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Effect of Protein Kinase C Modulators on the Osteoclast-like Cell Generation Induced by Osteotropic Hormones

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Protein kinase C (PKC) has been known to play an important role in the process of cell differentiation. The present study was undertaken to investigate the effect of modulators of PKC in the generation of osteoclast-like cells from mouse bone marrow cells. The marrow cells were collected from the femurs and tibiae of 4- to 6-week-old mice and cultured for 8 days. In order to examine the role of PKC in the generation of osteoclast-like cells induced by hormones, a PKC modulator, PMA or H7 was supplemented in the presence of PGE_2, 1,25-dihydroxyvitamin D_3, or PTH. After culture, the cells were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclast and the TRACP-positive multinucleated cells (MNCs) which have 3 or more nuclei were counted.

The present study showed that TRACP-positive MNCs were not formed in the presence of PMA and H7 alone. However, the generation of TRACP-positive MNCs induced by PGE_2 and 1,25-dihydroxyvitamin D_3 was stimulated by PMA at 10^{-9}-10^{-7} M. This augmentation of TRACP-positive MNC generation was inhibited by the presence of 10^{-5} M H7. The generation of TRACP-positive MNCs induced by PTH was suppressed by 10^{-9}-10^{-7} M PMA. This suppression of TRACP-positive MNC generation by 10^{-7} M PMA was completely reversed by 10^{-5} M H7. H7 at 10^{-5} M inhibited PGE_2-induced TRACP-positive MNC generation. These results suggest that PKC may play an important role in the generation of osteoclasts and may modulate the action of osteotropic hormones in the bone resorption.

Key words: *protein kinase C, PMA, H7, osteoclast-like cells, osteotropic hormones*

Introduction

Osteoclast, a major bone-resorbing cell, is hematopoietic in its origin and is formed by fusion of mononuclear precursors to become multinuclear giant cell (Ko and Bernard, 1981; Mundy and Roodman, 1987). There have been many studies on the recruitment, differentiation and activation of osteoclasts, but the exact mechanisms concerning these processes have not been elucidated.

The roles of osteotropic hormones and some growth factors in the differentiation and activation of osteoclasts have been extensively studied (Chambers, 1989; Nijweide *et al.*, 1986). Several lines of evidence indicate that cyclic adenosine 3',5'-monophosphate (cAMP) might be involved in the regulation of bone metabolism by osteotropic hormones.

Osteotropic hormones such as parathyroid hormone (PTH) and PGE_2 increase cAMP in osteoblasts and calcitonin which inhibits bone resorption increases cAMP production in osteoclasts (Wong, 1986; Wong and Kocour, 1983; Rifkin *et al.*, 1988).

In addition to cAMP, other components which originate from the other pathways of signal transduction induced by hormones and growth factors are involved in the regulation of bone resorption. Diacylglycerol (DAG) and inositol triphosphate (IP_3) generated by phospholipase C serve as messengers for the activation of protein kinase C (PKC) and the mobilization of intracellular Ca^{2+}, respectively. Recent studies have revealed that PTH, PGE_2 and calcitonin stimulate phosphoinositide pathway and that changes in the extracellular concentration of Ca^{2+} can affect the bone cell function, and calcium ionophore stimulates osteoclast generation in mouse bone marrow cell culture (Lorenzo and Raize, 1981; Stern *et al.*, 1982; DeBartolo *et al.*, 1982). Studies to observe the effects of PKC activators, such as phorbol 12-myristate 13-acetate (PMA) and PKC inhibitors on the bone resorption have shown different results depending on the concentration of PMA and experimental conditions including species difference, cell types and culture methods (Bos *et al.*, 1992; Abraham *et al.*, 1988;

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Lorenzo and Sousa, 1988; Murrills *et al.*, 1992; Teti *et al.*, 1992; Kukita *et al.*, 1992).

PKC was reported to be involved in the action of osteotropic hormones. PTH primarily affects osteoblasts and increases intracellular cAMP level, and PTH-stimulated osteoblasts may produce and release the substances that activate osteoclasts (Rodan and Martin, 1981; Chase and Aurbach, 1970). Intracellular Ca^{2+} and PKC are involved in the activation and desensitization of PTH-stimulated adenylate cyclase (Lowik *et al.*, 1985; Abou-Samra *et al.*, 1989; Freyaldenhoven *et al.*, 1992; Ikeda *et al.*, 1991; Fukayama *et al.*, 1992) and may mediate the action of PTH in osteoblasts. Moreover, PKC increases the number of receptors for 1,25-dihydroxyvitamin D_3 in the osteoblasts, and PKC inhibitor decreases bone resorption induced by 1,25-dihydroxyvitamin D_3 (van Leeuwen *et al.*, 1992a; 1992b).

PGE_2 causes local bone resorption and stimulates not only the production of cAMP but the mobilization of calcium and the metabolism of phosphoinositide (Dziak *et al.*, 1983; Farr *et al.*, 1984; Farndale *et al.*, 1988; Kozawa *et al.*, 1992). PKC, in turn, has the ability to down-regulate the production of cAMP by PGE_2 in human osteoblastic cell line (Hagel-Bradway *et al.*, 1991).

Calcitonin receptors on the osteoclasts are known to use cAMP as an intracellular second messenger. Recently, it has been shown that calcitonin activates both adenylate cyclase and PKC, and PMA increases the accumulation of cAMP induced by calcitonin (Nicholson *et al.*, 1987; Su *et al.*, 1992; Ransjo and Lerner, 1991).

All these results indicate that the activation of PKC affects the bone resorption and the function of osteoclasts, but its definitive function and relationship with cAMP pathway remains to be elucidated. To elucidate the role of protein kinase C in bone resorption, we examined the effects of PMA and H7, a PKC activator and an inhibitor, on the generation of osteoclast-like cells induced by osteotropic hormones.

Materials and Methods

Bone marrow cell cultures

Four- to six-week-old mice (ICR) were killed by cervical dislocation, and tibiae and femurs were aseptically isolated and dissected free of adhering tissues. The ends of bone were cut off with scissors and the marrow cavity was flushed with 1 ml α-minimum essential medium (α-MEM, GIBCO, Grand Island, NY) by slowly injecting at one end of the bone using a sterile 25G needle. The marrow cells were collected, washed twice with α-MEM, and cultured in 0.5 ml of α-MEM containing 10% fetal calf serum (GIBCO, Grand Island, NY) at 3.0×10^6 cell/ml in 24-well plates (Nunc, RosKilde, Denmark). Cultures were fed every 3 days by replacing 0.4 ml of old medium with fresh medium. All cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air. After 8 days, the cells adherent to the well surface were rinsed once with phosphate buffered saline (pH 7.4) and fixed with ethanol-acetone (50:50, v/v) for 1 min. Culture plates were dried at room temperature for 10 min, and the cells were stained for tartrate-resistant acid phosphatase (TRACP). The cells containing 3 or more nuclei were counted as multinucleated cells (MNCs).

Effect of PMA and H7 on osteoclast-like MNC generation

In experimental group, various concentrations of PMA (Sigma Chemical Co., St. Louis, MO), and/or H7 (1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine, Sigma) were added at the beginning of the culture and maintained throughout the culture period.

10^{-6} M PGE_2 (Sigma), 10^{-7} M 1,25-dihydroxyvitamin D_3 (BIOMOL, Plymouth Meeting, PA) and 100 ng/ml PTH (Sigma), all known to generate osteoclast-like MNCs in mouse bone marrow culture, were added at the beginning of the culture, alone or in combination with PMA or H7, and maintained.

Enzyme histochemistry for tartrate-resistant acid phosphatase

TRACP was used as a marker enzyme for osteoclasts. Staining for TRACP was performed according to the modified method of Burstone

Table 1. Effect of PMA, H7, PGE_2 1, 25-dihydroxyvitamin D_3 and PTH on the generation of TRACP-positive MNCs

Treatment	N	Number of TRACP (+) MBCs
No treatment	11	0
PMA 10^{-9} M	11	0.27 ± 0.29
PMA 10^{-8} M	11	0.63 ± 0.50
PMA 10^{-7} M	11	1.0 ± 0.45
H7 10^{-6} M	7	0
H7 10^{-5} M	7	0
PGE_2 10^{-6} M	4	190 ± 16.7*
1, 25-DHCC 10^{-7} M	4	113 ± 8.0*
PTH 100 ng/ml	4	85 ± 4.7*

Mouse bone marrow cells (1.5×10^6 cells/well) were cultured for 8 days with PMA, H7, PGE_2, 1, 25-dihydroxyvitamin D_3, or PTH. Cells were stained for TRACP and then TRACP-positive multinucleated cells were counted.
Values are mean ± S.E. (Student's *t*-test).
*p<0.01, compared to no treatment.
1, 25-DHCC denotes 1, 25-dihydroxycholecalciferol.

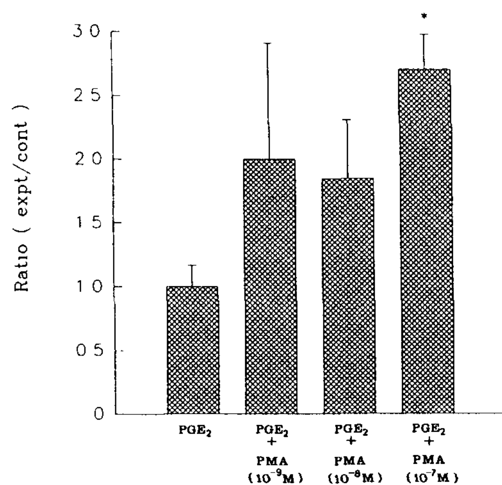

Fig. 1. Effect of PMA on the generation of TRACP-positive MNCs induced by PGE_2 (10^{-6} M). Mouse bone marrow cells (1.5×10^6 cells/well) were cultured for 8 days with osteotropic hormone-containing α-MEM with or without supplementation of PKC modulator(s). Values are mean ± S.E. of quadruplicate cultures and expressed as the ratio of number of TRACP-positive MNCs to corresponding mean of control (PGE_2 without PMA).
*p<0.01, compared to control

(Burstone, 1958). Fixed cells were incubated for 20 minutes at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate, and fast red violet LB salt (Sigma) as a stain for the reaction product in the presence of 20 mM sodium tartrate. TRACP-positive cells appeared as dark red cells.

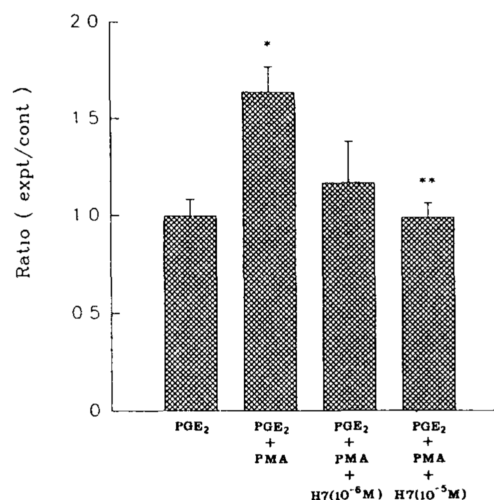

Fig. 2. Effect of H7 on the generation of TRACP-positive MNCs in the presence of PGE_2 (10^{-6} M) and PMA (10^{-7} M). Mouse bone marrow cells were cultured under the conditions as described in the legend to Fig. 1. Values are mean ± S.E. of quadruplicate cultures and expressed as the ratio of number of TRACP-positive MNCs to corresponding mean of control (PGE_2 without PMA and H7).
*p<0.05, compared to control
**p<0.05, compared to PGE_2+10^{-7} M PMA

Results

Osteoclast-like MNCs(TRACP-positive MNCs) were not formed in cultures with vehicle only (Table 1). However, well-known bone resorbing agents including PGE_2, 1, 25-dihydroxyvitamin D_3 and PTH increased the number of TRACP-positive MNCs (Table 1).

Initially, whether PMA is able to induce osteoclast-like cell generation in bone marrow cell was examined. As shown in Table 1, few TRACP-positive MNCs were formed in the presence of 10^{-9}-10^{-7} M PMA.

However, PMA potentiated the ability of PGE_2 and 1,25-dihydroxyvitamin D_3 to increase the generation of TRACP-positive MNCs. As shown in Fig. 1, the generation of TRACP-positive MNCs induced by PGE_2 (10^{-6} M) was significantly enhanced by 10^{-7} M PMA. At lower concentration of PMA (10^{-9}, 10^{-8} M), TRACP-positive MNC generation was augmented, but it was not statistically significant.

To examine further whether the augmentation of TRACP-positive MNC generation by 10^{-7} M PMA is

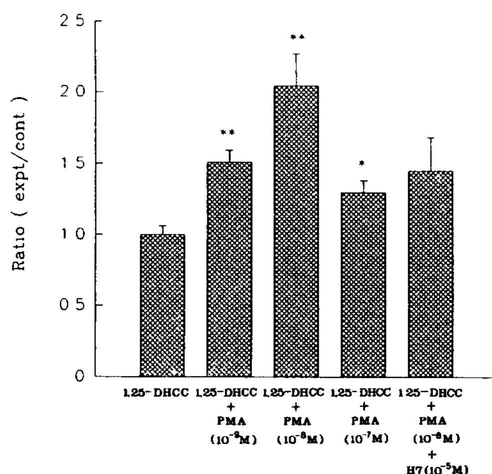

Fig. 3. Effect of PMA and H7 on the generation of TRACP-positive MNCs induced by 1, 25-dihydroxyvitamin D_3 (10^{-7} M). Mouse bone marrow cells were cultured under the conditions as described in the legend to Fig. 1. Values are mean±S.E. of quadruplicate cultures and expressed as the ratio of number of TRACP-positive MNCs to corresponding mean of control (1,25-dihydroxyvitamin D_3 alone).
*p<0.05, compared to control
**p<0.01, compared to control.
1,25-DHCC denotes 1, 25-dihydroxycholecalciferol.

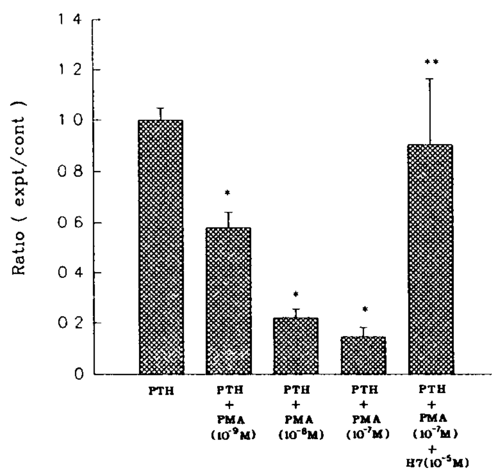

Fig. 4. Effect of PMA and H7 on the generation of TRACP-positive MNCs induced by PTH (100 ng/ml). Mouse bone marrow cells were cultured under the conditions as described in the legend to Fig. 1. Values are mean±S.E. of quadruplicate cultures and expressed as the ratio of number of TRACP-positive MNCs to correresponding mean of control (PTH alone).
*p<0.01, compared to control
**p<0.05, compared to PTH + PMA 10^{-7} M

related to the activation of PKC, the effect of H7, PKC inhibitor, was observed. As shown in Fig. 2, the augmentation of TRACP-positive MNC generation by 10^{-7} M PMA was inhibited in a dose-dependent manner by H7 and completely blocked by 10^{-5} M H7. These results indicated that the augmentation of TRACP-positive MNC generation by PMA was related to the activation of PKC.

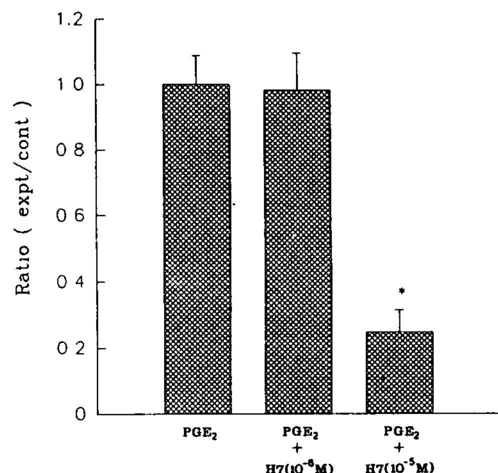

Fig. 5. Effect of H7 on the TRACP-positive MNCs generation induced by PGE_2 (10^{-6} M). Mouse bone marrow cells were cultured under the conditions as described in the legend to Fig. 1. Values are mean±S.E. of quadruplicate cultures and expressed as the ratio of number of TRACP-positive MNCs to corresponding mean of control (PGE_2 without H7).
*p<0.01, compared to control.

The generation of TRACP-positive MNCs induced by 1,25-dihydroxyvitamin D_3 (10^{-7} M) was stimulated by PMA at the concentration of 10^{-9}-10^{-7} M. The enhancement of the generation of TRACP-positive MNCs by PMA reached maximum at the concentration of 10^{-8} M (Fig. 3). The effect of PMA was inhibited by 10^{-5} M H7, but not completely (Fig. 3). These results indicated that PMA by itself was unable to induce osteoclast-like cell generation, but enhanced the effects of PGE_2 and 1,25-dihydroxyvitamin D_3.

On the contrary, the generation of TRACP-positive MNCs induced by PTH (100 ng/ml) was suppressed by 10^{-9}-10^{-7} M PMA in a dose-dependent manner (Fig. 4). The suppression of TRACP-positive MNC generation by 10^{-7} M PMA was completely reversed by 10^{-5} M H7 (Fig. 4).

To gain further insight into the role of PKC in osteoclast generation, the effect of H7 on the TRACP-positive MNC generation was examined. H7 at 10^{-5} M inhibited PGE_2-induced TRACP-positive MNC generation (Fig. 5). In the cultures treated with H7 (10^{-6}, 10^{-5} M) alone, TRACP-positive MNCs

were not formed (Table 1).

Discussion

PKC has been known to play a crucial role in the stimulation of several cellular functions by transducing extracellular signals into the cells in the cellular events (Nishizuka, 1984, 1986). PKC is a calcium- and phospholipid-dependent enzyme that is activated by diacylglycerol which is produced by receptor-mediated hydrolysis of inositol phospholipid (Nishizuka, 1984). Phorbol esters mimic the effects of endogeneous diacylglycerol in activating PKC, thus are widely used to study the involvement of PKC in the regulation of cellular function (Castagna *et al.*, 1982).

Osteotropic hormones such as PTH, PGE_2, and calcitonin activate the second messenger pathways in bone cells. They increase cAMP production and cause a rise in intracellular Ca^{2+}. The role of cAMP and Ca^{2+} signalling system in the bone resorption has been reported previously, but the specific role of PKC signalling pathway in hormone-induced bone resorption is still unclear.

The present study demonstrated that PMA had no effect on the osteoclast-like MNC generation in the mouse bone marrow cell cultures. This result is consistent with the previous reports that PMA alone had no effect on the osteoclast formation in the rat bone marrow cells (Kukita *et al.*, 1992) and mouse calvarial cells (Amano *et al.*, 1994).

However, it contradicted the reports that PMA increased the bone resorption in organ cultures (Abraham *et al.*, 1988; Lorenzo and Sousa, 1988; Ransjo and Lerner, 1990). This discrepancy may be due to the differences in the properties of cells and the assay systems used. The present study showed that H7 had no effect on osteoclast-like MNC generation. The result is also consistent with the reports that PKC inhibitors had no effect on basal bone resorption in fetal mouse calvaria culture (Bos *et al.*, 1992).

Taken together, these results indicated that PKC modulators were unable to induce osteoclast-like cell generation by themselves. In contrast, these agents were able to modulate the effects of osteotropic hormones on the generation of osteoclast-like cells. The generation of osteoclast-like MNCs induced by PGE_2 was enhanced by PMA while inhibited by H7. These findings are in accordance with the findings of Bos *et al.* (1992) who found that PKC inhibitor suppressed bone resorption induced by PGE_2. And potentiating effect of PMA was blocked by H7. These results suggest that stimulatory action of PGE_2 on osteoclast-like MNC generation is highly correlated with the activation of PKC in bone marrow cells.

1,25-dihydroxyvitamin D_3 has been demonstrated to increase the intracellular Ca^{2+} level and the generation of IP_3, and it also affects the PKC intracellular signalling system in various cell types (Lieberherr, 1987; Lieberherr *et al.*, 1989; Civitelli *et al.*, 1990; Martell *et al.*, 1987; Obeid *et al.*, 1990). The present studies demonstrated that the generation of osteoclast-like MNCs induced by 1,25-dihydroxyvitamin D_3 was increased by PMA. This result was different from previous findings that PMA stimulated the 1,25-dihydroxyvitamin D_3-induced osteoclast-like MNC generation at 10^{-9} M, but inhibited at 10^{-7} M in the rat bone marrow cultures (Kukita *et al.*, 1992). Kukita *et al.* (1992) suggested that inhibitory effect of PMA on osteoclast-like MNC generation was due to PKC down-regulation by PMA. Such down-regulation can be dependent on the incubation time and its sensitivity may be different according to the cell type. In this study, PMA at 10^{-8} M was more potent in stimulation of osteoclast-like MNC generation than 10^{-7} M, thus it can be suggested that mouse bone marrow cell is less sensitive to PKC down-regulation by PMA than rat bone marrow cells. The stimulatory effect of PMA on 1,25-dihydroxyvitamin D_3-induced osteoclast-like MNC generation was inhibited by H7. This suggests that the PKC may be functionally involved in the generation of osteoclast-like cells through potentiating the activity of 1,25-dihydroxyvitamin D_3.

In the case of PTH-induced osteoclast-like MNC generation, PMA inhibited the osteoclast-like MNC generation in a dose-dependent manner. This inhibitory effect of PMA on osteoclast-like MNC generation was blocked completely by H7. This results contradicted the reports that PKC inhibitor decreased PTH-induced bone resorption (Bos *et al.*,

1992), but is in accordance with the report of Ransjo and Lerner (1990). This discrepancy of PMA effect on PGE_2-, 1,25-dihydroxyvitamin D_3-, and PTH-induced osteoclast generation may be due to difference of cell type affected by each hormone in the bone marrow cell culture. Regarding this, PTH receptor is not found in osteoclasts and its effect of osteoclast generation is mediated by osteoblasts (Takahashi *et al.*, 1988). Also it is suggested that 1,25-dihydroxyvitamin D_3 receptor is present not on mature osteoclasts but on its progenitor cells (Chambers, 1988). At present, it remains to be elucidate whether the effect of PTH on osteoclast-like cell generation is mediated directly via PKC of osteclasts or mediated indirectly via PKC of the other cells of bone marrow cell culture such as stromal cells which have osteoblastic characteristics.

Conclusion

To investigate the role of PKC in the generation of osteoclast-like cells from their precursor cells, the effect of PMA and H7 on the generation of osteoclast-like MNCs induced by osteotropic hormones was examined. The femurs and tibiae were isolated from the 4- to 6-week-old mice and the marrow cavity was flushed with 1 ml of α-minimun essential medium by slow injection. The collected marrow cells were adjusted to 1.5×10^6 cells/well in 24-well plates and cultred for 8 days. In order to examine the role of PKC on osteoclast-like MNC generation induced by hormones, a PKC modulator, PMA or H7 was supplemented in the presence of PGE_2, 1,25-dihydroxyvitamin D_3, or PTH. After culture, cultured cells were stained for TRACP, a marker enzyme of osteoclasts, according to the modified method of Burstone (1958). The TRACP-positive MNCs, which have 3 or more nuclei, were counted.

The observed results were as follows.

1. TRACP-positive MNCs were not formed in the presence of PMA or H7.

2. The generation of TRACP-positive MNCs induced by PGE_2 (10^{-6} M) was significantly enhanced by PMA at 10^{-7} M. And the augmentation of TRACP-positive MNC generation was completely inhibited by 10^{-5} M H7.

3. The generation of TRACP-positive MNCs induced by 1,25-dihydroxyvitamin D_3 (10^{-7} M) was increased by PMA at the concentration of 10^{-9}-10^{-7} M, with the greatest effect at 10^{-8} M. And the effect of PMA was inhibited by 10^{-5} M H7 but not completely.

4. The generation of TRACP-positive MNCs induced by PTH (100 ng/ml) was suppressed by 10^{-9}-10^{-7} M PMA. And the suppression of TRACP-positive MNC generation by 10^{-7} M PMA was completely blocked by 10^{-5} M H7.

5. H7 at 10^{-5} M inhibited PGE_2-induced TRACP-positive MNC generation.

These results suggest that PKC may play an important role in the generation of osteoclasts and may modulate the action of osteotropic hormones in the bone resorption.

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