[14] In this clonal cell line, the most responsive elements are those with a polyhedral cell body and short cytoplasmic processes,[15] a morphologic type similar to the osteoblast precursors bearing PTH receptors observed in sections of normal bone.[13] Although the mechanisms underlying this heterogeneous $[Ca^{2+}]_i$ response are still under investigation, production of cAMP, the other arm of PTH signal transduction, has thus far never been studied in individual cells, mainly because of technical limitations. Barsony and Marx[16] described a cytochemical method for single-cell studies of cyclic nucleotide accumulation in microwave-fixed cells, but the technique does not allow real-time recording in live cells. The recent development of a new fluorescent indicator for cAMP[17] provides the most appropriate tool to verify whether the heterogeneity observed for the $[Ca^{2+}]_i$ responsiveness to PTH also applies to the cAMP pathway.

These studies were therefore designed to analyze cAMP responses to PTH in single UMR-106-01 osteoblast-like cells and in human osteoblasts isolated from trabecular bone specimens. Activation of the cAMP pathway was monitored by following the changes in fluorescence emission by the cAMP-sensitive probe, FlCRhR, microinjected in individual cells. Results indicate that, unlike $[Ca^{2+}]_i$ signals, cAMP responses to PTH are homogeneous from cell to cell in the transformed rat osteoblastic cells, UMR-106-01, whereas the more differentiated trabecular bone-derived human osteoblasts are poorly responsive to the hormone. Thus, substantial differences exist in the mechanisms whereby two signaling pathways are activated in individual PTH target cells.

MATERIALS AND METHODS

Chemicals

Synthetic bovine PTH(1–34) and bPTH(3–34) were purchased from Bachem, Inc. (Torrance, CA). [Tyrosine^{34}] bPTH(7–34)amide [bPTH(7–34)] was the kind gift of Drs. Michael Rosenblatt and Michael P. Caulfield (Merck, Sharp and Dohme Research Laboratories, West Point, PA). Bovine PTH(2–34) and propionyl bPTH(2–34) [pbPTH(2–34)] were synthesized by Dr. Kam F. Fok (Monsanto Co., St. Louis, MO). The chemical structure of these fragments was confirmed by amino acid composition analysis and mass spectrometry. PTH peptides were dissolved in 1 mM HCl and stored at −20°C. The murine C_α subunit of protein kinase A was synthesized in *Escherichia coli* by a recombinant DNA technique[18] and labeled with fluorescein 5′-isothiocyanate.[17] The R^{II} subunit was isolated from either porcine heart[19] (a gift of Susan Taylor) or *E. coli* expressing the murine $R^{II}\alpha$ subunit[20] (a gift of John Scott) and was labeled with carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester, as described.[21] The complex of the two labeled subunits C and R constitutes the cAMP probe (FlCRhR).[17]

Cell cultures

The clonal cell line UMR-106-01, kindly provided by Dr. Nicola C. Partridge (St. Louis University, St. Louis, MO), was used between passages 16 and 22. These cells were derived from the rat osteogenic sarcoma cell line UMR-106, which has been characterized as having an osteoblastic phenotype.[2,22] They were maintained in Dulbecco's modified minimum essential medium (DMEM) with Earle's salts, supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin, and 100 U/ml of penicillin.[23]

Human trabecular bone cells were prepared according to the method of Gehron Robey and Termine,[24] with modifications.[25] Surgical specimens of ribs, obtained by two human donors undergoing chest surgery at the Department of Cardiology of Washington University Medical Center, were split longitudinally and the marrow cavity rinsed with serum-free DMEM and Ham's F12. The trabecular bone was removed, minced, and washed several times in the same medium. The bone chips thus obtained were incubated with type IV collagenase (Sigma Chemical Co., St. Louis, MO) to release cells from the bone surfaces. The released cells were then seeded on plastic flasks and cultured in growth medium supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin, and 100 U/ml of penicillin. The bone chips were washed and cultured separately using the same medium employed for the collagenase-released cells. After a few days, cells began to grow out from the bone particles and within 3–4 weeks reached confluence. Thereafter, both collagenase-released cells and those outgrown from the bone chips were released with trypsin-EDTA (0.05–0.02%, respectively; Sigma Chemical Co.), counted, and seeded on round glass coverslips (25 mm diameter) housed on plastic Petri dishes in medium containing 10% fetal bovine serum. These cells stain positively for alkaline phosphatase and produce osteocalcin both constitutively and under stimulation with 1,25-dihydroxyvitamin D_3,[25] thus meeting two major criteria for the definition of an osteoblastic phenotype.

Measurement of cyclic AMP in single cells

Coverslips with semiconfluent (50–60% confluent) monolayers of UMR-106-01 or human osteoblastic (HOB) cells were placed in a custom-made holder and positioned on the stage of an inverted epifluorescence microscope (IM35; Zeiss, Okerkochen, Germany). The cells were bathed in Hank's buffered saline (GIBCO, Gaithersburg, MD) and maintained at a constant temperature of 33°C provided by a water circulation system. Individual cells were pressure injected with the cAMP indicator FlCRhR[17,26] using micropipettes with a tip of approximately 0.5 μm in diameter. A description of the microinjection methods has been published elsewhere.[21] Following microinjection, cells were allowed to recover for at least 30 minutes. After this time, only cells that appeared morphologically unperturbed by the injection procedure and in which FlCRhR fluorescence was largely restricted to the cytoplasm were used.

FlCRhR fluorescence was monitored using a high-speed laser scanning confocal microscope capable of dual emission ratio imaging.[27] Excitation was accomplished with the 488 nm line from an argon laser, and emission was simultaneously collected at 500–530 nm (fluorescein emission band) and at above 560 nm (rhodamine emission band). Individual fluorescence images were stored on an optical disk recorder (TQ-2028F; Panasonic, Secaucus, NJ) for later analysis. Following background subtraction, images corresponding to the 500–530/>560 nm ratio were generated and displayed in pseudocolors. In each color image, the scale at the right-hand side shows the relationship between pseudocolor and 500–530/>560 nm ratio. This ratio increased by 1.7-fold between zero and saturating cAMP concentration [cAMP] for the holoenzyme batches used here. Absolute calibration of ratio, or pseudocolor hues in terms of free [cAMP], proved problematic because these cells did not give consistent or stable responses to the cAMP antagonist R_pcAMPS, which ideally should simulate 0 [cAMP] as the reference point for calibration. Nevertheless, because cells typically increased their ratio by 1.5- to 1.6-fold upon effective stimulation, they must have gone from only slight to nearly complete activation of the kinase, which is half-maximally activated at 100–300 nM [cAMP].[17,21]

The intracellular distribution of the fluorescein-labeled C subunit of PKA was followed using the individual fluorescence images acquired at 500–530 nm. The amount of C subunit present in the nucleus relative to that in the cytoplasm was assessed by ratioing the average 500–530 nm fluorescence intensity in the nucleus to that in the cytoplasm. This measure provided an index of the relative distribution of C subunit independently of such factors as illumination intensity, change of focus, or photobleaching.

Data were quantitated by calculating the percentage change in the 500–530/>560 nm ratio compared with baseline and expressed as average ± standard deviation. Group means were compared using a nonpaired *t*-test.

RESULTS

Pseudocolor images of UMR-106-01 cells microinjected with FlCRhR during sequential exposure to 10^{-7} M bPTH(3–34) and PTH(1–34) are illustrated in Color Plate 1. No changes were detected following addition of the shortened PTH fragment to the bathing medium, but an increase in the 500–530/>560 nm emission ratio occurred after stimulation with bPTH(1–34), corresponding to an increase in free cytosolic [cAMP]. The increase of cAMP-dependent fluorescence ratio, which lasted for at least 25–30 minutes during continuous exposure to the hormone, was observed in virtually all cells (68 of 74) microinjected with FlCRhR and exposed to PTH(1–34). Cells that did not respond to the hormone also did not respond to forskolin, suggesting that they either had an insensitive or abnormal adenylyl cyclase or were severely damaged by the microinjection. Because the $[Ca^{2+}]_i$ response to PTH(1–34) is heterogeneous in UMR-106-01 cells and is correlated with specific morphologic profiles,[14] the cAMP probe was injected in cells of different shapes. Cyclic AMP responses were observed in all cells, independently of their morphology and confluence status. In particular, as exemplified in Color Plate 1, cells growing in clusters, which were found to be poorly responsive to PTH,[14] invariably responded to the hormone exposure with a prompt increase in cAMP. Although a full dose-response study was not performed, homogeneous responses were also observed with lower concentrations of PTH (not shown).

A time course of the PTH(1–34) effect on cAMP levels of UMR-106-01 cells is illustrated in Fig. 1. Tracings were obtained by averaging pixel values of the digital images in areas corresponding to the cytoplasm of each individual UMR-106-01 cell of Color Plate 1. FlCRhR fluorescence ratio did not change after addition of bPTH(3–34) but rapidly increased following exposure to bPTH(1–34), reaching a maximum approximately 1–3 minutes thereafter. In most experiments, cAMP levels remained elevated for at least 25–30 minutes. In some cases, a slow decline with time was observed. This is caused at least in part by photobleaching of fluorescein by the exciting light, which causes a decrease in the 500–530/>560 nm ratio. Figure 1 (bottom) illustrates the time course of the redistribution of the catalytic subunit of protein kinase A expressed as the ratio of brightness of the fluorescein-labeled subunit in the nucleus to that in the cytoplasm. In many cells, activation of PKA leads to long-term effects dependent on gene expression mediated through phosphorylation of CREB, which is highly localized to the nucleus.[28] As shown in previous work,[29] cAMP-dependent dissociation of holoenzyme allows movement of free catalytic subunit of PKA from the cytoplasm to the nucleus. This was also the case for UMR-106-01 cells. Addition of bPTH(3–34) did not increase [cAMP] or the nuclear translocation ratio. However, bPTH(1–34) led to a slow increase in the translocation ratio after sustained elevation of [cAMP].

In the continuous presence of hormone, [cAMP] remained elevated for at least 15 minutes. Removal of bPTH(1–34) from the bath was followed by a slow decline in [cAMP] to levels near baseline (Fig. 2). This decrease in fluorescence ratio could be distinguished from photobleaching by subsequent addition of PTH or forskolin (not shown) to elevate the fluorescence ratio again.

The cAMP response to PTH(1–34) was also studied in monolayers of HOB cells (Color Plate 2). These cells, unlike UMR-106-01 cells, showed no change in FlCRhR fluorescence ratio in response to PTH. A total of 10 HOB cells, isolated from two different donors, were successfully microinjected with FlCRhR. None of them showed detectable responses to either human PTH(1–34) (n = 2) or bovine PTH(1–34) (n = 8), but they all responded to prostaglandin E_2 (PGE_2), with an average increase in fluorescence ratio of 154 ± 27% of baseline. Forskolin elicited a small further increase (10 ± 8%) in the fluorescence ratio. As in the UMR-106-01, cells for microinjection were selected from confluent areas, as well as in sparse monolayers. Injections were also performed in individual, isolated cells. Figure 3 (top) illustrates the time course from the experiment in Color Plate 2. The rise of FlCRhR fluorescence ratio following stimulation with PGE_2 was rapid, reaching a plateau within 1–2 minutes after addition of the agonist. Further addition of forskolin only slightly increased FlCRhR fluorescence, suggesting that 3×10^{-6} M PGE_2 stimulated cAMP production to nearly maximal levels in these cells. Because the microinjected labeled PKA subunits become saturated at cAMP concentrations above 1 μM,[17] the fluorescence ratio probably does not discriminate between [cAMP] elevations above the

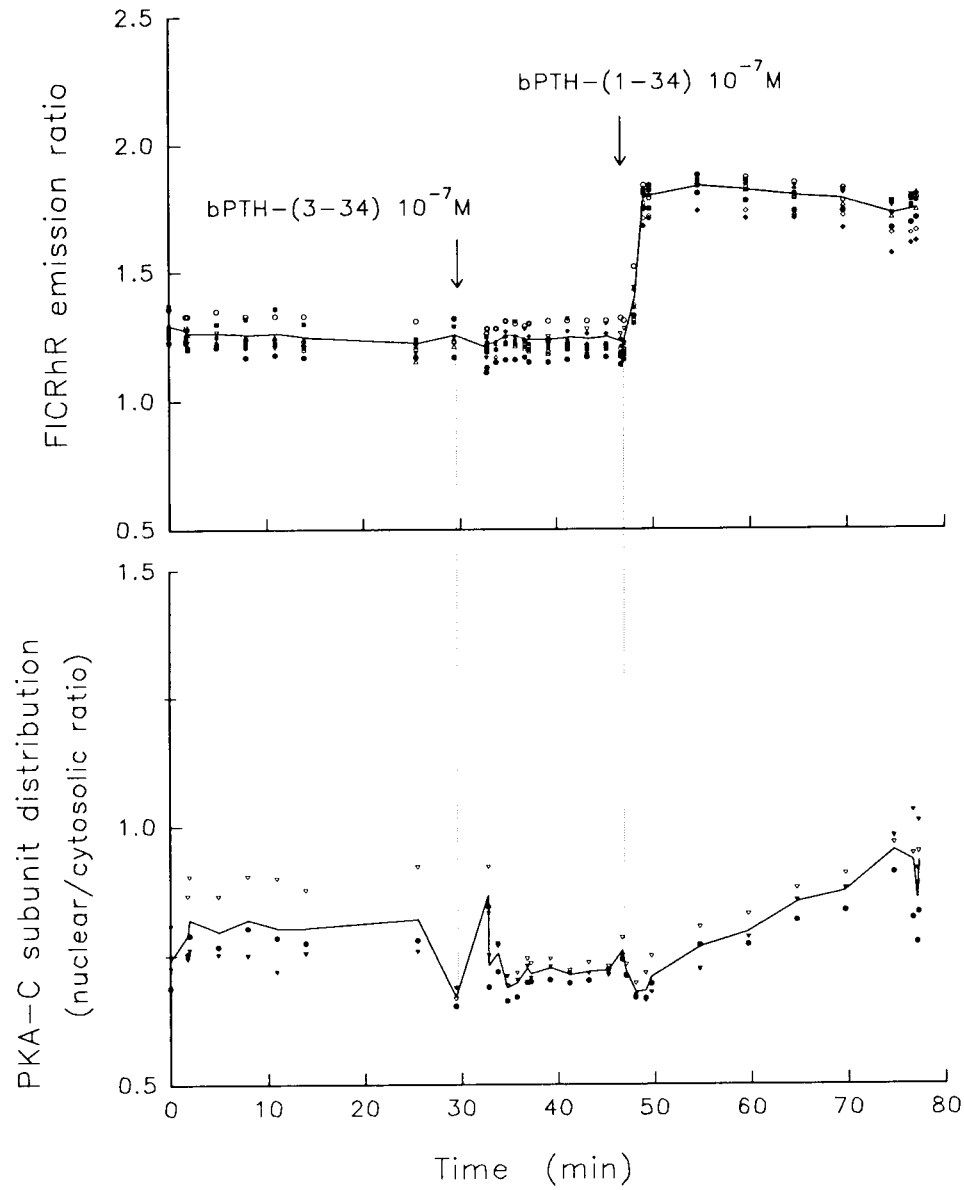
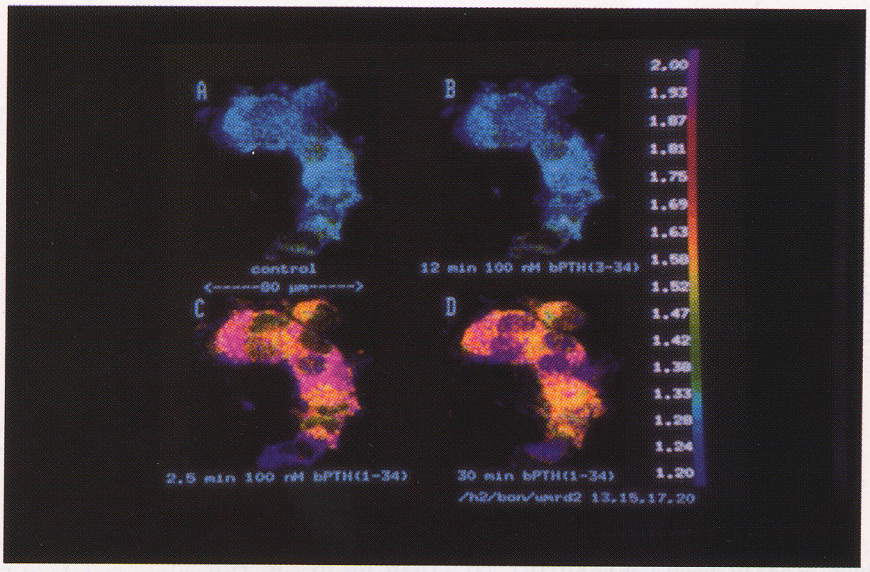


FIG. 1. Effect of subsequent stimulation with bPTH(3–34) and bPTH(1–34) on intracellular [cAMP] of UMR-106-01 cells. Data are relative to 10 individual cells taken from the experiment illustrated in Color Plate 1. (Top) Level of FICRhR emission ratio (cytoplasmic cAMP) versus time calculated as the average pixel value within regions corresponding to individual cells; (bottom) relative nuclear translocation of the C subunit of PKA, measured in three cells. Each symbol corresponds to a different cell. Lines are the average of fluorescence ratios obtained for all the cells at each time point.

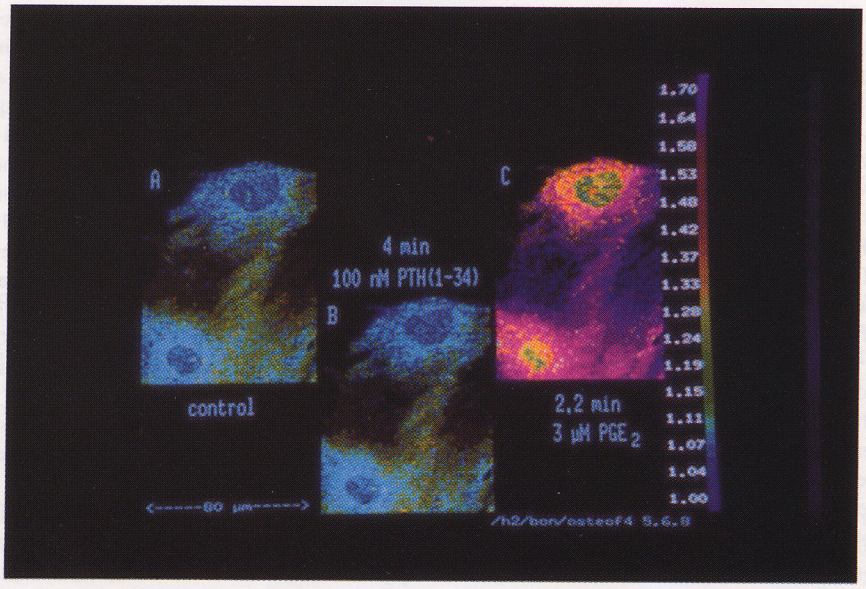
micromolar range, as are usually estimated by traditional biochemical assays for total cAMP.[23] Note, however, that FICRhR has the same affinity for cAMP as the native mammalian protein kinase A and therefore reports the free cAMP concentration within the physiologically important range.

The large size of HOB cells allowed monitoring of FICRhR fluorescence distribution in different areas of the cytoplasm of a single cell. Cyclic AMP-dependent fluorescence averaged over different cytoplasmic zones of one HOB cell revealed a uniform increase following PGE_2 exposure, with no evident subcellular gradients.

In populations of UMR-106-01 osteoblastic cells, the cAMP response can be dissociated from the $[Ca^{2+}]_i$ response by deletion of the first two amino acids at the N terminus of the PTH peptide sequence.[23] As described earlier, a concentration of 10^{-7} M of the 3–34 fragment was also unable to stimulate the production of cAMP at the single-cell level. At the same concentration, bPTH(2–34) and, under certain conditions, the propionyl derivative, pbPTH(2–34), retain a weak agonist activity at the cell population level.[23] To verify whether the loss of cell responsiveness to the shortened PTH fragments is caused by either loss of activity in each cell or a lower number of cells



COLOR PLATE 1. Pseudocolor video images of UMR-106-01 cells during exposure to PTH fragments. Osteogenic sarcoma cells grown on glass coverslips were mounted on the stage of a confocal laser scanning microscope and microinjected with FlCRhR, as detailed in Material and Methods. The color scale, in fluorescence ratio units (right), represents increasing levels of cAMP from blue (the lowest) to purple (the highest). (A) Resting state; (B) 12 minutes after exposure to bPTH(3–34); (C) 2.5 and (D) 30 minutes after addition of bPTH(1–34). The concentration of both fragments was 10^{-7} M.



COLOR PLATE 2. Pseudocolor video images of human trabecular bone osteoblasts loaded with FlCRhR during exposure to human PTH(1–34) and PGE_2. Osteoblastic cells were grown on glass coverslips and microinjected with FlCRhR, as detailed in Material and Methods. The color scale, in fluorescence ratio units (right), represents increasing levels of cAMP from blue (the lowest) to purple (the highest). (A) Resting state; (B) 4 minutes after exposure to 10^{-7} M hPTH(1–34); (C) 2.2 minutes after addition of 3×10^{-6} M PGE_2. The concentration of both fragments was 10^{-7} M.

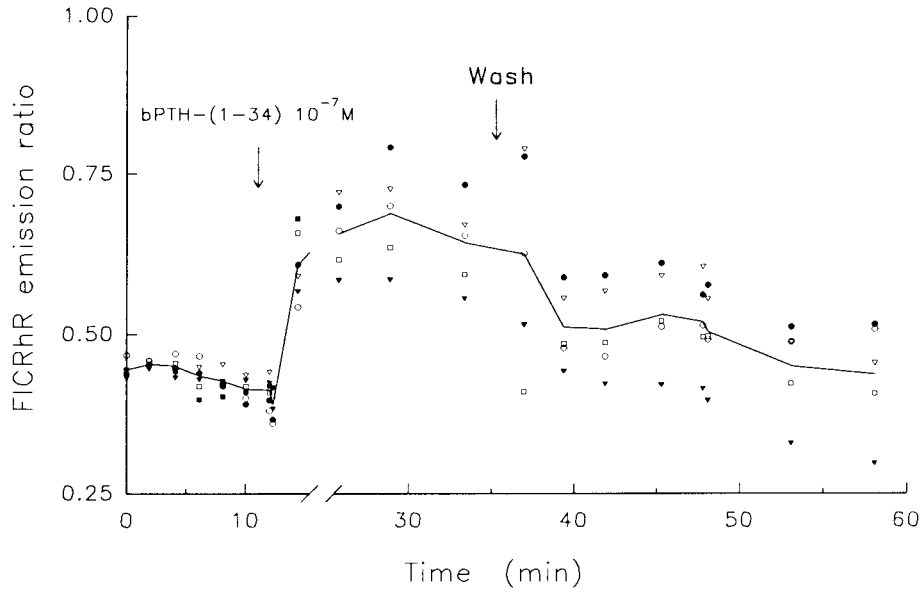


FIG. 2. Reversibility of cAMP stimulation by bPTH(1–34) in UMR-106-01 cells. Cells microinjected with FlCRhR were exposed to 10^{-7} M bPTH(1–34) and washed with fresh culture medium, as indicated, after approximately 25 minutes. The level of FlCRhR emission ratio in five individual cells is plotted with respect to time. Each symbol corresponds to a different cell. Lines are the average of fluorescence ratios obtained for all the cells at each time point.

responsive to the hormone, these PTH peptides were tested in their ability to stimulate cAMP in single cells. Consistent with the data obtained in cell populations,[23] deletion of the first amino acid decreased the potency of the hormone to elicit cAMP production in single cells. In particular, bPTH(2–34) induced smaller increases in FlCRhR fluorescence ratio than an equimolar dose of PTH(1–34) (67%; Fig. 4, top) in all of the injected cells (n = 26), suggesting a homogeneous lower amplitude of response in each cell, not a decrease in the fraction of responsive cells. Accordingly, the 2–34 fragment also induced nuclear translocation of the C subunit of PKA, although this effect appeared to be slower and less pronounced than that induced by bPTH(1–34) (Fig. 4, bottom). In contrast, pbPTH(2–34) had no effect on either FlCRhR fluorescence or C subunit translocation (Fig. 5). Bovine PTH(7–34) was also ineffective (not shown). The relative potencies of the fragments alone and their inhibitory activities on the bPTH(1–34) effect on FlCRhR fluorescence are illustrated in Table 1. Bovine PTH(1–34) added to the bathing medium after exposure to pbPTH(2–34), bPTH(3–34), or bPTH(7–34) (not shown) produced a change in FlCRhR ratio virtually identical in amplitude to that obtained without preexposure to the fragments. Therefore, within the time frame used in these studies, these shortened fragments did not inhibit the action of bPTH(1–34) on rapid cAMP production in osteoblastic cells.

DISCUSSION

PTH stimulates both a transient increase in $[Ca^{2+}]_i$ and the production of cAMP in UMR-106-01 osteogenic sarcoma cells. Previous studies demonstrated a nonuniform $[Ca^{2+}]_i$ response to PTH, which was dependent on cell morphology and receptor distribution.[14] Only 20–30% of these cells respond to PTH in asynchronous cultures.[14,23] Measurements of PTH-induced increases in cAMP production have been measured in bulk cell homogenates, precluding detection of cell-to-cell heterogeneity. Recent advances now allow fluorescence imaging of [cAMP] at the single-cell level.[17] Ideally, single-cell measurements of $[Ca^{2+}]_i$ and [cAMP] would be performed simultaneously. However, because of the spectral overlap of the available fluorescent probes for $[Ca^{2+}]_i$ with the wavelengths required for imaging of FlCRhR or the need for two excitation sources and three emission wavelengths, this type of experiment would be technically difficult. This study nonetheless demonstrates independent measurements of the cAMP response to PTH examined in single UMR-106-01 cells in asynchronous cultures.

In these experiments there was no apparent cell-to-cell heterogeneity in the cAMP response to PTH in single UMR-106-01 cells. PTH led to a rapid and sustained increase in [cAMP] in these cells, regardless of their morphology and confluence state. The observation of homogeneous cAMP responses to PTH, although not totally unexpected, establishes a substantial difference in the activation of the two second messenger systems by the hormone. Although PTH activates the Ca^{2+} pathway in a minority of cells, the response of the cAMP pathway is present in the entire population. The discrepant response patterns between the cAMP and $[Ca^{2+}]_i$ signaling systems cannot be related to the lack of PTH receptors in some cells, because receptors are present in all UMR-106-01 cells, albeit unevenly distributed among the various morphologic types.[15] However, because PTH receptors are expressed at a lower density in those cells that commonly do not exhibit a $[Ca^{2+}]_i$ response,[14] it is possible that a higher number of receptors and/or signal transducing elements is required for activation of the phospholipase C pathway than is necessary for adenylyl cyclase stimulation.

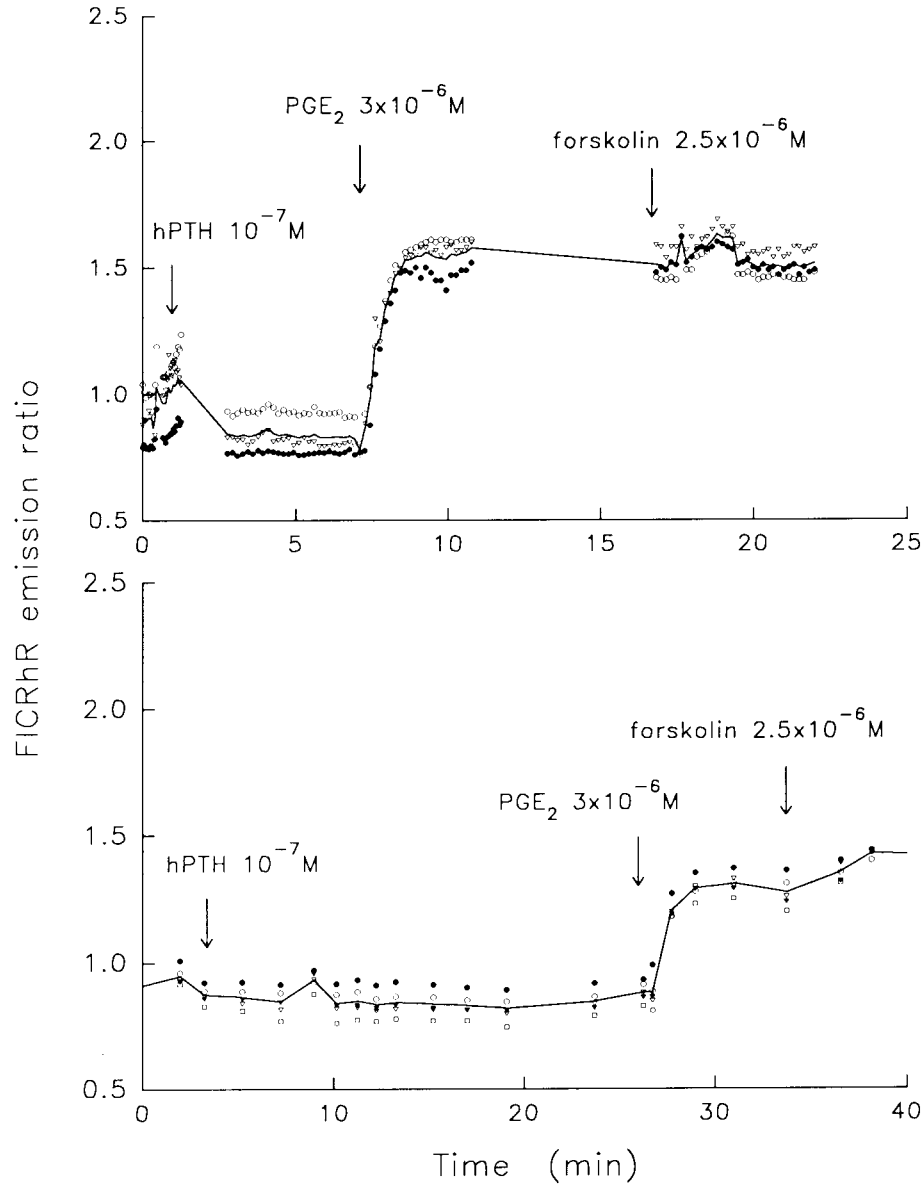


FIG. 3. Effect of subsequent stimulation with hPTH(1–34), PGE_2, and forskolin on intracellular cAMP of human osteoblastic cells (HOB). Cells were prepared as described in Material and Methods, and hormones were added to the bathing medium when indicated by the arrows. (Top) Level of cytoplasmic cAMP (FICRhR emission ratio) in three individual cells of the experiment illustrated in Color Plate 2. Each symbol corresponds to a different cell. Lines are the average of fluorescence ratios obtained for all the cells at each time point. (Bottom) cAMP level in three different cytosolic areas on a single HOB cell.

Alternatively, the different pattern of interactions downstream of the PTH receptors during the different phases of the cell cycle[30] may explain the observed differences. Accordingly, production of cAMP appears to represent a universal response to PTH, whereas $[Ca^{2+}]_i$ transients are confined to a particular cell subtype and to the *S* phase of the cell cycle.[31] Thus, one can speculate that the pattern of second messengers produced by each cell after hormonal stimulation reflects the way that cell is programmed or is able to regulate itself to enact a specific function.

The rapid loss of adenylyl cyclase stimulation by PTH fragments shortened at the N terminus is consistent with previous data on cell populations, wherein cAMP was determined using a radioimmunoassay.[23] Monitoring [cAMP] in single cells now demonstrates that the loss of potency of these peptides in activating adenylyl cyclase is caused by a lower amplitude of response in each cell, rather than a lower number of responsive cells. Of the fragments studied in the current work, only bPTH(2–34) retained partial agonist activity on cAMP production. The other shorter fragments, bPTH(3–34) and bPTH(7–34), were not effective, supporting earlier work.[32,33] However, these data contrast with previous reports of a weak agonist

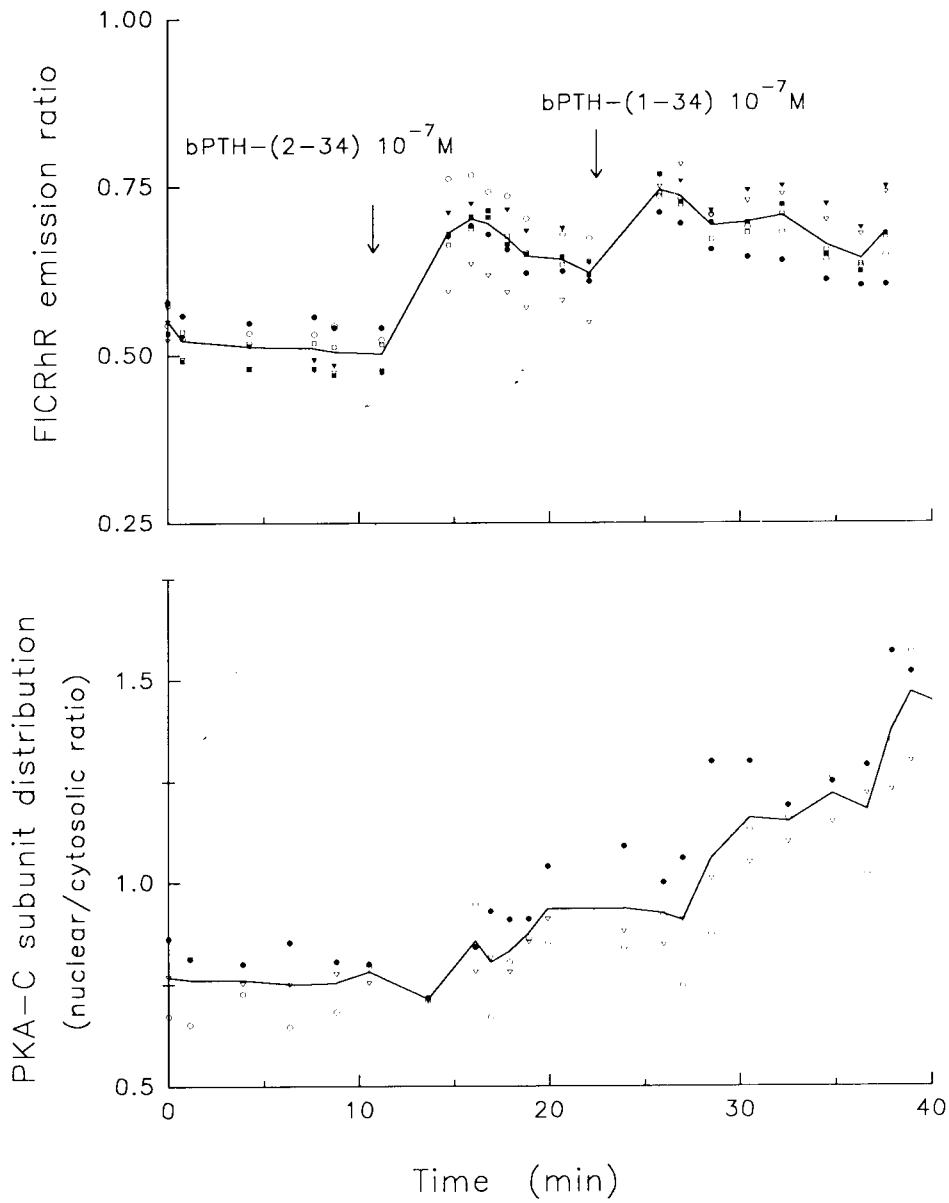


FIG. 4. Effect of subsequent stimulation with bPTH(2–34) and bPTH(1–34) on intracellular cAMP of UMR-106-01 cells. Cells were prepared as described in Material and Methods, and hormones were added to the bathing medium when indicated by the arrows. (Top) Level of FlCRhR emission ratio in four individual cells; (bottom) relative nuclear translocation of the C subunit of PKA, measured in three cells. Each symbol corresponds to a different cell. Lines are the average of fluorescence ratios obtained for all the cells at each time point.

activity on cAMP production by bPTH(3–34) at high doses (10^{-6} M) and in the presence of phosphodiesterase inhibitors and/or pertussis toxin, which all together possibly raise the sensitivity of a radioimmunoassay for cAMP to nonphysiologic extremes.[34,35]

In the present studies, cell exposure to pbPTH(2–34), PTH(3–34), or PTH(7–34) did not block the action of PTH(1–34) on cAMP production, as indicated by FlCRhR fluorescence. Whereas PTH(7–34) has been shown to be a poor inhibitor of cAMP production induced by PTH(1–34), when used at the dose of 10^{-7} M,[36] previous data obtained using biochemical assays demonstrated that 10^{-7} M PTH(3–34) peptides were able to inhibit PTH(1–34)-stimulated adenylyl cyclase activity and cAMP production in renal membranes[32,37] and in UMR-106 cell clones.[35,38] The reason for such a discrepancy between the fluorometric and the biochemical data may be related to the fact that biochemical assays, normally performed in the presence of a phosphodiesterase inhibitor, measure the total cAMP accumulation integrated over a period of several minutes, whereas the technique used in the present study gives real-time information

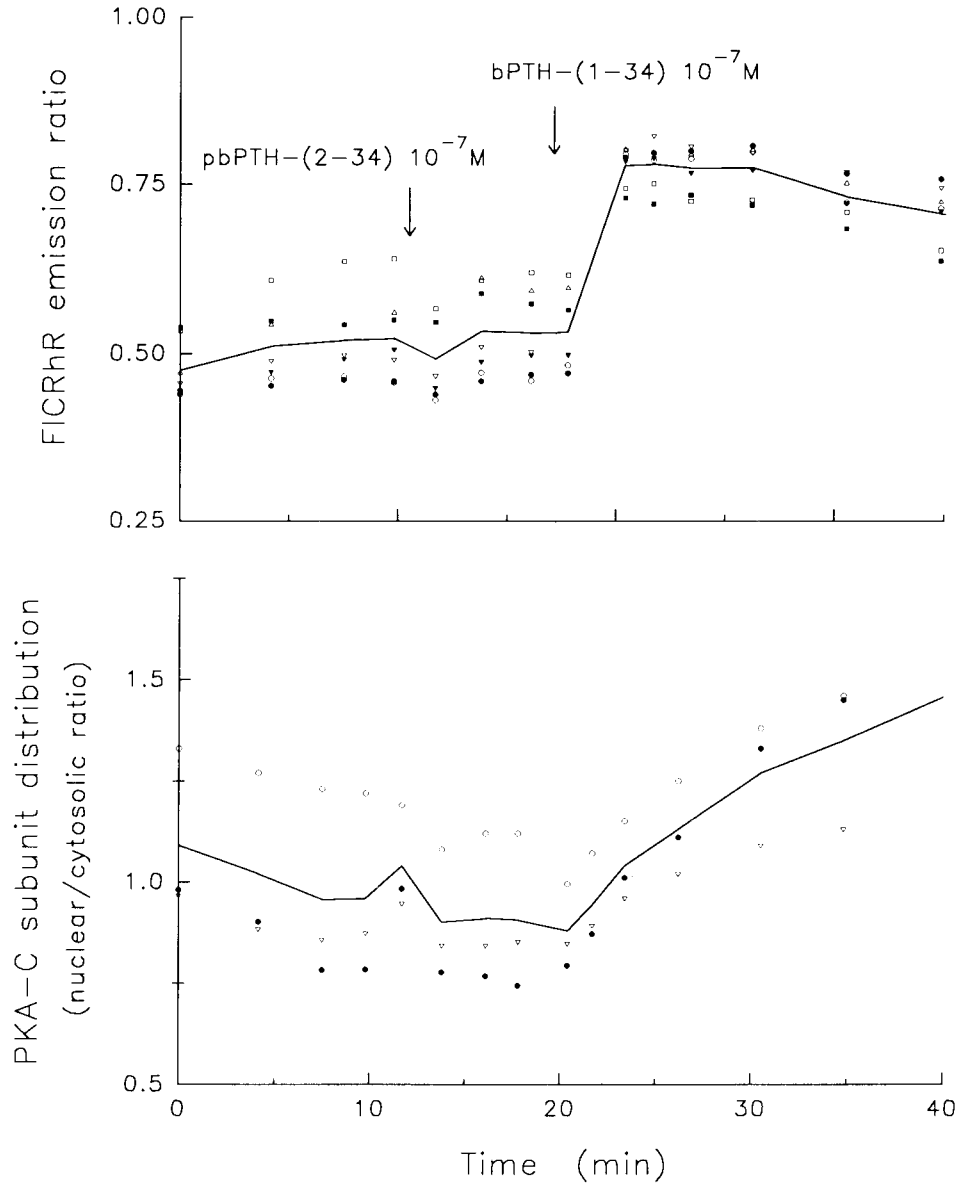


FIG. 5. Effect of subsequent stimulation with propionyl-bPTH(2–34) and bPTH(1–34) on intracellular cAMP of UMR-106-01 cells. Cells were prepared as described in Material and Methods, and hormones were added to the bathing medium when indicated by the arrows. (Top) Level of FICRhR emission ratio in four individual cells; (bottom) relative nuclear translocation of the C subunit of PKA, measured in three cells. Each symbol corresponds to a different cell. Lines are the average of fluorescence ratios obtained for all the cells at each time point.

on cytosolic free cAMP concentrations in live cells. It should also be noted that using biochemical assays, 3–34 fragments of PTH at 10^{-7} M concentration can only partially inhibit adenylyl cyclase activation by equimolar doses of bPTH(1–34) in UMR-106-01 cells,[35] but at higher molar ratios the fragment is a more effective inhibitor.[38] Furthermore, PTH receptor desensitization is complete only after 2 h exposure to the peptide.[39] These findings lend support to the present data, which clearly indicate that bPTH(1–34) is able to induce significant adenylyl cyclase stimulation and PKA activation in the presence of equimolar doses of PTH fragments lacking the first two amino acids at the N terminus.

The lack of responsiveness of HOB cells to PTH may seem surprising, because cAMP production stimulated by PTH is considered one of the major markers of osteoblastic phenotype.[40,41] Indeed, positive responses to PTH have been reported in cells isolated from trabecular bone using isolation procedures similar to ours.[24,42,43] However, osteoblastic cultures obtained by the collagenase digestion of trabecular bone or outgrown from bone particles may not yield phenotypically homogeneous osteoblasts in all cell isolates. In our hands, PTH-stimulated cAMP production detected by radioimmunoassay is widely variable and quantitatively lower than the response in clonal cell lines. Furthermore, $[Ca^{2+}]_i$ responses are usually

TABLE 1. POTENCY OF PTH PEPTIDES IN STIMULATING CAMP PRODUCTION, AND INHIBITING BPTH(1–34)-INDUCED CAMP RELEASE IN SINGLE UMR-106-01 OSTEOGENIC SARCOMA CELLS[a]

	First stimulation: peptide		*Second stimulation: 10^{-7} M bPTH(1–34)*	
Peptide	*% of Baseline*	n	*% of Baseline*	n
Control	—	—	145 ± 17[b]	15
bPTH(1–34)	148 ± 4[b,c]	3	147 ± 6	3
bPTH(2–34)	130 ± 11[c]	26	151 ± 14[d]	21
pbPTH(2–34)	100 ± 3	19	148 ± 18[d]	19
bPTH(3–34)	99 ± 3	11	146 ± 7[d]	11

[a]Cells were grown on glass coverslips to subconfluence and injected with FICRhR on the stage of a confocal epifluorescence microscope, as described in Materials and Methods, and exposed, in succession, to one of the peptides listed on the left, followed by 10^{-7} M bPTH(1–34). Data represent the percentage increase from baseline (normalized to 100) of FICRhR fluorescence ratio at peak or 5 minutes after the addition of the peptide to the bathing medium (when no changes were detected).

[b]Significantly different from baseline.

[c]Concentration 10^{-8} M.

[d]Significantly different from the change induced by the first stimulation ($p < 0.01$, t-test for unpaired samples).

not seen in these cells (manuscript in preparation). Although the cells analyzed in these studies were derived from only two donors, the absence of a cAMP response is contrary to the contention that all osteoblastic cells are obligatory PTH target cells, as classically assumed.[41] Although the physiologic basis for the poor sensitivity of these cells to PTH is still unclear, it is possible that more differentiated osteoblasts that reside on the bone surface—from which our HOB are derived—do not express receptors for PTH in significant numbers, as suggested by studies in sections of normal bone.[13] PTH receptors have been demonstrated to be present at a high density in cells away from the bone surfaces, representing a less differentiated osteoblast precursor.[13] Accordingly, the high degree of PTH response, along with certain morphologic features, seems to indicate that the UMR-106-01 cells may reproduce a phenotype closely related to the PTH-responsive osteoblast precursors of normal bone.[15] Single-cell analyses of phenotypic characteristics and hormonal responsiveness in cells derived from normal bone should help elucidate the significance of a changing hormonal sensitivity during the process of osteoblast differentiation.

In summary, we have made the first real-time recordings of intracellular cAMP levels in individual osteoblastic cells during hormonal stimulation and established that the cAMP response to PTH(1–34) is homogeneous in single UMR-106-01 cells. On the contrary, the hormone does not appear to affect [cAMP] significantly in trabecular bone-derived human osteoblasts. PTH-receptor coupling to alternate signal transduction pathways, such as phospholipase C, may therefore occur independently of cAMP activation, which shows much less population heterogeneity than $[Ca^{2+}]_i$ signaling.

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