Parathyroid hormone and prostaglandin E₂ stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblast cultures

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Parathyroid hormone (PTH) and prostaglandin E₂ (PGE₂) are physiological agonists which stimulate bone cells to resorb bone, a process by which the mineralized extracellular bone matrix is dissolved. Bone resorption has a key role in the maintenance of plasma calcium levels. It has been established that both PTH and PGE₂ activate adenylate cyclase in osteoblasts, but it is apparent that (1) the two agents have qualitatively different effects on osteoblasts, and (2) the generation of cyclic AMP cannot account for all the effects of PTH on bone cell metabolism. Others have demonstrated that PTH and PGE₂ may also elevate intracellular calcium levels, but the mechanism by which this is achieved has not been fully defined. Here we have investigated the effects of PTH on neonatal mouse osteoblasts in culture and shown that physiological concentrations of the hormone (50 nM) caused a small increase (22%) in total inositol phosphates accumulation, with a larger increase (40%) in inositol trisphosphate. We found that this activation occurred at lower concentration than was necessary to activate adenylate cyclase. PGE₂ was a more effective activator of inositol phosphates accumulation than PTH, causing up to 300% increase in the total inositol phosphates after 30 min. Both PTH and PGE₂ stimulated cyclic AMP accumulation, but the activation of adenylate cyclase by forskolin did not enhance inositol phosphates production. We conclude that both PTH and PGE₂ stimulate phosphoinositide turnover in mouse osteoblasts and suggest that this mechanism may contribute to their elevation of intracellular calcium in bone cells.

INTRODUCTION

Bone resorption, a complex process which includes mobilization of the mineral phase of bone matrix to maintain the plasma calcium concentration, is regulated by PTH and other physiological agents such as vitamin D and prostaglandins. However, osteoclasts, the cells responsible for bone resorption at bone surfaces, are thought not to possess PTH receptors, and the hormone's action on these cells is mediated by the major bone forming cells, osteoblasts. In addition, osteocytes may resorb bone under direct stimulation by PTH. It has been suggested that the action of PTH may be mediated by the production and secretion of prostaglandins. These interactions within bone tissue have been summarized elsewhere (Sledge, 1985; Nijweide *et al.*, 1986; Raisz & Martin, 1984).

The cellular effects of PTH on osteoblast-like cells include stimulation of proliferation (Plas *et al.*, 1985; McDonald *et al.*, 1986), inhibition of proliferation at high concentrations (Partridge *et al.*, 1985), elevation of [Ca]_i in some but not all osteoblast-like cell lines (Lowik *et al.*, 1985; Boland *et al.*, 1986) and the activation of adenylate cyclase (Chase & Aurbach, 1970). The latter action is insufficient to account for all of the effects of

PTH (Lerner *et al.*, 1986a; Conaway *et al.*, 1987; Ransjo & Lerner, 1987) and it has been proposed that osteoblasts possess two different receptors for PTH (Lowik *et al.*, 1985). In support of this proposal, Yamaguchi *et al.* (1987) have demonstrated that PTH can elevate [Ca]_i by cyclic AMP-dependent and -independent pathways.

It is well established that, like PTH, PGE₂ stimulates adenylate cyclase in osteoblasts, but its activity is not wholly coincident with that of PTH. For example, Boland *et al.* (1986) have shown that PTH but not PGE₂ caused an elevation of [Ca]_i in osteoblast-like cells. In murine osteoblast cultures (similar to those used here) [Ca]_i was elevated by both PGE₂ and PTH, only the elevation caused by PTH was dependent on the presence of extracellular calcium (Lieberherr, 1987).

In many cell types, ligands acting through a discrete subclass of their receptors evoke a rise in intracellular calcium levels and stimulate the turnover of inositol-containing lipids (Michell, 1975). Hydrolysis of phosphatidylinositol 4,5-bisphosphate, the initial ligand-activated reaction in this pathway, generates inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and diacylglycerol, both of which may serve as intracellular messengers (Berridge, 1984; Berridge & Irvine, 1984). Thus, diacylglycerol may activate protein kinase C (Nishizuka, 1984)

Abbreviations used: ANOVA, analysis of variance; [Ca]_i, intracellular calcium concentration; DMEM, Dulbecco's modification of Eagle's medium; GroPIns, glycerophosphoinositol; IBMX, 3-isobutyl-1-methylxanthine; InsP, D-*myo*-inositol monophosphate; InsP₃, D-*myo*-inositol trisphosphate; InsP₄, D-*myo*-inositol tetrakisphosphate; PGE₂, prostaglandin E₂; PtdIns(4,5)P₂, phosphatidylinositol bisphosphate; PTH, parathyroid hormone.

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and $\text{Ins}(1,4,5)\text{P}_3$ has been shown to mediate the release of calcium from intracellular stores (Streb *et al.*, 1983, 1984). Furthermore, additional messengers may be generated by the metabolism of either of these putative second messengers.

In the present work we have addressed the question of whether PTH and PGE_2 can activate phosphoinositide breakdown in osteoblasts.

EXPERIMENTAL

Materials

PTH was the gift of Dr. Zanelli, National Institute of Biological Standards and Control, London, to the Eastman Dental Hospital. PGE_2 was from Sigma, and forskolin was from Hoechst. Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum were from Flow Laboratories. *myo*-[2- ^3H]inositol, [2,5- ^3H]cyclic AMP and [6- ^3H]thymidine were from Amersham.

Cell culture

Osteoblasts were obtained by sequential proteolytic digestion of neonatal mouse calvariae as described by Heath *et al.* (1984). The cells were seeded at a density of $(3-4) \times 10^5$ cells per 30 mm petri dish and maintained in DMEM containing 10% foetal calf serum in a humidified atmosphere of CO_2/air (1:19) at 37 °C.

Measurement of inositol phosphates accumulation

Inositol phosphates accumulation was measured by a method based on that of Berridge *et al.* (1982). Briefly, after 5-7 days in culture the cell monolayers were incubated with *myo*-[2- ^3H]inositol (10 $\mu\text{Ci}/\text{ml}$) for 24 h. The medium was removed and the monolayers washed with three changes of 'dissection medium', a modification of BGJ medium designed to maintain physiological pH in air (Reynolds, 1976), containing 5 mM-LiCl. The cells were incubated for a further 25 min before hormone additions were made in a negligible volume (typically 10 μl). The cell monolayers were maintained throughout at 37 °C in LiCl-containing 'dissection medium'. After 30 min exposure to hormone, the medium was removed and 1.0 ml of chloroform/methanol (1:2, v/v) was added to each dish. After 3 min this was transferred to a polypropylene vial, 330 μl of chloroform and 330 μl of water added, and the vials were centrifuged to separate the phases. The upper aqueous phase was added to a 250 μl bed volume column of Dowex 1-X8 (formate form) which was then washed with 5×5 ml of water to elute [^3H]inositol. The final 5 ml wash contained background levels of radioactivity. Four 2 ml portions of 5 mM-sodium tetraborate containing 60 mM-sodium formate were applied to the columns and the eluates pooled (GroPIIns). The total inositol phosphates (InsPs) were then eluted from the columns with 4×2 ml of 1.0 M-ammonium formate in 0.1 M-formic acid. Where inositol phosphates were resolved, 4×2 ml of 0.2 M-ammonium formate in 0.1 M-formic acid, and 0.4 M-ammonium formate in 0.1 M-formic acid, were used to elute inositol phosphate (InsP) and inositol bisphosphate (InsP₂) respectively, prior to elution of higher inositol phosphates (InsP₃) with 4×2 ml of 1.0 M-ammonium formate in 0.1 M-formic acid. The fractions were evaporated to dryness at 70 °C and re-dissolved in 0.5 ml of water. Optiphase Safe scintillant was added (5 ml) and radioactivity (d.p.m.) estimated by liquid scintillation.

Cyclic AMP determinations

After 7 days in culture, osteoblast monolayers were washed with dissection medium and incubated for 25 min. IBMX (0.1 mM) was added 5 min before the addition of PTH and the incubations were terminated after a further 5 min. Cell extraction and the determination of cyclic AMP was performed as described in detail by Murray & Farndale (1985).

Cell proliferation

Osteoblasts, prepared as described above, were seeded into 96-well microtitre plates at 1.5×10^4 cells per well. After 4 days, the foetal calf serum was reduced to 2%, and PTH, PGE_2 or other agents were included in the medium. After a further 18 h, 10^6 d.p.m. of [^3H]thymidine was added to each well, and the cultures were harvested 6 h later. The wells were washed twice and the medium replaced with 100 μl of ice-cold trichloroacetic acid (5% w/v), and left for 20 min at 5 °C. Labelled DNA was eluted with 100 μl of 0.1 M-NaOH, neutralized with 500 μl of 0.5 M-acetic acid, and estimated by liquid scintillation.

RESULTS

Table 1 shows the result of an experiment testing the effect of a range of PTH concentrations on inositol phosphates accumulation in osteoblasts. In unstimulated cells, the level of total InsPs, which includes InsP, InsP₂ and InsP₃, did not change during the experimental period. At a concentration of 50 nM, PTH caused a small but significant increase in total InsPs accumulation. At both lower and high concentrations, the elevation of total InsPs was not significant. These data suggest that the response to PTH may be biphasic. In separate experiments, the effect of PTH on the amount of cyclic AMP generated in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX was measured (Table 1). Under these conditions maximal stimulation of adenylate cyclase occurred at 500 nM-PTH, an order of magnitude higher than that required to cause a significant increase in inositol phosphates accumulation.

The pooled results from nine experiments showed that the increase in total InsPs accumulation evoked by 50 nM-PTH, although small (22% higher than controls) was significant ($P < 0.01$, two-way ANOVA), and the results from experiments (Table 2) in which the inositol phosphates were resolved showed a significant increase (40% over controls) in InsP₃ but not InsP₂ or InsP. The increase in InsP₃ in these experiments was sufficient to account fully for the increased total InsPs (Table 1). By comparison, foetal calf serum evoked a large increase in total inositol phosphates (mean of 4-fold over control), and also increased the radioactivity in the GroPIIns fraction (mean 5.4-fold; results not shown). PTH, however, had no effect on GroPIIns accumulation.

We conclude that PTH caused an average 40% increase in InsP₃ accumulation in osteoblasts. This effect, although small, is of similar magnitude to responses reported for a number of ligands acting on other cells (Berridge *et al.*, 1982; Wakelam *et al.*, 1986). Furthermore, Charest *et al.* (1985) found that adrenaline evoked a 30% increase in InsP₃ in hepatocytes and concluded that this was sufficient to produce a maximal increase in the intracellular calcium concentration and activation of phosphorylase.

Table 1. Mouse osteoblast response to PTH

The response of mouse osteoblasts to increasing doses of PTH is shown. [^3H]inositol-pre-labelled cultures were treated with PTH in the presence of 5 mM-LiCl for 30 min and inositol phosphates were determined as described in the Experimental section. Cyclic AMP was determined in cultures incubated with 0.1 mM-IBMX for 10 min, and then incubated with the indicated dose of PTH for a further 5 min. Results are the means (and S.E.) of triplicate determinations. *, Higher than control, $P < 0.05$, ** $P < 0.01$.

[PTH]	^3H in total InsPs (d.p.m./dish)	Cyclic AMP (pmol/dish)
Control	1248 (75)	2.04 (0.16)
5 nM	1408 (98)	2.31 (0.13)
50 nM	1548 (68)*	3.00 (0.41)*
500 nM	1295 (121)	24.50 (2.18)**
5 μM	1381 (19)	20.50 (2.35)**

Table 2. Inositol trisphosphate accumulation in mouse osteoblasts

The results of three experiments, in which [^3H]inositol-prelabelled mouse osteoblasts were treated with 50 nM-PTH for 30 min and InsP₃ was determined as described in the Experimental section, are shown. Values shown are means (and S.E.). * $P < 0.05$, ** $P < 0.02$, using two-sample *t*-tests. There was no increase in GroPIns, InsP or InsP₂ in these experiments.

Expt. no.	<i>n</i>	^3H in InsP ₃ fraction (d.p.m./dish)	
		Control	50 nM-PTH
1	5	272 (13)	368 (26)**
2	10	297 (30)	367 (13)*
3	4	646 (64)	938 (61)**

We examined the effect of PGE₂ on phosphoinositide metabolism and found a large, dose-dependent stimulation of total InsPs accumulation (mean 300 % increase at 25 μM -PGE₂) together with an elevation of the GroPIns fraction. Separation of the inositol phosphates after 30 min exposure to 10 μM -PGE₂ revealed that the increase in total InsPs was restricted to the InsP fraction (Fig. 1). After shorter exposure to PGE₂ (3 min), significant elevation of both InsP₂ and InsP₃ was observed. During this experiment, control levels of InsP did not change significantly, although both InsP₂ and InsP₃ levels rose so that the control and PGE₂-treated samples had converged by 10 min. Perturbation of monolayers of cells in cultures by handling procedures is known to stimulate prostaglandin synthesis (Samuelsson *et al.*, 1975), and it is possible that the rise in InsP₂ and InsP₃ levels in unstimulated cells was caused by this effect.

An examination of a range of concentrations of PGE₂ showed that its effect on total inositol phosphate accumulation was coincident with the activation of adenylate cyclase. We therefore challenged parallel cultures of osteoblasts with forskolin, an agent which is known to activate the catalytic subunit of adenylate

cyclase. It was found that, although forskolin produced a rapid and large increase in cyclic AMP, it had no effect on basal levels of total inositol phosphates accumulation (Fig. 2); indeed a small attenuation of the foetal-calf-serum-stimulated InsPs fraction was observed with forskolin (results not shown). These data suggest that the formation of inositol phosphates in response to PGE₂ was not secondary to cyclic AMP production.

It has been suggested that the action of PTH on osteoblasts may be mediated by prostaglandin production (see Raisz & Martin, 1984). We therefore conducted an experiment in which osteoblasts were incubated with and without 14 μM -indomethacin and challenged with PTH. This showed that the significant elevation of the InsP₃ fraction produced by PTH was abolished by indomethacin (Table 3).

Table 4 shows the incorporation of [^3H]thymidine into osteoblast cultures, after 4 days in culture, i.e. at confluence. PTH inhibited the incorporation of radiolabel into the cultures, in agreement with the work of Partridge *et al.* (1985). Thus, at concentrations which elevated both inositol phosphates and cyclic AMP production, PTH inhibited proliferation. However, in many cell types activation of phosphoinositide turnover is associated with mitogenic stimulation (Berridge, 1984). Both forskolin and PGE₂ caused marked inhibition of [^3H]thymidine incorporation into osteoblast cultures. These results suggest that the elevation of cyclic AMP levels causes potent inhibition of mitogenesis in murine osteoblasts.

DISCUSSION

PTH has not hitherto been shown to activate phosphoinositide hydrolysis in bone cells, although a small PTH-induced increase in InsP₃ in renal tubules has been reported (Hruska *et al.*, 1987). Recent work has suggested that prostaglandins may also stimulate phosphoinositide breakdown in other tissues (Mene *et al.*, 1987; Davis *et al.*, 1987). Here we have shown that both PTH and PGE₂ elevate the accumulation of inositol phosphates and cyclic AMP in murine osteoblast cultures. We suggest that InsP₃ production contributes to the elevation of [Ca]_i evoked by PTH and PGE₂ previously observed in osteoblast-like cells (Lowik *et al.*, 1985; Boland *et al.*, 1986; Yamaguchi *et al.*, 1987; Lieberherr, 1987). The elevation of [Ca]_i may result from the mobilization of intracellular calcium stores by Ins(1,4,5)P₃, as observed in other cell types (see Berridge & Irvine, 1984); alternatively, Ins(1,4,5)P₃ and/or Ins(1,3,4,5)P₄ may mediate the entry of extracellular calcium at the plasma membrane (Kuno & Gardner, 1987; Irvine & Moore, 1986).

It is known that in some tissues a single ligand may activate both adenylate cyclase and phosphoinositide hydrolysis; 5-hydroxytryptamine has been reported to do so in blowfly salivary glands (Berridge & Heslop, 1981) as has glucagon in hepatocytes (Wakelam *et al.*, 1986). In these reports it was concluded that the agonist's effects were mediated by different sub-classes of receptors. In the present work the stimulation of PtdIns(4,5)P₂ hydrolysis and adenylate cyclase by PGE₂ occurred over the same concentration range. In contrast, the dose-dependences of PTH-stimulated adenylate cyclase and PtdIns(4,5)P₂ breakdown were different. At a concentration of 500 nM, PTH caused maximal stimulation of adenylate cyclase, but had no significant effect on total

Fig. 1. Time course of inositol phosphates accumulation in response to 10 μM-PGE₂.

Confluent monolayers of mouse osteoblasts were prelabelled with [³H]inositol and treated, in the presence of 5 mM-LiCl, with 10 μM-PGE₂ for the indicated times. Inositol phosphates were determined as described in the Experimental section. ○, Controls; ▲, PGE₂-treated samples. Mean values (d.p.m. ± S.E., *n* = 4) are plotted versus time. **P* < 0.05 or smaller, using two-sample *t*-tests.

Fig. 2. Total inositol phosphates and cyclic AMP levels in response to increasing doses of PGE₂.

Four confluent dishes (for InsP) or three (for cyclic AMP) for each determination were treated with PGE₂ at the following doses: (a) 25 nM, (b) 250 nM, (c) 2.5 μM or (d) 25 μM. Forskolin (50 μM) in 0.1% ethanol, or 0.1% ethanol, were also tested. InsPs or cyclic AMP were determined as described in the Experimental section. The results shown are the mean (± S.D.) and the least significant difference from control values indicated (*) yielded *P* < 0.02 using two-sample *t*-tests. Other differences were non-significant.

InsPs production. A preliminary experiment has shown that the effect of PTH was blocked by indomethacin, suggesting that the PTH-stimulated PtdIns(4,5)P₂ breakdown is mediated by prostaglandin production. Yang *et al.* (1987) have shown that in chick calvarial cultures, PGE₂ was the major eicosanoid produced in response to PTH. Further work is necessary to discover whether this also applies to murine osteoblasts.

The observation that forskolin did not elevate InsPs accumulation shows that activation of adenylate cyclase is not sufficient to activate the phosphoinositide pathway. The converse relationship also seems unlikely, since, in contrast with the results obtained with PGE₂, PTH at a level sufficient for maximal stimulation of phosphoinositide hydrolysis was virtually without effect on cyclic AMP levels. Further work will be necessary to determine whether the activation of arachidonate metabolism is secondary to elevation of cyclic AMP levels (as indicated by Lerner *et al.*, 1986b).

It is interesting that the activation of inositol phosphate production by PTH was not temporally parallel with that caused by PGE₂; the increase in radiolabel associated with InsPs was mainly in the InsP₃ fraction after 30 min treatment with PTH, whereas, after a similar period of incubation with PGE₂, InsP predominated. Yang *et al.* (1987) have shown in chick calvarial cultures that PTH caused a significant increase in PGE₂ production after 60, but not 10, min; intervening times were not tested. Thus, the onset of PtdIns(4,5)P₂ hydrolysis following PTH stimulation of mouse osteoblasts may be delayed by the intervening activation of arachidonate metabolism.

It is clear from the present work that both PTH and PGE₂ activate phosphoinositide hydrolysis, and that

Table 3. Action of indomethacin and PTH on inositol phosphates

Osteoblasts were incubated with or without 14 μ M-indomethacin for 55 min, during the last 30 min of which 50 nM-PTH was added where indicated. Inositol phosphates were separated as described in the Experimental section. Values (d.p.m./dish) are means (and S.E.) of six replicates. *Different from control ($P < 0.05$), using two-sample t -test.

Treatment	GroPIns	InsP	InsP ₂	InsP ₃
Control	1341 (60)	856 (20)	371 (16)	272 (13)
Indomethacin	1248 (43)	776 (30)	343 (30)	281 (28)
PTH	1485 (154)	895 (72)	445 (66)	368 (26)*
PTH + indomethacin	1202 (94)	710 (27)	295 (32)	270 (56)

Table 4. Effect of PTH, PGE₂ and forskolin on thymidine incorporation

The incorporation of [³H]thymidine into mouse osteoblasts cultured in microtitre plates is shown. After 96 h PTH, PGE₂ or forskolin at the indicated dose was added to the wells, and the serum concentration was reduced to 2%. Where shown, serum levels were restored to 10%. After a further 18 h, radiolabel was added and estimated 6 h later as described in the Experimental section. The values shown are the ratio of experimental to control radioactivity for each treatment. There were typically ten wells per group. * $P < 0.02$, ** $P < 0.01$ for the difference from control values, comparing d.p.m. by two-sample t -tests. Ethanol controls, where tested, had no effect.

Treatment	[³ H]Thymidine incorporation relative to controls
Expt. 1: PTH	
Control	1.00
Serum	2.19**
5 nM	0.96
50 nM	0.76**
500 nM	0.61**
5 μ M	0.89**
Expt. 2: PGE ₂	
Control	1.00
Serum	1.81**
25 nM	0.90
250 nM	0.88*
2.5 μ M	0.84**
25 μ M	0.75**
Expt. 3: Forskolin	
Control	1.00
Serum	1.52**
0.1 nM	1.01
10 nM	0.63**
1 μ M	0.70**
0.1 mM	0.44**

inositol phosphates produced by either ligand may be of sufficient magnitude to contribute to the elevation of [Ca]_i observed by others. We suggest that phosphoinositide hydrolysis may play a functional role in the bone-resorptive activity of these hormones.

We are grateful to Mark Biffen for helpful discussion. This work was supported by the Medical Research Council and an award from the Elmore Medical Research Fund to S.R.P.

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Received 18 August 1987/5 February 1988; accepted 1 March 1988