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Protein Kinase C Modulator Effects on Parathyroid Hormone-Induced Intracellular Calcium and Morphologic Changes in UMR 106-H5 Osteoblastic Cells

Michael Babich,[1]* **Lisa R.P. Foti,**[1] **and Kevin L. Mathias**[2]

[1]University of Illinois College of Medicine, Rockford, Illinois
[2]Spectronics Instruments, Inc., Rochester, New York

Abstract The effects of parathyroid hormone (PTH) on 1,4,5-inositol triphosphate (1,4,5-IP_3) and intracellular free calcium (Ca_i^{2+}) in osteoblasts are variable, whereas adenylate cyclase activity is consistently stimulated. Cyclic AMP is considered a mediator in the contractile effects of PTH on osteoblasts, but the regulation and role of Ca_i^{2+} remains unclear. Recent studies indicate that protein kinase C (PKC) inhibits PTH-stimulated Ca_i^{2+} increases in osteoblastic cells. Therefore, the objectives of this study were to determine the effects of PKC modulators and PTH on UMR 106-H5 rat osteoblastic cell morphology, and the relationship between cell shape and PTH-induced Ca_i^{2+} changes. In suspended cells loaded with the calcium indicator dye fura-2, pretreatment with PKC inhibitors calphostin C (100 nM × 1 h) and H-7 (30 μM × 18 h) potentiated the effects of 1 μg/ml bPTH(1-84) on Ca_i^{2+} (83% increase over basal) by 1.4- and 1.65-fold, respectively. In comparison, PTH (10 ng–1 μg/ml) was without significant effect on adherent cell Ca_i^{2+} as measured by single-cell image analysis, although another in vitro bone resorbing agent, thrombin (10 U/ml), produced an acute 3-fold increase in the ratio (R) of emission (~λ510 nm) detected and optimized at λ348/374 nm (i.e., Ca-bound dye/free dye) in control cells. Phase-contrast microscopy revealed PKC inhibitor-treated cells changed from a spread configuration to a stellate form with retracting processes or cell rounding and a collapse of actin stress fibers. Within 1 h of PTH addition, PKC inhibitor-treated cells continually became extended/respread up to 3 h with an associated increase in actin stress fibers that was preceded by an acute 1.6-fold Ca_i^{2+} increase. In contrast, control or PKC activator-treated cells (phorbol 12,13-dibutyrate or 12-O-tetradecanoylphorbol-13-acetate; TPA) contracted/retracted within 5 min in response to PTH. A role for Ca_i^{2+} in PTH-induced cell spreading was further indicated by a contractile response to PTH when PKC-inhibitor-treated cells were loaded with the intracellular calcium chelator dimethyl BAPTA (3 μM × 30 min). PTH-induced Ca_i^{2+} increases in adherent PKC inhibitor-treated cells were also associated with a 1.8-fold 1,4,5-IP_3 increase as measured by mass assay. The data suggest PKC contributes to UMR 106-H5 cell morphology and selectively regulates signal pathways activated by PTH to promote either cell contraction (cAMP) or extension (1,4,5-IP_3/Ca_i^{2+}). J. Cell. Biochem. 65:276–285.

Key words: morphology; calcium imaging; osteoblasts; inositol phosphate; inositol 1,4,5-triphosphate; thrombin

A generally accepted hypothesis on bone resorption [Rodan and Martin, 1981; Baron, 1996] suggests that factors which cause the bone forming osteoblasts to contract and separate from one another will enable osteoclasts, the cells responsible for resorption, to gain access to the mineralized matrix of bone. Subsequent osteoblast stimulation by hormones or cytokines may then lead to the production of specific signals to either activate osteoclasts or to promote the growth of bone. The classic bone regulatory agent, parathyroid hormone (PTH), produces cyclic AMP-dependent osteoblast contractions [Miller et al., 1976; Egan et al., 1991] that presumably favor bone resorption. However, PTH produces both catabolic and anabolic effects on bone [Dempster et al., 1993]. The ability of calcium ionophores to increase polymerized actin and tubulin in osteoblasts [Lomri and Marie, 1990] suggests that intracellular free calcium (Ca_i^{2+}) contributes to the bone-

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*Correspondence to: Michael Babich, UIC College of Medicine, 1601 Parkview Avenue, Rockford, IL 61107-1897. Email: michaelb@uicvm.uic.edu

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forming effects of PTH via increased cell spreading. However, the cellular mechanisms that regulate the diverse effects of PTH on osteoblasts and bone are unclear, but are likely to involve regulation of second-messenger production.

Protein kinase C (PKC) is a candidate regulator of hormone-signaling in the osteoblast. In suspended parental and subclonal UMR 106-H5 rat osteosarcoma cells, PKC activating drugs completely block PTH-stimulated Ca_i^{2+} increases, whereas PTH-stimulated adenylate cyclase activity is enhanced [Merritt et al., 1992; Babich et al., 1994]. In light of the diverse effects of PTH on bone and osteoblastic cell shape, the objectives of this study were to determine (1) the effects of PKC modulators and PTH on UMR 106-H5 cell morphology, and (2) the role of Ca_i^{2+} in PTH-induced changes in cell shape.

METHODS

Materials

PKC modulating drugs were: TPA (12-O-tetradecanoylphorbol-13-acetate), PDBu (phorbol 12,13-dibutyrate), calphostin C and 4α-phorbol purchased from Calbiochem (San Diego, CA); H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] and H-1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide) from Seikagaku America, Inc. (Rockville, MD); and staurosporine from Boehringer Mannheim Corp. (Indianapolis, IN). Fura-2, AM, and 5,5-dimethyl BAPTA, AM, were purchased from Molecular Probes (Eugene, OR), and mouse anti-chicken actin monoclonal antibody (C4 clone) and FITC-conjugated goat anti-mouse IgG were from ICN (Costa Mesa, CA). Bovine PTH(1-84) was provided by the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture.

Cell Morphology

UMR 106-H5 osteoblast-like rat osteosarcoma cells maintained in culture as previously described [Babich et al., 1990] were grown in 6-well culture dishes and treated with PKC modulating drugs prior to PTH addition. The drugs and PTH were continuously present and photographs were taken under phase contrast microscopy (134×) to document morphologic changes. In some experiments the intracellular calcium chelator dimethyl BAPTA AM (3 μM × 30 min) was loaded into cells prior to PTH addition. Preliminary work indicated this was sufficient to completely prevent both thrombin- and PTH-stimulated Ca_i^{2+} increases in suspended UMR 106-H5 cells, whereas PTH-stimulated adenylate cyclase is not significantly affected (Babich and Nissenson, unpublished observations).

Actin was visualized in cells grown on glass coverslips and treated identical to those used above. In some experiments MC3T3-E1 osteoblastic cells derived from normal mouse calvaria [Kodama et al., 1981] were included to compare with actin changes in transformed UMR 106-H5 cells. The cells were rinsed with TBS (0.01 M tris-HCl, 0.15 M NaCl), fixed for 10 min with ice-cold acetone:methanol (1:1) and either immediately used for fluorescent antibody studies or stored at −20°C until use. The fixed cells were incubated with TBS containing 2% goat serum (15 min at 37°C), washed with TBS, and incubated with mouse anti-chicken actin antibody (1:40 dilution × 2 h) followed by FITC-conjugated goat anti-mouse IgG (1:100 dilution × 1 h). Preliminary control studies indicated that almost no detectable fluorescence was detected with the FITC-conjugated secondary antibody alone or in the presence of nonspecific mouse IgG. An Olympus-10ADS camera-mounted to an Olympus BH-2 reflected fluorescence light microscope was used to photograph actin (165×).

Measurement of Ca_i^{2+}

Adherent UMR 106-H5 cells grown on glass coverslips were loaded with the calcium indicator dye fura-2 AM (3 μM × 30 min) [Grynkiewicz et al., 1985]. The ratio (R) of emission (~λ510 nm) was detected and optimized at λ348/374 nm (i.e., Ca^{2+}-bound dye/free dye). The outline of fura-2 emission was similar to the actual cell boundaries as determined by changing to cell imaging under light microscopy before and after experiments. Individual cells were identified using a Nikon (Melville, NY) DIAPHOT TMD inverted microscope with a Nikon 40× dry fluor objective and DM400 dichroic mirror block. The ICCD system included a Dage-MTI GENIISYS intensifier and CCD-72 camera, with an AMINCO-Bowman Series 2 Luminescence Spectrometer (SLM-AMINCO, Rochester, NY) as an excitation source for these experiments. A randomized

quartz fiber optic cable was used to transmit excitation light to the microscope. The Image-1/FL package (Universal Imaging Corporation, Westchester, PA) was used for image capture, cell identification and tracking, and ratiometric analyses. The colorimetric scale derived from the software program was empirically assigned (e.g., blue and red correspond to relatively low and high ratios, respectively) and used to photograph the images of different Ca^{2+}-bound/unbound fura-2 ratios.

In some experiments, UMR 106-H5 cells were suspended and loaded with the intracellular calcium indicator fura-2 AM as previously described [Babich et al., 1991] to measure Ca_i^{2+}. PTH-induced changes in suspended cell Ca_i^{2+} were assessed by comparing the peak Ca_i^{2+} concentration with basal Ca_i^{2+} values.

Measurement of 1,4,5-IP_3

Total cellular 1,4,5-IP_3 content was determined by a commercially available mass assay system (Amersham, Arlington Heights, IL). The protocol provided by the vendor was essentially followed, with some necessary modifications to ensure the standard curve, and unknowns were performed under similar conditions. Briefly, UMR 106-H5 cells grown to ~60% confluence in 10-cm culture dishes were incubated for specified times with modified medium (MEM-growth medium containing 2% heat inactivated fetal calf serum, 0.1 mM LiCl, and PKC modulating drugs or vehicle) followed by exposure to reaction medium (MEM with 0.05% BSA, 1 mM LiCl, hormone or vehicle, and 10 mM HEPES, pH 7.4). The reaction was stopped by aspiration of the medium, immediate addition of ice-cold 4% $HClO_4$ (3 ml), and placing the dishes on ice for 20 min. Cell lyzates scraped into microcentrifuge tubes were then centrifuged (2,000*g* × 15 min at 4°C), and the resulting supernatants were neutralized with 10 N KOH prior to assay for 1,4,5-IP_3 content. Stock 1,4,5-IP_3 standards were diluted with a buffer comprised of 0.1 volume reaction MEM/0.9 volume 4% $HClO_4$ and neutralized with 10 N KOH. The revised dilution conditions were required because we predetermined (1) the standards would otherwise be assayed at pH ~8.5, whereas the unknown samples are at pH ~7.7, and (2) pH will affect the apparent affinity of the binding protein for 1,4,5-IP_3 (i.e., k_D base ≥ neutral > acid). In addition, the cell treatment and conditions (e.g., ~60% cell confluence) were selected to duplicate the adherent Ca_i^{2+} measurement conditions as close as possible. It should be noted, however, that increased variability in hormone-stimulated 1,4,5-IP_3 was observed in preliminary experiments with cells at higher confluence.

TABLE I. Effects of PKC Modulating Drugs on PTH-Stimulated Ca_i^{2+} [a]

	Ca_i^{2+} fold increase[b]
Calphostin C	1.40 ± 0.25* (17)
H-7	1.65 ± 0.20* (17)
H-1004	1.05 ± 0.15 (3)
TPA	0.03 ± 0.03* (7)

[a]Following treatment with calphostin C (100 nM × 1 h), H-7 (30 μM × 18 h), H-1004 (30 μM × 18 h), or TPA (100 nM × 10 min), UMR 106-H5 cells were suspended to measure Ca_i^{2+} as described in Methods.
[b]Mean ± SE (N); the data were calculated vs. the peak Ca_i^{2+} effect of 1 μg/ml bPTH(1–84) alone in paired cell samples (i.e., PTH alone = 1.0).
*$P < 0.01$ vs. PTH alone, Dunnett's t-test for comparison with control means.

RESULTS

Acute exposure of osteoblastic cells to PKC activator drugs have recently been shown to block PTH-stimulated Ca_i^{2+} increases and potentiate PTH-stimulated adenylate cyclase activity [Merritt et al., 1992; Babich et al., 1994]. To extend the reported observations on PKC-regulated PTH-induced Ca_i^{2+} increases, the effects of PKC inhibitors were examined. PKC inhibitors (H-7, calphostin C shown in Table I) promoted the peak Ca_i^{2+} transient that results from a maximal effect of PTH on intracellular calcium mobilization (i.e., basal vs. PTH Ca_i^{2+} = 120 vs. 220 nM). By comparison, the PKC activator TPA abolished the Ca_i^{2+} response to PTH and H-1004, a relatively inactive H-7 analog, was without effect. The results support the idea that PKC regulates PTH receptor-signaling.

The ability of PTH to promote osteoblast contraction has been linked to decreased actin and myosin polymerization and appears to be mediated by cyclic AMP [Egan et al., 1991], whereas Ca_i^{2+} has been implicated in cellular extension or respreading [Lomri and Marie, 1990]. Thus, different signaling pathways may mediate opposing morphologic changes invoked by PTH. However, there is no direct evidence for Ca_i^{2+}-dependent cellular respreading in response to PTH. Therefore, the effects of PTH

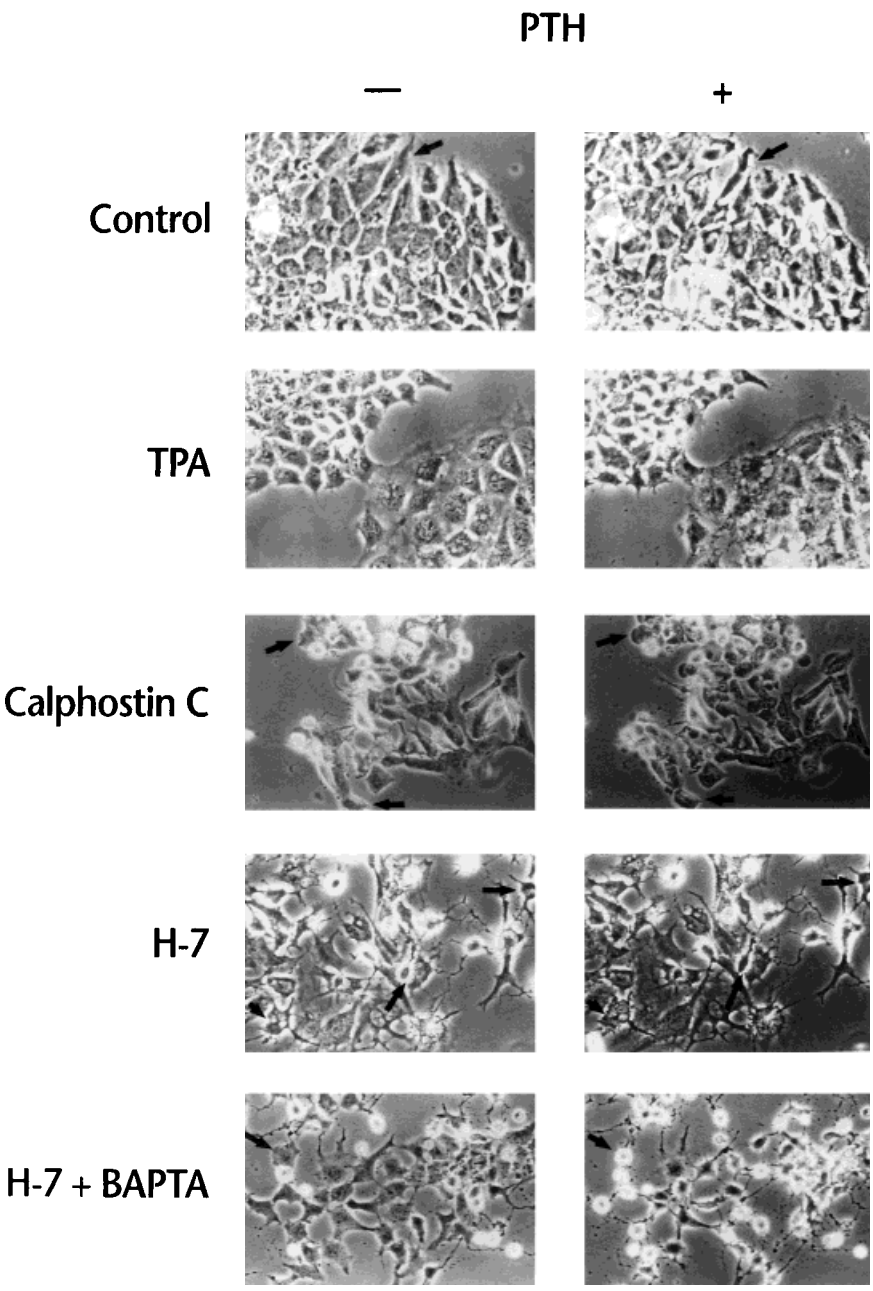

Fig. 1. Effects of bPTH(1-84) and protein kinase C modulating drugs on UMR 106-H5 cell morphology. **Control:** PTH (300 ng/ml)-induced control cell contraction at 3 h. **TPA:** PTH-induced contraction at 3 h in cells pretreated with TPA (100 nM × 15 min). **Calphostin C:** Cells precontracted by calphostin C (100 nM × 4 h) become respread in response to PTH within 1 h. **H-7:** Cells precontracted by treatment with H-7 (30 μM × 18 h) become respread upon PTH addition. **H-7 + BAPTA:** Similar to H-7, but the cells were loaded with BAPTA (3 μM × 30 min) prior to PTH addition. Arrows follow the effects on representative cells.

and PKC modulators on UMR 106-H5 cell morphology, and the relationship between cell shape and PTH-induced Ca_i^{2+} changes were examined. Prior to PTH addition, control cells were generally cuboidal and spread out (Fig. 1) with well-defined cell-cell contact (i.e., zero time). Time course photographs indicate PTH (10 ng/ml–1 μg/ml; 300 ng/ml shown) evoked cellular contraction and retraction (e.g., follow arrows) within 15 min and continued up to 3 h. TPA alone did not appear to affect cell shape, but the contractile response to PTH was observed as early as 3 min (3 h shown). Pretreatment of cells with calphostin C (10–100 nM × 4 h) or

H-7 (100 μM × 4 h or 30 μM × 18 h as shown), which rapidly reduces actin microfilament bundles in Swiss 3T3 and PTK2 cells [Birrell et al., 1989], produced a stellate form with retracting processes and cell rounding. However, it was noted that calphostin C produced a more rapid transition from cuboidal, to stellate, then rounded or collapsed cells, than H-7 (e.g., Fig. 1). Differences in the rate and extent of cellular retraction and contraction presumably reflect differences in the specificity and potency of the various PKC inhibitors. Thus, calphostin C was included because it is among the most specific and potent PKC inhibitors known [Tamaoki, 1991] but lacks an appropriate inactive control analog such as H-1004 is to H-7. In contrast to control and TPA-treated cells, subsequent addition of PTH to PKC inhibitor–treated cells produced respreading of stellate or rounded cells. Similar results were obtained with other PKC activators and inhibitors (PdBu, 300 nM; staurosporine, 100 nM), whereas the relatively inactive control drugs 4α-phorbol (≤300 nM) and H-1004 (≤100 μM) were without effect (not shown).

In agreement with the phase-contrast photographs, PTH likewise produced opposite effects on the actin staining patterns of control and PKC inhibitor–treated UMR 106-H5 cells (Fig. 2, top). Control cell actin stress fibers or radial patterns became collapsed and perinuclear upon exposure to PTH. PKC inhibitors (calphostin C shown) led to fewer filaments and collapsed actin, but PTH addition eventually produced a greater amount of actin stress fibers associated with the re-extended morphology. However, the time course for PTH-induced F-actin restoration in PKC-inhibited cells appeared to vary and, in some instances, cellular contraction occurred in cells that appeared morphologically similar to control cells (not shown), presumably reflecting differential effects of PKC inhibitors on individual cells. Nevertheless, the overall effect of PTH on cells pre-contracted with PKC inhibitors was to elicit cellular respreading associated with actin reassembly. Conducting the same experiments with MC3T3-E1 cells revealed similar effects on actin organization occur in non-tumorigenic cells (Fig. 2, bottom). If anything, the effects of calphostin C were more extensive on MC3T3-E1 cells because the majority of cells had already become rounded and sloughed off by the same time photographs were taken to compare with UMR 106-H5 cells. Moreover, PTH addition to calphostin C–treated MC3T3-E1 cells prevented the loss of cells in addition to producing a respeading effect.

PTH is thought to produce cAMP-dependent osteoblast contractions, but a role for Ca_i^{2+} is unclear. Therefore, PTH-induced cellular respreading was tested in PKC inhibitor-treated cells loaded with the intracellular calcium chelator dimethyl BAPTA AM (Fig. 1, H-7 treated cells shown). BAPTA produced a slightly more stellate appearance of UMR 106-H5 cells but, in contrast to H-7 treatment alone, began to contract or retract extensively upon PTH addition. The data suggest increased Ca_i^{2+} is prerequisite for PTH-induced respreading of precontracted cells.

To directly test whether PTH elicits Ca_i^{2+} increases in PKC inhibitor-treated cells, single-cell image analysis was performed. PTH did not detectably raise control cell Ca_i^{2+} (Fig. 3, left column), but did produce contractile responses as shown earlier (i.e., Fig. 1). However, control cell Ca_i^{2+} significantly increased in response to thrombin (positive control). In contrast to control cells, PKC inhibitor-pretreated cells displayed a higher basal Ca_i^{2+} and unmasked an ability for PTH to raise adherent cell Ca_i^{2+} (Fig. 3, right column). The Ca_i^{2+} response generally peaked within 50 s with a return to baseline at 3 min. However, the onset and duration of PTH-induced Ca_i^{2+} increases were variable, presumably due to either incomplete PKC inhibitor effects on some cells or a distinct cell cycle dependent phenomenon. We also observed that within 20–60 min, a downward refocus (~3 μm) was sometimes necessary to restore optimal visualization of the cell outlines, which may reflect PTH-induced cell broadening/flattening of cells precontracted with PKC inhibitors (not shown). The data collectively suggest PKC regulates PTH-stimulated Ca_i^{2+} signaling that mediates extended cell morphology.

The reported effects of PTH on osteoblastic cell 1,4,5-IP_3 accumulation are variable and generally small, which is similar to PTH-stimulated Ca_i^{2+} increases. Therefore, the ability of PTH to raise 1,4,5-IP_3 in the Ca_i^{2+}-responsive PKC inhibitor-treated cells used above was determined. PTH did not detectably raise control cell 1,4,5-IP_3 accumulation (Table II), as expected from the lack of effect on adherent cell Ca_i^{2+}. In contrast, PTH produced 2.1- and 1.8-fold 1,4,5-IP_3 increases in H-7 and calphostin C–treated cells, respectively. It was

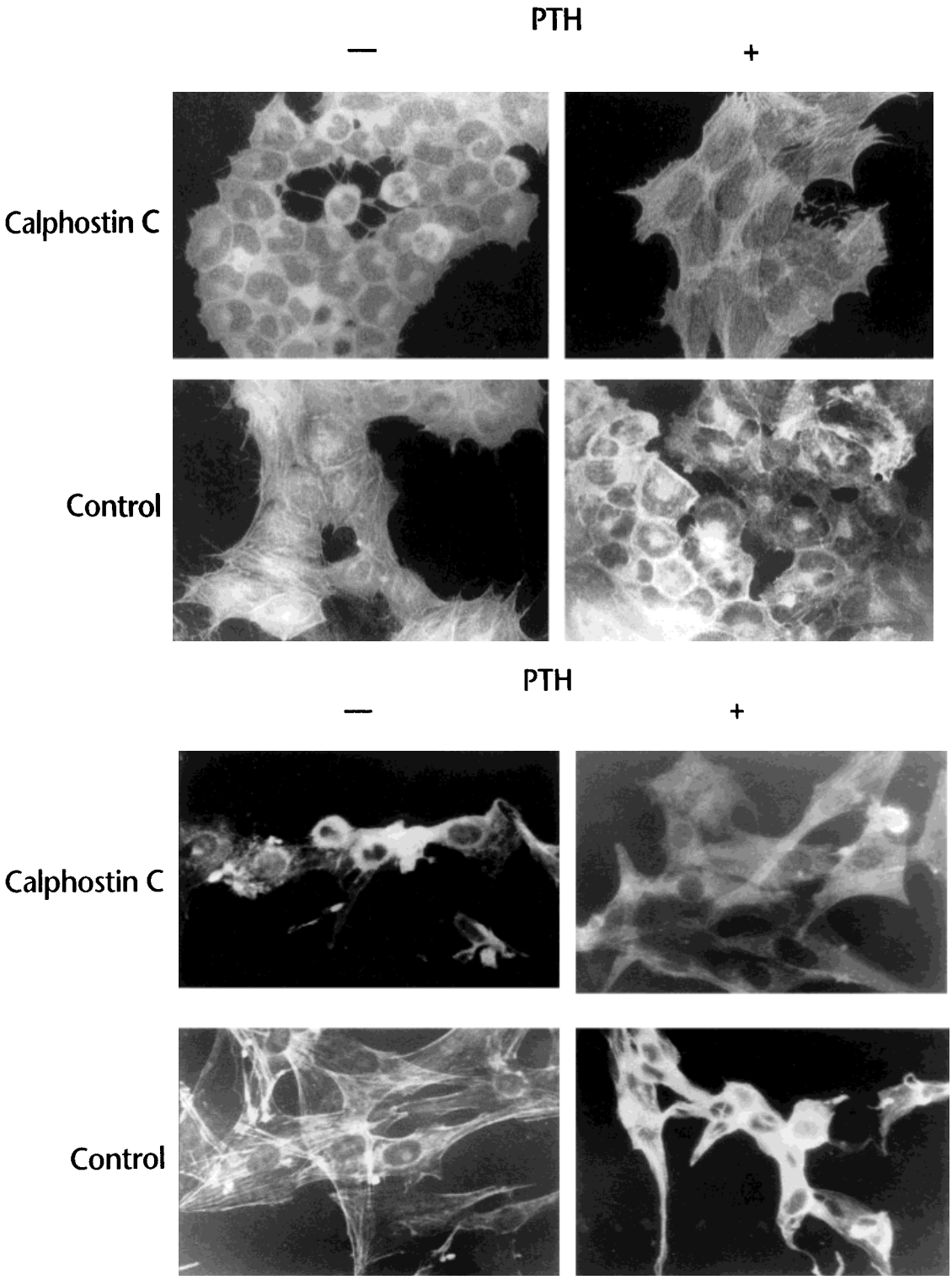

Fig. 2. Effects of calphostin C and PTH on actin organization. Control or PKC inhibitor (calphostin C, 100 nM × 4 h)-treated UMR 106-H5 cells (**top**) and MC3T3-E1 cells (**bottom**) were exposed to either vehicle or PTH (300 ng/ml × 1 h) prior to immunocytochemical staining for actin as described under Methods.

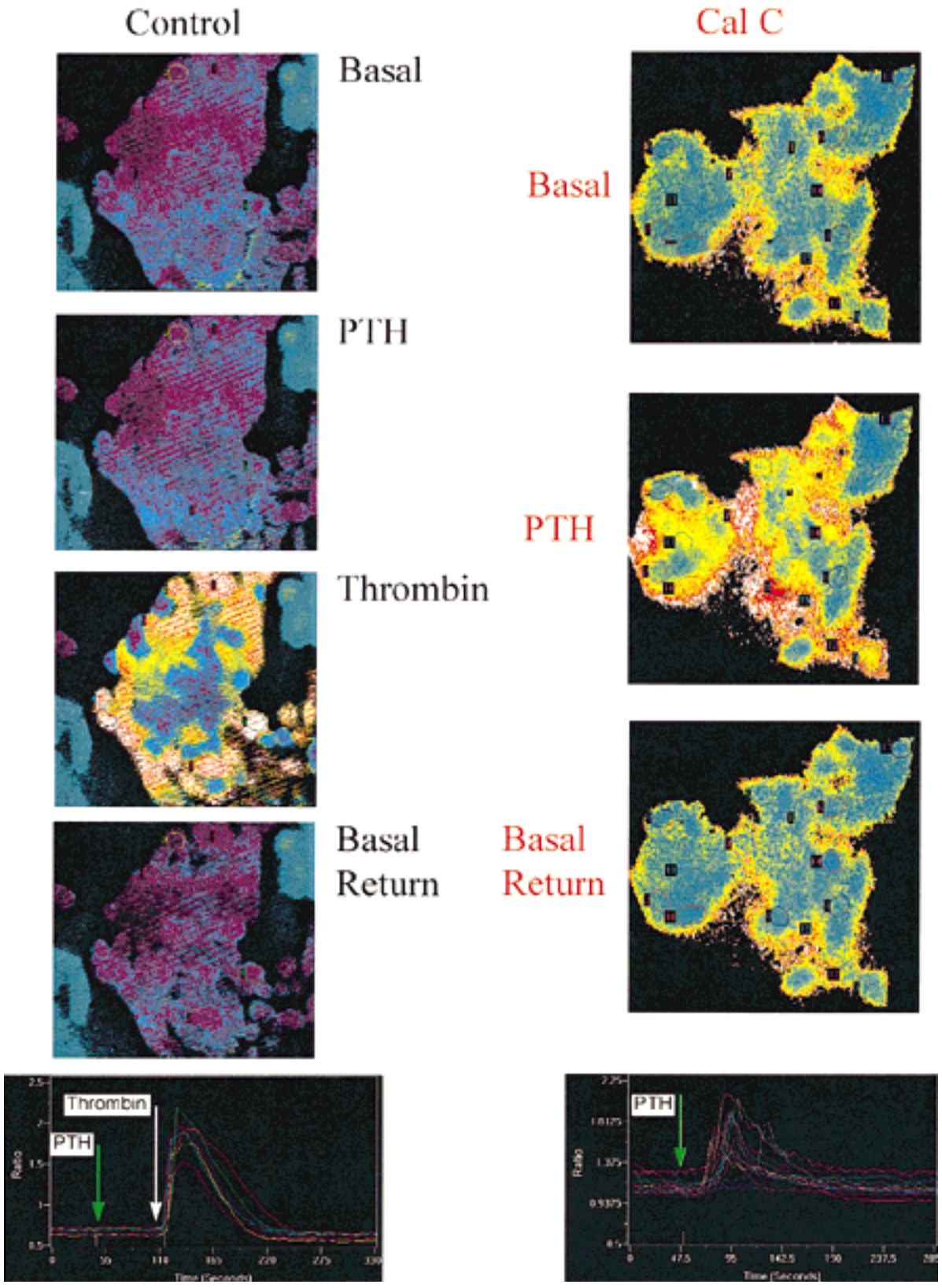

Fig. 3. PTH raises Ca_i^{2+} in PKC inhibitor-treated adherent UMR 106-H5 cells. The effects of PTH on Ca_i^{2+} were monitored by the ratio of fura-2 emission vs. time. Cell images reflect Ca_i^{2+} at the following times: basal (zero time), PTH (300 ng/ml $\times$ 1 min), thrombin (10 U/ml peak), and return to basal (~10 min following PTH addition). The arbitrary color scale represents relative ratio changes (blue $\rightarrow$ green $\rightarrow$ yellow $\rightarrow$ orange $\rightarrow$ red $\rightarrow$ white: range = $\leq$100 nM to $\geq$1,000 nM Ca_i^{2+}). The time vs. R plot is representative for 7 and 15 cells marked (dotted outlines) at zero time for control and calphostin C (100 nM $\times$ 4 h), respectively. The percent increase over basal Ca_i^{2+} produced by PTH in calphostin C treated cells = 59.3 $\pm$ 8. The data are representative of $\geq$20 total cells analyzed from each of three different preparations.

TABLE II. PTH-Stimulated 1,4,5-IP_3 Accumulation in PKC Inhibitor-Treated UMR 106-H5 Cells

	1,4,5-IP_3, pmol/mg protein[a]		
	Control	H-7 (30 μM × 18 h)	Calphostin C (100 nM × 4 h)
Vehicle	6.4 ± 2.0	3.1 ± 1.0**	4.5 ± 1.0
bPTH (1–84)	5.9 ± 1.5	6.3 ± 2.3*	7.9 ± 2.7*

[a]Following 45-s exposure to 300 ng/ml bPTH(1–84) or vehicle, 1,4,5-IP_3 was measured in adherent cells treated identical to those used for single-cell Ca_i^{2+} imaging. The data represent the mean ± SE of duplicate determinations from each of five (control) or four (H-7, calphostin C) different preparations.
*$P < 0.05$ vs. corresponding vehicle, Tukey-Kramer method for multiple comparisons between means.
**$P < 0.05$ vs. vehicle-treated control cells.

also noted that H-7 treatment alone inhibited basal 1,4,5-IP_3 accumulation. The results are in agreement with the notion that PTH-induced intracellular calcium mobilization, when detected, is associated with or preceded by PLC stimulation.

DISCUSSION

The present work demonstrates an interplay between osteoblast morphology, PKC activity, and PTH receptor-signaling. Constitutive or drug-enhanced PKC activity favors an extended cell morphology and is permissive to PTH-induced cell contraction and retraction. The potentiating effects of PKC activators on PTH-induced cellular contractions, as well as adenylate cyclase activity [Babich et al., 1994], are consistent with previous reports that PTH produces cAMP-dependent osteoblast contractions [Miller et al., 1976; Egan et al., 1991]. In addition, the contractile effects of PTH on PKC inhibitor-treated cells loaded with a calcium chelator presumably results from the effects of unopposed PTH-stimulated cAMP production. The present study also implicates that an alternative pathway (i.e., 1,4,5-IP_3/Ca_i^{2+}) may lead to opposite morphologic effects by PTH. Exposure of UMR 106-H5 cells to PKC inhibitors produced cell contraction/retraction and unmasked an ability for PTH to raise Ca_i^{2+} and elicit cell respreading and extension. An ability of PTH to induce cell extention or relaxation has also been observed in primary cultures of mouse osteoblasts [Lomri and Marie, 1988] and vascular smooth muscle cells precontracted by depolarization [Wang et al., 1993]. However, a link between different PTH-stimulated signaling pathways and opposite morphologic effects within the same cells has not been reported.

Another observation worth noting is that PTH-stimulated Ca_i^{2+} increases were associated with PKC inhibitor-treated adherent cells that displayed elevated basal Ca_i^{2+} levels. The data are consistent with another study [Wiltink et al., 1993] in which PTH was more effective at raising Ca_i^{2+} in parental UMR 106 cells and primary rat osteoblastic cultures with higher resting Ca_i^{2+} values. It is currently unclear how basal Ca_i^{2+} is elevated or if it is relevant to facilitating PTH effectiveness. Although several speculations may be invoked (e.g., elevated basal Ca_i^{2+} concentrations lowering the threshold for Ca_i^{2+}-induced intracellular calcium release) other work is required to define the mechanisms involved.

The ability of an intracellular calcium chelator [Egan et al., 1991] and calcium ionophore [Lomri and Marie, 1990] to decrease and increase osteoblastic cell actin polymerization, respectively, support the present findings that Ca_i^{2+} is involved in cellular extension. However, the mechanism by which PTH-stimulated Ca_i^{2+} increases lead to an extended morphology is unknown. In contrast to the present findings, Tram et al. [1993] and Murray et al. [1995] proposed that calcium activated cysteine proteases (e.g., calpains) are involved in PTH-induced osteoblastic cell contractions. The discrepancy regarding a Ca_i^{2+} role in osteoblast contraction vs. extension/respreading may be explained on the basis that the calcium signal induced by PTH, as measured in individual osteoblastic cells [e.g., Babich et al., 1991; Civitelli et al., 1992; Wiltink et al., 1993], appears to be compartmentalized from the calcium pools and/or second messengers activated by other agonists. Therefore, agonists may not uniformly activate cysteine proteases or elicit the same cellular responses (e.g., changes in morphology, cell growth, etc.). For example, thrombin is effective at raising control cell Ca_i^{2+} and is mitogenic [Babich et al., 1990], whereas PTH is antiproliferative and, unlike thrombin, can be manipulated by PKC to elicit different effects on signaling and morphology [e.g., present work; Merritt et al., 1992; Babich et al., 1994; Sugimoto et al., 1994). The different Ca_i^{2+} responses to thrombin vs. PTH in adherent control cells also support earlier work that the two hor-

mones use distinct pathways to mobilize intracellular calcium [Babich et al., 1991; Wiltink et al., 1993]. It is possible that PKC inhibitors alter the response of second messenger target proteins, the crosstalk between signal transduction pathways, and the cytoskeleton such that compartmentalized Ca_i^{2+} increases stimulated by PTH lead to an opposite cellular respreading effect, rather than a contractile effect that results from both cAMP and calpain action in control cells. Furthermore, it is unclear whether both cAMP and calpain act in synergy, or whether calpain activity is regulated by either cAMP or PKC. More studies are needed to define the regulation and roles of different second messengers generated in response to PTH.

Current data indicate PKC exerts a regulatory effect via phosphorylation of key signaling components or the PTH receptor itself. In support, PKC activators produce >2-fold increases in basal phosphorylation of the PTH/PTHrP receptor expressed in human embryonic kidney cells [Blind et al., 1995, 1996]. The data also suggest a regulatory role of PKC on the cytoskeleton. PKC inhibitor-treatment leads to a stellate configuration and some elongated processes that correspond to an ability of PTH to raise Ca_i^{2+} in adherent cells. The findings are in agreement with single-cell image analyses of adherent parental UMR 106 cells [Civetelli et al., 1992] in which PTH is more likely to raise Ca_i^{2+} in cells that are similar in morphology to PKC inhibitor-treated cells. Moreover, the effects of PTH on Ca_i^{2+} appear to be cell cycle-dependent [Bizzarri, et al., 1994]. Presumably, the PKC modulators used in the current work affect the cell cycle as well as cell shape, which is a phenomenon associated with the cell cycle.

The results also suggest the existence of a cellular factor(s) that is regulated by PKC and modulates both PTH receptor-signaling and cytoskeletal architecture. In this regard, (1) PTH is more effective at raising Ca_i^{2+} in spherically shaped suspended osteoblastic cells than in adherent cells [e.g., see also Schöfl et al., 1991; Babich et al., 1991]; (2) PKC inhibitors potentiate the ability of PTH to raise Ca_i^{2+}, whereas PKC activators completely block the Ca_i^{2+} response while potentiating PTH-stimulated adenylate cyclase activity [i.e., Merritt et al., 1992; Babich et al., 1994]; (3) osteoblast adhesion to type I collagen likewise modulates the Ca_i^{2+} and cAMP responses to PTH [Green et al., 1995]. The effects by type I collagen were mediated by PKC, which is consistent with the idea that an interplay exists between PKC activity, Ca_i^{2+} signaling, and osteoblast adhesion or morphology.

PTH is a potent and consistent stimulator of adenylate cyclase activity in target cells [Dunlay and Hruska, 1990; Civitelli et al., 1994], but the effects on 1,4,5-IP_3 accumulation and Ca_i^{2+} are generally variable. The purpose for such cellular control of the 1,4,5-IP_3/Ca_i^{2+} signaling pathway remains uncertain. It is likely that regulation of PTH-stimulated second-messengers will affect the physiologic response of osteoblasts and ultimately bone remodeling (e.g., contraction to favor resorption vs. respreading to favor formation). Conceivably, there are cellular factors that affect morphology, interact with key signal transduction components, and are PKC substrates. Other work is required to identify such factors, and to determine whether the anabolic vs. catabolic effects of PTH on bone are a consequence of altered PTH receptor-signaling and shape changes in osteoblasts.

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