Humoral Factors of Ascites Sarcoma 180 Stimulate Osteoblastic UMR 106-01 Cell Proliferation and Bone Resorption via Signal Transduction Pathways, Which Are Clearly Different from Those of Parathyroid Hormone

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ABSTRACT—Ascites sarcoma 180 (S180A) is a transplantable tumor that induces hypercalcemia in tumor-bearing mice and stimulates bone resorption in cultured neonatal mouse calvaria without parathyroid hormone (PTH)-like activity. The serum-free conditioned media of S180A cell cultures (S180A-CM) stimulated [3H]thymidine incorporation (178.3% of the control) and inhibited alkaline phosphatase activity (39.0% of the control) in the osteoblastic osteosarcoma cell line UMR 106-01, contrary to PTH. To investigate signal transduction by S180A-CM, we determined the levels of intracellular free calcium ([Ca2+]i), inositol 1,4,5-triphosphate (IP3), 1,2-diacylglycerol (DAG), phosphatidylcholine (PC) and protein kinase (PK) C activity in UMR 106-01 cells. PTH and PTH-related protein (PTHrP), both potent bone-resorbing factors (BRFs), caused an increase in [Ca2+]i and stimulated IP3 production, whereas S180A-CM had little or no effect on these parameters. On the other hand, S180A-CM stimulated DAG production, accompanied by PC breakdown, and the translocation of PKC activity from the cytosol to the membrane fraction. Sphingosine, a specific PKC inhibitor, inhibited bone-resorbing activity (BRA) in S180A-CM more effectively than PTH or PTHrP-stimulated resorption. H-7, an inhibitor of both cAMP-dependent PKA and PKC, completely inhibited BRA in S180A-CM. These results suggest that BRFs of S180A-CM stimulate osteoblastic cell proliferation and bone resorption via two signal transduction pathways, which are different from those of PTH: 1) activation of PKC by DAG resulting from PC hydrolysis and 2) activation of PKA subsequent to prostaglandin E2 production by bone.

Keywords: Ascites sarcoma 180, Bone resorption, Diacylglycerol production, Phosphatidylcholine breakdown, Protein kinase C

Hypercalcemia frequently occurs in advanced malignancies. Accelerated bone resorption is believed to be the primary cause of this hypercalcemia. It has been proposed that several bone-resorbing factors (BRFs), including parathyroid hormone-related protein (PTHrP), transforming growth factors (TGFs), interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), are involved in this syndrome. Of these, PTHrP is the most important factor responsible for bone changes in humoral hypercalcemia of malignancy. It is well-known that PTHrP binds to PTH receptors and mimics the action of PTH. Therefore, PTHrP is believed to resorb bone via the same pathway as PTH.

There have been many reports concerning the mechanism by which PTH induces bone resorption. Rodan and Martin (1) raised the hypothesis that osteoblast is the main target cell for PTH, and the activity of osteoclasts is regulated indirectly via cell-to-cell communication. This hypothesis has been strongly supported by various reports. Silve et al. (2) reported that receptors for PTH were present in osteoblasts but not in osteoclasts. McSheehy and Chambers (3) showed that PTH stimulated resorption by isolated osteoclasts only when they were co-cultured with osteoblasts. Löwik et al. (4) showed evidence that PTH acted on osteoblasts, as well as many other cells (5), via two second messengers: cAMP and intracellular free calcium ([Ca2+]i). Reid et al. (6) and Yamaguchi et al. (7) also showed that PTH induced an acute transient increase in [Ca2+]i in osteoblastic cells. Furthermore, Civitelli et al. (8) demonstrated that PTH
produced a transient, dose-related increase in inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG), resulting in the activation of protein kinase (PK) C and an increase in \([\text{Ca}^{2+}]_i\). With regard to the notion that these second messengers are implicated in bone resorption, several observations have been reported: 1) both cholera-toxin and forskolin, potent stimulators of cAMP in bone tissue, stimulated \(4^{5}\text{Ca}\) release from neonatal mouse calvarial bones (9); 2) the bone resorptive effects of PTH (10) and 1\(_\alpha\)(OH) vitamin D₃ (11) can be inhibited by the 'calcium channel blocker' verapamil; and 3) the calcium ionophore A23187 stimulates bone resorption (12).

We previously reported (13) that 1) plasma Ca and Pi levels are elevated in ascites sarcoma 180 (S180A)-bearing mice, whereas hypophosphatemia was observed in PTHrP-producing tumors; 2) The serum-free conditioned media of S180A cell cultures (S180A-CM) exhibited dose-dependent bone-resorbing activity (BRA); and 3) S180A-CM failed to stimulate cAMP production in either UMR 106-01 cells or neonatal mouse calvaria at concentrations that stimulated bone resorption, suggesting that S180A-CM does not contain PTHrP. We also reported that S180A cells did not express mRNA for PTHrP based on Northern blot analysis (14).

The purpose of our study is to investigate the mechanism by which the BRFs, which lack PTH-like activity, stimulate bone resorption, where the PTH-like activity is defined as the ability to stimulate cAMP production in osteoblastic cells (15). S180A-CM contains TGF\(_\beta\), IL-1\(\alpha\), IL-6 and other unidentified factors, as reported previously (14). The first two of these factors are known to have no effect on cAMP production and are thought to act as local stimulators of bone resorption, whereas PTH and PTHrP increase renal tubular Ca reabsorption in addition to their local resorptive effects on bone. In the present study, we examined the effects of S180A-CM on intracellular second messengers, i.e., \([\text{Ca}^{2+}]_i\), IP₃, DAG, phosphatidylcholine (PC) and PKC, in comparison with PTH, PTHrP and TGF\(_\beta\). We also examined the effects of inhibitors of PKA and/or PKC on S180A-CM-induced bone resorption.

MATERIALS AND METHODS

Cells and animals

UMR 106-01 cells, a clonal rat osteosarcoma cell line, were generously provided by Dr. Nicola C. Partridge (Pediatric Research Institute, St. Louis University, MO, USA). Cells were maintained as monolayer cultures in Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui Pharmaceutical Co., Tokyo) and 10% fetal bovine serum (FBS; Whittaker Bioproducts, Inc., Walkersville, MD, USA). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air, and the cells were passaged at confluence using 0.05% trypsin-5 mM EDTA. The S180A-bearing mice were generously provided by Dr. Akio Hoshi (National Cancer Center Research Institute, Tokyo). This tumor was transplanted weekly in ddY mice by i.p. injections of ascites containing 4–5 × 10⁶ cells.

Sources of chemicals

Recombinant human transforming growth factor\(_\beta\) (TGF\(_\beta\)) was purchased from Gibco (Grand Island, NY, USA); Synthetic parathyroid hormone (PTH; human, 1–34) and parathyroid hormone-related protein (PTHrP; human, 1–34 amide) were purchased from Peptide Institute, Inc. (Osaka); \[^{3}H\]thymidine (\[^{3}H\]thymidine, specific activity: 15.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA); \[^{3}H\]glycerol (\[^{2}H\]glycerol, specific activity: 15.0 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA); Fura-2 acetoxymethyl ester (Fura-2/AM) was purchased from Dojin Laboratories, Kumamoto; sphingosine (2-amino-4-octadecene-1,3-diol) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA); and H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) was purchased from Seikagaku Co. (Tokyo).

Preparation of S180A-conditioned medium

Cells obtained from the ascites of S180A-bearing mice, which were negative for non-specific esterase-staining (\(\alpha\)-naphthyl esterase kit, Sigma Chemical Co.), were suspended in serum-free DMEM (10⁶ cells/ml) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 48 hr. Conditioned media were dialyzed against 1% acetic acid and concentrated by ultrafiltration with an Amicon YM 10 membrane (molecular weight cutoff at 10 kDa) in a stirred cell (Amicon, Inc., Beverly, MA, USA). The supernatant was lyophilized and extracted with 1 M chloride) was purchased from Seikagaku Co. (Tokyo).

\[^{3}H\]Thymidine incorporation and alkaline phosphatase (ALPase) activity

UMR 106-01 cells were grown to confluence in DMEM–10% FBS, and the medium was then replaced with serum-free DMEM. Twenty-four hours later, portions of the control or test preparations were added, and then the cells were cultured for another 24 hr. During the last 2 hr of the experiment, the cells were pulsed with 0.5 \(\mu\)Ci/well of \[^{3}H\]thymidine, and the radioactivity in the acid-insoluble fraction was counted in a liquid scintilla-
tion spectrometer. ALPase activity was determined spectrophotometrically by measuring the amount of p-nitrophenol released from p-nitrophenylphosphate at 37°C after 30 min (16).

**Measurement of \([Ca^{2+}]_i\)**

UMR 106-01 cells were grown to confluence in DMEM – 10% FBS and then maintained in serum-free medium for 24 hr prior to use. Cells were removed from flasks by a 20-min exposure to 5 mM EDTA in PBS at 37°C and loaded with 3 μM Fura-2/AM (17) in Hepes-buffered DMEM at 37°C for 30 min. These cells were washed 3 times with Hepes-buffered salt solution (HBSS; 148 mM NaCl, 5.8 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose and 10 mM Hepes, pH 7.35), suspended in HBSS, and then transferred to a cuvette (1.2–1.5 × 10⁶ cells/cuvette) fitted to an intracellular Ca²⁺ analyzer (Model CAF-100; Japan Spectroscopic Co., Tokyo). The cells were stirred continuously, reagents were added directly to the cuvette, and the fluorescence intensity was recorded at an excitation wavelength of 340 and 380 nm and an emission wavelength of 500 nm at 37°C. Values of \([Ca^{2+}]_i\) were calculated from the observed fluorescence intensity with excitation at 340 nm (F) according to the formula: \([Ca^{2+}]_i = K_d \cdot (F - F_{min})/(F_{max} - F)\). \(K_d\), the dissociation constant of the Fura-2 • Ca²⁺ complex, has been shown to be 224 nM in the cytosolic environment (18). Finax was obtained by lysing the cells with Triton X-100 (final concentration of 0.1%), and Fₘᵢₙ was obtained by further adding EGTA (final concentration, 1 mM). Due to the short duration of the experiments, no corrections were made for leakage of Fura-2 to the extracellular buffer.

**Analysis of IP₃**

Confluent cultures of UMR 106-01 cells were serum-starved for 24 hr. Cells were preincubated with serum-free DMEM containing 10 mM LiCl. After 10 min, stimulators were added and incubated for 15 sec, and then the media were removed by aspiration. The cells were washed with ice-cold PBS and with 5% trichloroacetic acid with occasional sonication on ice. The extracts were centrifuged, and the supernatants were washed 4 times with diethyl ether saturated with distilled water to remove trichloroacetic acid. Diethyl ether was then evaporated by exposure to N₂ gas. The supernatants were brought to pH 7 with 10% NaHCO₃, and aliquots were used for IP₃ analysis using an IP₃ assay kit (Amersham, Arlington Heights, IL, USA).

**Analysis of DAG**

UMR 106-01 cells were grown to confluence in 35-mm dishes in DMEM– 10% FBS and then labeled with [³H]-glycerol (1 μCi/dish) in serum-free DMEM for 48 hr. Culture media were removed, and then the cells were washed 4 times with HBSS and left in HBSS for more than 10 min at 37°C. Cultures were incubated with stimulators for the indicated times at 37°C and the reactions were terminated by adding 3.75 volumes of ice-cold chloroform/methanol (1 : 2, V/V). The lipids were then extracted by the method of Bligh and Dyer (19) and separated on a layer of Silica Gel 60 (E. Merck, Darmstadt, Germany) developed with chloroform/methanol/acetic acid (65 : 15 : 5, V/V/V). The lipids were identified by staining with iodine vapor and scraped from the plates. \(R_f\) values of the known standards PI, PA and DAG were 0.167, 0.65 and 0.98, respectively.

**Analysis of PC**

UMR 106-01 cells grown in 35-mm dishes were labeled with [³H]choline (1 μCi/dish) in DMEM–10% FBS for 72 hr, and the media were then replaced with fresh DMEM–10% FBS. After 48 hr, culture media were removed, and then the cells were washed 4 times with HBSS and left in fresh HBSS for 10 min at 37°C. Cultures were incubated with stimulators, and the reactions were terminated by aspirating HBSS and adding methanol/10 mM glycine (5 : 2, V/V). After 15 min, cells were scraped from the dishes, and lipids were extracted and separated on a layer of Whatman LK6D developed with chloroform/methanol/acetic acid/water (100 : 50.7 : 16 : 12, V/V/V/V). The lipids were identified by staining with iodine vapor and scraped from the plates. \(R_f\) values of the known standards lysophosphatidylcholine (LysoPC), sphingomyelin (Sph), PC, PA and 1-stearoyl-2-arachidonoyl-sn-glycerol (DAG) were 0.381, 0.483, 0.653, 0.864 and 0.98, respectively.

**Measurement of PKC activity**

Confluent cultures of UMR 106-01 were serum-starved for 24 hr and incubated with stimulators for the indicated times at 37°C. Then the cells were homogenized in Tris buffer (50 mM Tris-HCl, 0.25 M sucrose, 5 mM EDTA, 10 mM EGTA, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine and 0.3% β-mercaptoethanol) on ice and separated into the membrane fraction and cytosol fraction by centrifuging at 105,000 × g for 60 min. PKC activity in the obtained fractions was determined using a Protein Kinase C Enzyme Assay System (Amersham).

**Bone resorption**

One-day-old mice (CD-1; Nippon Charles River Co., Atsugi) were injected subcutaneously with 1.5 μCi of ⁴⁵CaCl₂ (New England Nuclear). Four days later, their
Calvaria were excised and divided into two halves by dissecting along the midsagittal suture. The bones were put on a nylon mesh in 24-well culture plates and precultured for 24 hr in BGJb medium (Gibco, NY, USA) containing 0.2% bovine serum albumin (BSA, Seikagaku Co.) and antibiotics. After preculturing, the medium was replaced with fresh medium (BGJb - 0.2% BSA) containing either the control or test preparations and cultured for an additional 96 hr. Bone resorption was assessed as the percentage of $^{45}$Ca released into the medium over 96 hr.

**Statistical analyses**

Data are expressed as the mean±S.D. The statistical significance of difference between the control and experimental groups was determined by Student’s $t$-test.

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**Fig. 1.** Effects of bone-resorbing factors on DNA synthesis (open column) and ALPase activity (filled column) in UMR 106-01 cells. Confluent UMR 106-01 cells in 24-well plates were serum-starved for 24 hr and then treated with PTH (2 x $10^{-7}$ M), S180A-CM or TGFβ (40 ng/ml) for another 24 hr. Data represent the mean±S.D. (n=6). **P<0.01, ##P<0.01: significantly different from the individual control. TdR: thymine deoxyribonucleoside, PNP: p-nitrophenol.**

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**Fig. 2.** Effects of neomycin and EGTA on PTH-induced changes in [Ca$^{2+}$], in UMR 106-01 cells. Confluent UMR 106-01 cells were serum-starved for 24 hr and harvested using 5 mM EDTA. The cells were loaded with 3 pM Fura-2/AM and resuspended in Hepes-buffered salt solution (148 mM NaCl, 5.8 mM KCl, 2 mM CaCl$_2·$2H$_2$O, 1 mM MgCl$_2·$2H$_2$O, 5 mM glucose and 10 mM Hepes; pH 7.35). PTH (2 x $10^{-7}$ M) and EGTA (1 mM) were added directly to the cell suspensions at times indicated by the arrows. Neomycin (5 mM) was added prior to the start of the experiments in (b) and (c).
RESULTS

Effects of S180A-CM on UMR 106-01 cells

As shown in Fig. 1, incorporation of [3H]thymidine into quiescent UMR 106-01 cells was stimulated by S180A-CM (178.3% of the control) or TGFα (186.7% of the control) and inhibited by PTH (66.4% of the control). In contrast, the ALPase activity of UMR 106-01 cells was inhibited by S180A-CM (39.0% of the control) or TGFα (31.3% of the control), and it was stimulated by PTH (113.4% of the control).

Effects of bone-resorbing factors on the intracellular second messengers

To elucidate the involvement of intracellular second messengers in the signal transduction in UMR 106-01 cells, we have determined the changes in [Ca\(^{2+}\)]\(_i\), IP3, DAG, PC and PKC induced by BRFs. We used the BRFs, PTHrP, S180A-CM and TGFα, at concentrations that stimulated bone resorption to the same extent as PTH in all of the experiments. Figure 2 shows the typical pattern of the change in [Ca\(^{2+}\)]\(_i\) when PTH (2 \times 10^{-7} M) was added alone or in combination with neomycin and EGTA. Thus, PTH induced a transient rise in [Ca\(^{2+}\)]\(_i\), which returned to near-resting levels within 1 min (Fig. 2a). As shown in Fig. 2b, the PTH-induced rise in [Ca\(^{2+}\)]\(_i\) was partially reduced (47% of PTH alone) by pretreatment with neomycin, which blocks phosphoinositide metabolism (20). Furthermore, this increase was inhibited more effectively (17% of PTH alone) by subsequent addition of EGTA, which selectively chelates extracellular Ca\(^{2+}\) (Fig. 2c). These data revealed that PTH stimulates both the influx of extracellular Ca\(^{2+}\) and the release of Ca\(^{2+}\) from endoplasmic reticulum, which is associated with phosphoinositide metabolism, suggesting that some of the actions of PTH might be mediated by intracellular messengers other than cAMP. Figure 3 shows the effects of the BRFs, PTH, PTHrP, S180A-CM and TGFα on [Ca\(^{2+}\)]\(_i\) in UMR 106-01 cells. PTHrP (5 \times 10^{-7} M) caused an increase in [Ca\(^{2+}\)]\(_i\) to a level comparable to PTH (PTH and PTHrP raise [Ca\(^{2+}\)]\(_i\) to 323.3 ± 20.6 and 292.6 ± 13.2 nM, respectively). On the other hand, S180A-CM caused a very slight increase in [Ca\(^{2+}\)]\(_i\) transiently, although the response was much smaller than that induced by PTH or PTHrP. As shown in Fig. 4, PTH or PTHrP significantly increased IP3 formation at 15 sec after treatment, whereas S180A-CM had no effect.

Fig. 3. Effects of PTH, PTHrP, S180A-CM and TGFα on [Ca\(^{2+}\)]\(_i\) in UMR 106-01 cells. Conditions are the same as those in the legend of Fig. 2. PTH (2 \times 10^{-7} M), PTHrP (5 \times 10^{-7} M), S180A-CM and TGFα (40 ng/ml) were added directly to the cell suspensions at times indicated by the arrows.

Fig. 4. Effects of PTH, PTHrP and S180A-CM on IP3 production in UMR 106-01 cells. Confluent UMR 106-01 cells in 35-mm² dishes were serum-starved for 24 hr and preincubated with 10 mM LiCl for 10 min. These cultures were treated with stimulators for 15 sec and used for IP3 analysis using an IP3 assay kit. Data represent the mean ± S.D. (n=4). **P<0.01: significantly different from the control.
These results suggest that S180A-CM does not enhance phosphatidylinositol (PI) turnover.

Therefore, we determined the DAG level in UMR 106-01 cells. We found that both S180A-CM and TGFα increased DAG production within a few minutes, and a significant increase was observed at 5 min after treatment (140.0% and 157.4% of the control, respectively) (Fig. 5). These results, indicating that S180A-CM can increase DAG production in the absence of accelerated PI turnover, lead us to presume that S180A-CM enhanced the conversion of PC to DAG. As expected, both S180A-CM and TGFα significantly lowered the PC levels in UMR 106-01 cells at 2 min (84.7% and 86.0% of the control, respectively) and 5 min (76.2% and 74.0% of the control, respectively) after treatment, as shown in Fig. 6. In this experiment, after 72 hr of labeling with [3H]choline, the radioactivities incorporated into the choline-containing lipids, LysoPC, Sph and PC were...
0.67±0.09%, 18.3±2.1% and 62.6±4.2% of the total radioactivity taken up by cells prior to stimulator addition, respectively. In addition to the decrease in PC, the level of Sph was also decreased at 2 and 5 min after treatment with S 180A-CM (89.4% and 79.2% of the control, respectively). Furthermore, S180A-CM and TGFa caused a rapid and transient decrease in PKC activity in the cytosol (79.8±10% and 70.0% of the control, respectively, after 10 min; Fig. 7, lower panel) that was associated with a transient increase in PKC activity in the membrane fraction (159.5±10% and 169.7% of the control, respectively, after 10 min; Fig. 7, upper panel).

Table 1. Effects of sphingosine or H-7 on various type of bone-resorbing factor-induced bone resorption

|             | PTH | PTHrP | S180A-CM | TGFα |
|-------------|-----|-------|----------|------|
| None        | 2.40| 2.48  | 2.47     | 2.60 |
| Sphingosine | 1.95| 2.04  | 1.51     | 1.53 |
| H-7         | 1.06| 1.02  | 0.96     | 1.01 |

Values are the T/C ratios of the percentages of 45Ca release, as calculated from the results in Fig. 8.

**Effects of sphingosine or H-7 on BRA in S180A-CM**

Figure 8 shows the effects of BRFs on cumulative 45Ca release from mouse calvaria over 96 hr in culture. Bone-resorbing activities in S180A-CM, TGFα, PTH and PTHrP were partially inhibited by the addition of 20 μM sphingosine, a specific PKC inhibitor. These BRAs were completely inhibited by 50 μM H-7, which inhibits both PKA and PKC. Table 1 shows the treated-over-control ratios (T/C ratio) of the percentage of 45Ca release, as calculated from the results in Fig. 8. Sphingosine inhibited BRA in S180A-CM more effectively than it inhibited PTH- or PTHrP-stimulated resorption.

**DISCUSSION**

It is well-recognized that BRFs, such as PTH and 1α,25(OH)2 vitamin D3, bind to specific receptors in osteoblasts and that the activation of osteoblasts, via increased formation of second messengers, leads to a rapid stimulation of existing osteoclasts and to a delayed increase in the recruitment of new osteoclasts. In the present study, we investigated transmembrane signal transduction of BRFs derived from a non-PTHrP-producing tumor, S180A, using osteoblastic UMR 106-01 cells, on the assumption...
that S180A stimulates bone resorption through a primary action on osteoblasts. In our previous study, S180A-CM failed to stimulate cAMP production in either UMR 106-01 cells or neonatal mouse calvaria (13). However, S180A-CM stimulated cAMP production by calvaria over a longer culture period (> 6 hr), presumably due to the secondary effect of prostaglandin (PG) E2 synthesized by calvaria, because this effect of S180A-CM was completely blocked by indomethacin (14). Therefore, the signal transduction pathways for the primary effect of S180A-CM and the secondary effect due to PGE2 production should be considered separately. We had expected that S180A-CM would cause a transient increase in [Ca2+], in UMR 106-01 cells by itself, i.e., in the absence of cAMP production, because the effects of BRFs on osteoblasts were thought to be mediated by two signal transduction systems: cAMP and intracellular free calcium (4). Unexpectedly, the change in [Ca2+] induced by S180A-CM was very slight compared to that with PTH or PTHrP. In addition, the production of IP3, which mobilizes Ca2+, was not stimulated at all by S180A-CM, in contrast to PTH or PTHrP. On the other hand, S180A-CM stimulated the production of DAG, one of the intracellular second messengers, concomitant with a decrease in PC content. This decrease in PC content led us to expect that phospholipase (PL) A2 would liberate arachidonic acid from PC, as well as DAG production. Arachidonic acid itself is known to activate PKC and to stimulate Ca2+ release from intracellular stores (21, 22). If we also consider our finding that S180A-CM had little effect on [Ca2+], arachidonic acid might not play a role in signal transduction in the primary effect of S180A-CM on bone. It is assumed that the primary function of DAG is to activate PKC, although DAG has other functions independent of this kinase. Likewise, some authors have reported that DAG can activate PKC in the absence of an increase in [Ca2+], and have suggested that PC is the source of DAG (23–27). In contrast, Leach et al. showed that DAG produced from PI, but not PC hydrolysis, was associated with the activation of PKC, using α-thrombin stimulation of IIC9 fibroblasts (28). These discrepant results may be due to the differences in the cells and agonists used. In the present study, S180A-CM caused a rapid (< 10 min) decrease and increase in PKC activity in the cytosol and membrane fractions, respectively. Abou-Samra et al. (29) have shown that PTH causes translocation of PKC from the cytosol to the membrane in rat osteosarcoma cell line ROS 17/2.8. The translocation of enzyme activity to the membrane fraction is known to reflect activation of the enzyme (50, 31), and once bound to the membrane, it is believed to phosphorylate several proteins.

Fig. 9. Proposed mechanism of signal transduction of bone-resorbing factors in osteoblastic cells. Ac, adenylate cyclase; PIP, phosphatidylinositol 4-phosphate; PIP-PLC, P1-specific PLC; PC-PLC, PC-specific PLC; PA, phosphatidic acid; (+), stimulatory effect; *, S180A-CM stimulates PGE2 production.
different responses after 'PKC activation'. In the present study, we were unable to distinguish the PKC subspecies because 1) 1 mM Ca was added to the assay mixture and 2) the PKC subspecies-specificity of the synthetic substrate peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH) used in the assay is uncertain, although it has been shown to be specific for PKC but not for other kinases such as PKA, phosphorylase kinase hexokinase or myosin light chain kinase (35, 36). It is of great interest that, despite these differences in the effects on cell proliferation and differentiation and in the signal transduction pathways, both PTH and S180A-CM finally resorb bones. On the other hand, the effects of TGFα on the second messengers in osteoblastic cells resembled those of S180A-CM. Regarding the signaling by IL-1, discrepant observations have been reported by other authors: 1) IL-1 does not stimulate PI turnover or an increase in [Ca2+]i, (37) and 2) IL-1 produces a transient and acute elevation in [Ca2+]i, in rat osteoblast osteosarcoma cells (38). To investigate the mechanism of bone resorption induced by S180A-CM in more detail in comparison with known local factors such as TGFα and IL-1, we must use purified fractions of S180A-CM. In our preliminary experiments using the peak fractions of BRA coeluted with TGFα activity or IL-1 activity, the latter didn’t cause [Ca2+]i transients at all, whereas the former caused a slight rise in [Ca2+]i. It is very important to investigate the involvement of second messengers other than cAMP, [Ca2+]i, IP3 and DAG in S180A-CM-induced bone resorption.

Furthermore, the BRA of S180A-CM was substantially inhibited by sphingosine, a PKC inhibitor (39), and the degree of this inhibition was greater than that on PTH- or PTHrP-stimulated bone resorption. This latter finding is in agreement with that of Civitelli et al. (40), who reported that PTH signal transduction in the control of osteoblastic growth and bone resorption was mediated mainly by cAMP, although activation of the Ca2+/PKC message system was necessary for a full hormonal response. The BRA of S180A-CM was completely inhibited by H-7, an inhibitor of both PKA and PKC (41, 42). Our unpublished data, which show that S180A-CM stimulates the production of PGE2 even in the presence of H-7, indicates that H-7 suppresses the activation of PKA induced by PGE2, but not PGE2 production itself. Thus, the BRA of S180A-CM appears to be mediated by two signal transduction pathways: one in which PKC is activated by DAG resulting from PC hydrolysis by PLC and/or PLD, which is indomethacin-insensitive, and another in which PKA is activated subsequent to PGE2 production by calvaria (Fig. 9). This hypothesis is supported by the fact that BRA of S180A-CM is inhibited only partially by indomethacin alone (14) and completely inhibited by the simultaneous addition of indomethacin and sphingosine (K. Suzuki et al., unpublished data). Moreover, S180A-CM had mitogenic activity on both osteoblastic UMR 106-01 cells and osteoclasts in calvaria (13, 14), and the BRA of S180A-CM was substantially inhibited by 5 mM hydroxyurea (45Ca release %: control, 27.54±1.39; S180A-CM, 53.14±11.18; S180A-CM+hydroxyurea, 38.39±3.06), which suggests that a stimulatory effect on the proliferation of osteoblasts and/or osteoclasts participates in S180A-CM-induced bone resorption. This may explain why S180A-CM requires a longer period of time (> 72 hr) to resorb bones, whereas 45Ca release by PTH was evident within 24 hr under the same experimental conditions. These observations are consistent with the finding that the prolonged activation of PKC by sustained high levels of DAG derived from PC, compared with phosphatidylinositol 4,5-bis-phosphate (PIP2)-generated DAG may be important for a subset of PKC-mediated events, such as mitogenesis or cellular differentiation (27), at least in osteoblasts.

In conclusion, S180A-CM stimulated [3H]thymidine incorporation and inhibited ALPase activity in osteoblastic UMR 106-01 cells, contrary to PTH. S180A-CM stimulated DAG production, accompanied by PC breakdown, and PKC activity in the absence of an increase in IP3 production in UMR 106-01 cells. S180A-CM-induced bone resorption was inhibited partially by sphingosine, a specific PKC inhibitor, and completely inhibited by H-7, an inhibitor of both PKA and PKC. Along with PKC activation, cAMP-PKA activation subsequent to PGE2 production by bone is also responsible for BRA in S180A-CM.

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