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Intracellular Signaling Mechanism of Bradykinin in Osteoblast-Like Cells: Comparison with Prostaglandin E_2

HARUHIKO TOKUDA, JUN KOTOYORI,
YUTAKA OISO AND OSAMU KOZAWA*

*First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, and *Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Aichi 480–03, Japan*

Abstract. Bradykinin is recognized to be involved in the process of bone resorption in chronic inflammatory diseases. We previously reported that prostaglandin E_2 (PGE_2), known as a potent bone resorbing agent, induces phosphoinositide hydrolysis, cAMP production and Ca^{2+} influx in osteoblast-like MC3T3-E1 cells, and these dose-dependencies are different to one another. To clarify the signaling mechanism of bradykinin, we compared the intracellular signaling system of bradykinin with that of PGE_2 in these cells. Bradykinin stimulated Ca^{2+} influx dose-dependently in the range between 0.1 nM and 0.1 μM even in the presence of nifedipine, a Ca^{2+} antagonist that inhibits the voltage-dependent L-type Ca^{2+} channel. The maximum effect of bradykinin (0.1 μM) on Ca^{2+} influx was almost as great as that of PGE_2 (0.5 μM). Bradykinin had little effect on cAMP accumulation, while PGE_2 significantly stimulated it. Bradykinin stimulated the formation of inositol phosphates much less strongly than PGE_2. Bradykinin stimulated inositol 1, 4, 5-trisphosphate [Ins(1, 4, 5)P_3] formation dose-dependently between 0.1 nM and 0.1 μM, and the dose-dependent curves of bradykinin-induced Ca^{2+} influx and Ins(1, 4, 5)P_3 were similar. However, the maximum effect of PGE_2 (10 μM) on Ins (1, 4, 5) P_3 formation was about 2-fold higher than that of bradykinin (0.1 μM). These results suggest that bradykinin induces Ca^{2+} influx independent of the voltage-dependent L-type Ca^{2+} channel and phosphoinositide hydrolysis in a similar dose-dependent manner in osteoblast-like cells.

Key words: Bradykinin, Prostaglandin E_2, Phosphoinositide, Calcium, Osteoblast.
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BRADYKININ is well-known as a mediator in inflammatory reactions and is formed by the cleavage of high molecular weight kininogen by kallikrein in the areas of inflammation [1]. In chronic inflammatory diseases such as rheumatoid arthritis and periodontitis, characterized by the destruction of mineralized tissue, it has been recognized that bradykinin is involved in the process of bone resorption [2, 3]. In *in vitro* studies, it has recently been reported that bradykinin stimulates the production of prostaglandins in osteoblasts [4, 5], including osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [6, 7]. As for the signal transduction mechanism of bradykinin in osteoblasts, it has been reported that bradykinin induces phosphoinositide (PI) hydrolysis and causes an increase in intracellular Ca^{2+} [5, 8]. It is generally accepted that in response to a variety of agonists, phosphoinositides are hydrolyzed by phospholipase C, resulting in the formation of diacylglycerol and inositol phosphates (IPs). Among these products, diacylglycerol and inositol 1, 4, 5-trisphosphate [Ins (1, 4, 5)P_3] serve as messengers for the activation of protein kinase C and the mobilization of Ca^{2+} from the intracellular Ca^{2+} store, respectively [9, 10].

Prostaglandin E_2 (PGE_2) is known as a potent

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Correspondence to: Dr. Osamu KOZAWA, Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Aichi 480–03, Japan

bone resorbing agent [11]. There is increasing evidence that the effects of PGE_2 are mediated through both cAMP production and PI hydrolysis in osteoblasts [12–15]. As far as PI hydrolysis is concerned, we found that it is mediated by a pertussis toxin-sensitive GTP-binding protein in MC3T3-E1 cells [16]. Moreover, in recent studies [17, 18], we have demonstrated that PGE_2 induces PI hydrolysis, cAMP production and Ca^{2+} influx in these cells, and these dose-dependencies are different to one another. We have also shown that both PGE_2-induced cAMP production and Ca^{2+} influx are autoregulated due to the activation of protein kinase C, resulting from PI hydrolysis in these cells [17, 18]. However, the details of intracellular signaling of various bone resorbing agents has not yet been clarified.

In the present study, we investigated the effects of bradykinin on Ca^{2+} influx, cAMP accumulation and PI hydrolysis compared with those of PGE_2 in osteoblast-like MC3T3-E1 cells. Our results suggest that bradykinin induces Ca^{2+} influx independent of the voltage-dependent L-type Ca^{2+} channel and PI hydrolysis in a similar dose-dependent manner in osteoblast-like cells.

Materials and Methods

Materials

myo-[2-^{3}H]Inositol (81.5 Ci/mmol), $^{45}CaCl_2$ (10–40 mCi/mg) and D-*myo*-1, 4, 5 inositol trisphosphate [^{3}H]assay system were purchased from Amersham Japan (Tokyo, Japan). Bradykinin was from Peptide Institute Inc. (Minoh, Japan), and PGE_2 from Sigma Chemical Co. (St. Louis, MO, USA). Nifedipine was provided by Bayer Pharmaceutical Co. (Osaka, Japan). The cAMP radioimmunoassay kit was provided by Yamasa Shoyu Co.(Chiba, Japan). Other materials and chemicals were obtained from commercial sources. PGE_2 was dissolved in ethanol, and nifedipine in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide in the culture medium was 0.1%, and this did not affect either Ca^{2+} influx or cAMP, IPs and Ins (1, 4, 5)P_3 formation.

Cell culture

MC3T3-E1 cells were generously provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4) were seeded into 35-mm diameter dishes in 2 m*l* of α-MEM containing 10% FBS. After 5 days, the medium was exchanged for 2 m*l* of α-MEM containing 0.3% FBS. For the experiment on the formation of IPs, the medium was exchanged for 2 m*l* of inositol-free α-MEM containing 0.3% FBS. The cells were used for experiments 48 h thereafter.

Assay for $^{45}Ca^{2+}$ influx

The cultured cells were washed twice with 1 m*l* of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$ and 5.5 mM glucose] and preincubated in 1 m*l* of the assay buffer containing 0.01% bovine serum albumin (BSA) at 37°C for 20 min. For the last 10 min, the cells were pretreated with 0.1 μM nifedipine. The cells were then stimulated by bradykinin or PGE_2 containing 5 μCi of $^{45}Ca^{2+}$ for various periods. After washing four times with 1 m*l* of the cold assay buffer containing [ethylenebis-(oxyethylenenitrilo)] tetraacetic acid, the reaction was immediately terminated by adding 1 m*l* of 0.1% sodium dodecyl sulfate. The radioactivity of the lysate was determined.

Assay for cAMP

The cultured cells were pretreated with 0.5 mM 3-isobutyl-1-methylxanthine at 37°C for 10 min in 1 m*l* of the assay buffer containing 0.01% BSA. The cells were then stimulated by various doses of bradykinin or 10 μM PGE_2. After washing with 1 m*l* of the assay buffer, the reaction was immediately terminated by adding 1 m*l* of 90% n-propanol, and then the intracellular cAMP was extracted [19]. cAMP in the extracts was measured by a radioimmunoassay kit.

Assay for the formation of IPs

The cultured cells were labeled with myo-[2-^{3}H]inositol (2 μCi/dish) for 48 h. The labeled cells were pretreated with 10 mM LiCl at 37°C for 10 min in 1 m*l* of the assay buffer containing 0.01%

BSA. The cells were then stimulated by various doses of bradykinin or 10 μM PGE_2 for 10 min. The reaction was terminated by 15% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to a column of Dowex AG1-X8 formate form. The radioactive IPs were then eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate [20, 21].

Assay for the formation of Ins(1, 4, 5)P_3

The experiments were performed as described under "Assay for the formation of IPs" except that unlabeled cells were used. Ins(1, 4, 5)P_3 in the supernatant was measured by an Ins(1, 4, 5)P_3 assay system.

Statistics

Data are presented as the mean±SD of triplicate determinations. Data were analyzed by analysis of variance followed by *t* test and a $P<0.05$ was considered significant.

Results

Time-dependent effect of bradykinin on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells

Bradykinin (0.1 μM) significantly stimulated $^{45}Ca^{2+}$ influx even in the presence of 0.1 μM nifedipine, a Ca^{2+} antagonist that inhibits the voltage-dependent L-type Ca^{2+} channel [22], in MC3T3-E1 cells (Fig. 1). The $^{45}Ca^{2+}$ influx stimulated by bradykinin gradually increased up to 10 min.

Dose-dependent effect of bradykinin on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells

Bradykinin stimulated $^{45}Ca^{2+}$ influx in a dose-dependent manner in the range between 0.1 nM and 0.1 μM in these cells (Fig. 2). The maximum effect of bradykinin was observed at 0.1 μM. EC_{50} on the $^{45}Ca^{2+}$ influx stimulated by bradykinin was about 8 nM.

Effect of bradykinin on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells: comparison with that of PGE_2

In a recent study [18], we have shown that PGE_2 stimulates Ca^{2+} influx in a dose-dependent manner, attaining the maximum at 0.5 μM and a dose of PGE_2 above 0.5 μM causes less than maximal stimulation in MC3T3-E1 cells. EC_{50} on the $^{45}Ca^{2+}$ influx by PGE_2 is 0.1 μM. In this experiment, we compared the effect of bradykinin with that of PGE_2 on $^{45}Ca^{2+}$ influx in these cells. Bradykinin (0.1 μM) was almost as potent as 0.5 μM PGE_2 in the effect on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells (Fig. 3).

Effect of bradykinin on cAMP accumulation in MC3T3-E1 cells: comparison with that of PGE_2

We previously reported that PGE_2 stimulates

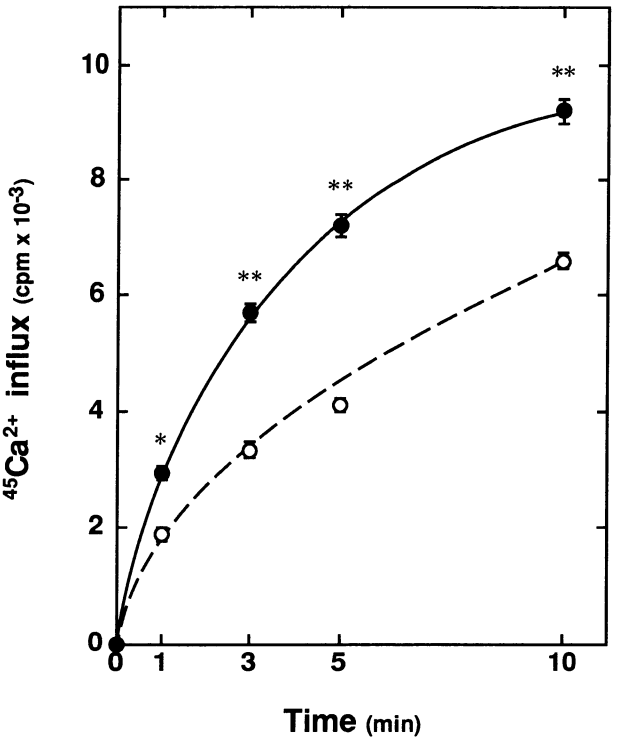

Fig. 1. Time-dependent effect of bradykininon $^{45}Ca^{2+}$ influx in MC3T3-E1 cells. The cultured cells were stimulated by 0.1 μM bradykinin (●) or vehicle (○) with 5 μCi of $^{45}Ca^{2+}$ for the indicated periods, and then the $^{45}Ca^{2+}$ influx was determined. Each value represents the mean±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P<0.05$; **, $P<0.01$ compared with control.

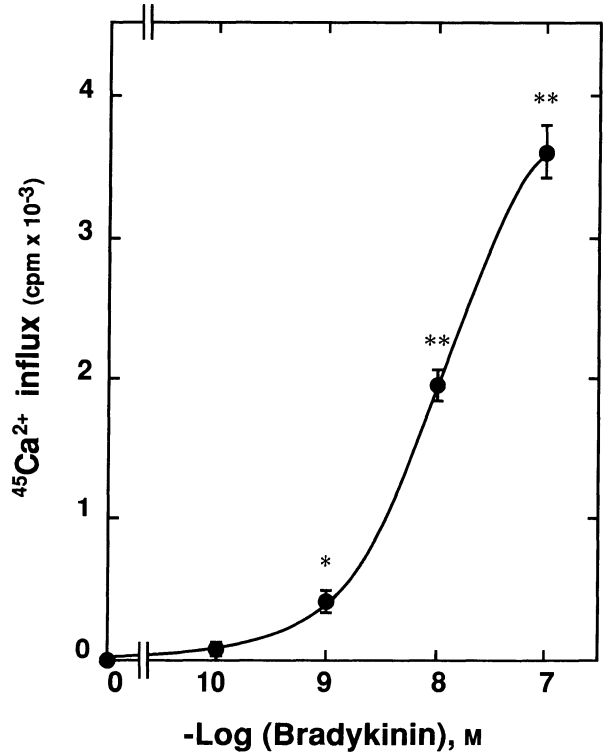

Fig. 2. Dose-dependent effect of bradykinin on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells. The cultured cells were stimulated by various doses of bradykinin with 5 μCi of $^{45}Ca^{2+}$ for 5 min, and then the $^{45}Ca^{2+}$ influx was determined. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P<0.05$; **, $P<0.01$ comapred with control.

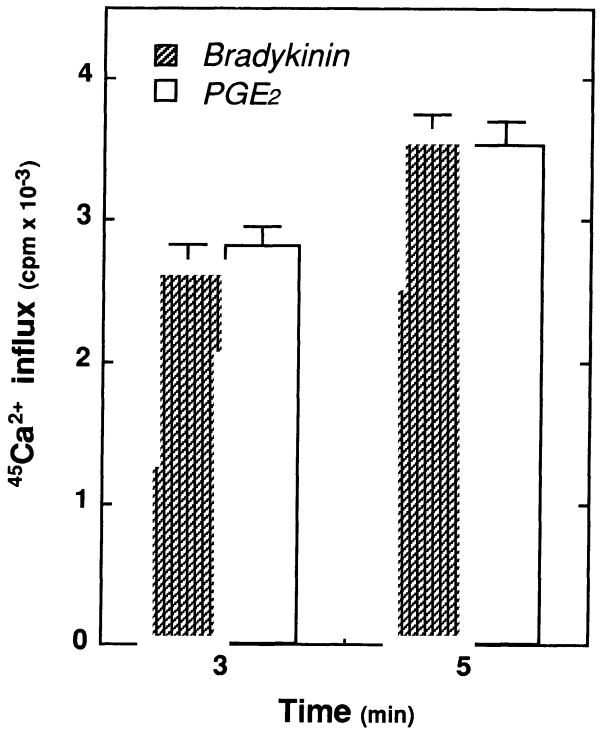

Fig. 3. Effects of bradykinin and PGE_2 on $^{45}Ca^{2+}$ influx in MC3T3-E1 cells. The cultured cells were stimulated by 0.1 μM bradykinin or 0.5 μM PGE_2 with 5 μCi of $^{45}Ca^{2+}$ for the indicated periods, and then the $^{45}Ca^{2+}$ influx was determined. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

cAMP accumulation dose dependently in the range between 1 nM and 10 μM in MC3T3-E1 cells and that cAMP accumulation reaches a peak at 5 min and decreases thereafter [17]. As shown in the previous study, 10 μM PGE_2 caused a significant increase in cAMP accumulation, while bradykinin had little effect on cAMP accumulation in MC3T3-E1 cells (Table 1).

Effects of bradykinin on the formation of IPs and Ins(1, 4, 5) P_3 in MC3T3-E1 cells: comparison with those of PGE_2

We previously showed that PGE_2 stimulates PI hydrolysis in a dose-dependent manner in the range between 1 nM and 10 μM in MC3T3-E1 cells [17]. The maximum effect of PGE_2 is observed at 10 μM and EC_{50} on the inositol trisphosphate formation by PGE_2 is 0.8 μM. We therefore next examined the effect of bradykinin on PI hydrolysis in these cells. Bradykinin stimulated the formation of IPs and Ins(1, 4, 5) P_3 in a dose-dependent manner in the range between 0.1 nM and 0.1 μM (Fig. 4). The maximum effect of bradykinin was at 0.1 μM and EC_{50} on the Ins(1, 4, 5) P_3 formation by bradykinin was 8 nM. However, the maximum effect on IPs formation observed at 0.1 μM was much less than that of PGE_2 at 10 μM in these cells (Table 1). In addition, the formation of Ins(1, 4, 5) P_3 induced by both bradykinin and PGE_2 reached a peak at 30 sec and decreased thereafter (data not shown). The maximum effect of PGE_2 observed at 10 μM was about 2-fold higher than that of bradykinin at 0.1 μM in MC3T3-E1 cells (Table 1).

Table 1. Effects of bradykinin and PGE_2 on cAMP accumulation, IPs formation and Ins (1, 4, 5) P_3 formation in MC3T3-E1 cells

Effector	cAMP accumulation	IPs formation	Ins (1, 4, 5)P_3 formation
	pmol/dish	cpm/dish	pmol/dish
Control	30±12	576±121	5.1± 1.1
Bradykinin (0.1 μM)	37±19	1,936±262*	26.9 ±4.2*
PGE_2 (10μM)	560±72*	35,328±998*	52.3± 5.5*

The cultured cells were stimulated by 0.1μM bradykinin or 10 μM PGE_2 for 5 min, 10 min and 30 sec in the assays of cAMP accumulation, IPs formation and Ins(1, 4, 5)P_3 formation, respectively. Each value represents the mean±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, Significantly different from control ($P<0.01$).

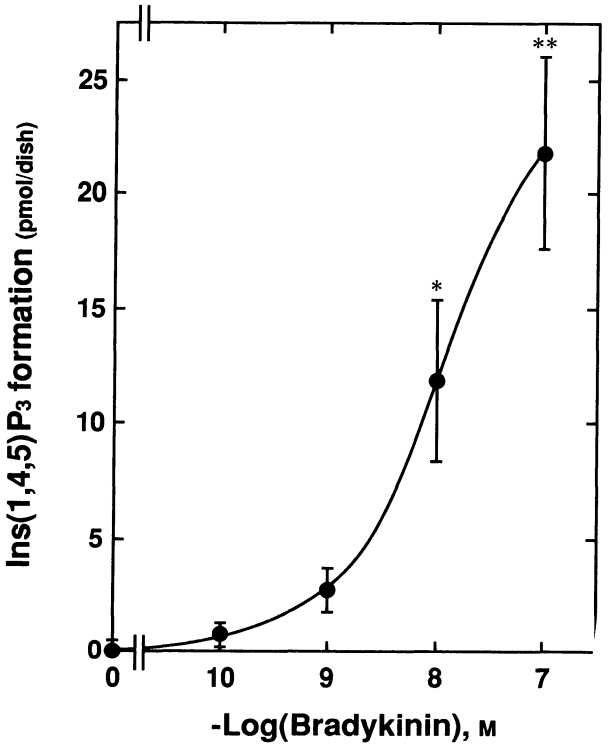

Fig. 4. Dose-dependent effect of bradykinin on Ins (1, 4, 5) P_3 formation in MC3T3-E1 cells. The cultured cells were stimulated with various doses of bradykinin for 30 sec. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean±SD of triplicate determinations. Similar results were obtained with two additional and differnet cell preparations. *, $P<0.05$, **, $P<0.01$ comapred with control.

Discussion

We previously reported that PGE_2, known as a potent bone resorbing agent [11], induces Ca^{2+} influx in addition to cAMP production and PI hydrolysis in osteoblast-like MC3T3-E1 cells [16–18], and the dose-dependent curves of PGE_2-induced PI hydrolysis, cAMP production and Ca^{2+}influx are different to one another [16–18]. We have also demonstrated that both PGE_2-induced cAMP production and Ca^{2+} influx are autoregulated due to the activation of protein kinase C, resulting from PI hydrolysis [17, 18]. In the present study, we compared the intracellular signaling systems of two bone resorbing agents, PGE_2 and bradykinin [2, 3], in these cells. We showed that bradykinin potently stimulated Ca^{2+}influx even in the presence of nifedipine, a Ca^{2+}antagonist that inhibits the voltage-dependent L-type Ca^{2+} channel, time- and dose-dependently like PGE_2. Bradykinin had little effect on cAMP accumulation, while PGE_2 significantly stimulated it. We also demonstrated that bradykinin markedly induced the formation of IPs, but the effect was much less potent than PGE_2 in these cells. It is recognized that PI hydrolysis by phospholipase C results in the formation of diacylglycerol and IPs. Among these products, diacylglycerol and Ins(1, 4, 5)P_3 serve as messengers for protein kinase C and mobilization of Ca^{2+} from the intracellular Ca^{2+} store, respectively [9, 10]. We also showed that the effect of bradykinin (0.1 μM) on Ca^{2+} influx was as great as 0.5 μM PGE_2, and that the effect of PGE_2 on Ins(1, 4, 5)P_3 formation was about 2-fold higher than that of bradykinin in these cells. In view of these results, it is possible that mobilization of Ca^{2+} by bradykinin from the intracellular Ca^{2+} store is less than that by PGE_2. As for PI hydrolysis by bone resorbing agents, we previously showed that a pertussis toxin-sensitive GTP-binding protein is involved in the coupling of PGE_2 receptor to phospholipase C in MC3T3-E1 cells [16]. It has recently been reported that bradykinin stimulates PI hydrolysis in a pertussis toxin-insensitive manner in these cells [5]. In addition, we showed here that bradykinin stimulated both Ca^{2+}influx and PI hydrolysis, and that the dose response curves of Ca^{2+} and Ins(1, 4, 5)P_3 formation induced by bradykinin seem to be similar. On the other hand, we previously showed that the dose-dependent curves of Ca^{2+} influx and

inositol trisphosphate formation induced by PGE_2 are different [18]. Taking account these findings, the intracellular signaling systems of these two bone resorbing agents seem to be quite different to each other in osteoblast-like MC3T3-E1 cells.

It has been reported that bradykinin stimulates the production of prostaglandins in osteoblast-like cells including MC3T3-E1 cells [4, 5]. Prostaglandins are known to be synthesized from arachidonic acid, which is released from esterified stores of phospholipids [23]. It is recognized that the release of arachidonic acid is a rate-limiting step in prostaglandins biosynthesis [24], and that the activities of cellular enzymes such as phospholipase A_2 and phospholipase C are dependent on the intracellular Ca^{2+} level [25, 26]. It is therefore most likely that bradykinin mobilizes Ca^{2+} from both the intracellular store and extracellular space in osteoblasts, resulting in a change in bone metabolism.

In conclusion, our results strongly suggest that bradykinin induces Ca^{2+} influx independent of the voltage-dependent L-type Ca^{2+} channel and PI hydrolysis in a similar dose-dependent manner in osteoblast-like cells.

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