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Mediators of the Biphasic Responses of Bone to Intermittent and Continuously Administered Parathyroid Hormone

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Abstract Parathyroid hormone (PTH) has biphasic effects on bone: continuous treatment is catabolic whereas intermittent treatment is anabolic. The mechanism(s) responsible for these differing effects are still unclear, partly because of the previous non-availability of a model system in which effects on both formation and resorption indices could be studied concomitantly. In cultured marrow cells from 6-week old C57BL/6 mice, we demonstrated that 4 days of intermittent PTH treatment increased mRNA for osteoblast differentiation markers (Runx2, alkaline phosphatase (AP), and type I procollagen (COL1A1) whereas continuous treatment resulted in production of large numbers of TRAP-positive multinucleated osteoclasts. Although IGF-I mRNA did not increase after intermittent treatment, it was consistently higher than after continuous treatment, and the addition of an anti-IGF-I neutralizing antibody prevented the increase in bone formation indices observed with intermittent treatment. By contrast, after continuous treatment, gene expression of RANK ligand (RANKL) was increased and that of osteoprotegerin (OPG) was decreased, resulting in a 25-fold increase in the RANKL/OPG ratio. In this model system, the data suggest that intermittent PTH treatment enhances osteoblast differentiation through an IGF-I dependent mechanism and continuous PTH treatment enhances osteoclastogenesis through reciprocal increases in RANKL and decreases in OPG. J. Cell. Biochem. 89: 180–190, 2003.

Key words: osteoblast differentiation; Runx2; insulin-like growth factor-I (IGF-I); rank ligand; osteoprotegerin

Parathyroid hormone (PTH) exerts biphasic effects on osteoblastic cells, depending on dose, duration of exposure, and differentiation stage of the cells. However, the mechanism(s) responsible for inducing the differing effects of PTH when administered continuously or intermittently remain a long-standing enigma. Previous studies in a variety of systems have shown that intermittent PTH has diverse effects on osteoblast lineage cells, and has independent effects on bone formation. In particular, intermittent administration increases bone mass by increasing formation over resorption whereas continuous administration induces bone loss through large increases in resorption. [Tam et al., 1982; Hock and Gera, 1992]. Its effects differ depending on the differentiation stage of the cells [Isogai et al., 1996] and on the time and duration of treatment [Ishizuya et al., 1997; Schiller et al., 1999]. A 6-h pulse of treatment was found by Ishizuya et al. to lead to increased mineralized nodule formation in cultures of rat osteoblastic cells, while Dobnig and Turner [1997] found that after a single injection of PTH, the vast majority of the hormone was cleared from circulation within 1 h, and by 6 h, circulating levels were not significantly different from controls. The differential response may be partly due to temporal regulation of the expression and activity of the PTH/PTHrP receptor (PTH1R) [McCauley et al., 1996; Kondo et al., 1997]. However, under the right conditions, PTH can increase alkaline phosphatase (AP) and osteocalcin expression

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and mineralization in vitro and bone apposition in vivo. Intermittent PTH has also been reported to increase both the proliferation and differentiation of bone marrow osteoprogenitors in vivo [Nishida et al., 1994] and to decrease apoptosis in vitro [Jilka et al., 1999] while causing a transient increase in apoptosis in vivo [Stanislaus et al., 2000]. At least some of the anabolic effects of intermittent PTH have been shown to be mediated by IGF-I [Canalis et al., 1989; Ishizuya et al., 1997], and IGF-I transcript and polypeptide levels increase both in vitro and in vivo following intermittent PTH administration [McCarthy et al., 1989; Pfeilschifter et al., 1995]. Conversely, IGF-I has been shown to suppress expression of the PTH/PTHrP receptor via the MAP kinase pathway [Kawane and Horiuchi, 1999], suggesting the presence of a regulatory feedback loop. Other studies have implicated TGFβ_1 and TGFβ_2 [Wu and Kumar, 2000], OPG and RANKL [Horwood et al., 1998; Lee and Lorenzo, 1999; Kanzawa et al., 2000] and the PKA [Greenfield et al., 1995; Halladay et al., 2002] and PKC [Kaji et al., 1996] signaling pathways, but results have not been consistent between investigators or between model systems, and measurements have not been made of both the anabolic and catabolic responses concurrently in the same model system. However, a definition of these mechanisms has become even more important as intermittent therapy with PTH(1-34) has now been approved by American and European regulatory agencies as a formation-stimulating regimen for the treatment of osteoporosis.

A major obstacle to identifying the metabolic mediators of the biphasic responses of bone to PTH has been the previous non-availability of a model system in which both the anabolic and catabolic effects could be studied concomitantly. Thus, differences in responses to the two modes of PTH administration are confounded by differences between the model systems. We report here the use of a mouse marrow model in which intermittent PTH administration increases osteoblast differentiation, and continuous PTH administration leads to induction of osteoclast formation. Using this model, our data suggest that the effects of PTH on increasing bone formation indices are mediated through an IGF-I-dependent mechanism but that the effects on resorption indices are mediated through an increase in the gene expression ratio of RANKL/OPG.

MATERIALS AND METHODS

Cell Culture

Animal protocols were approved by the Mayo Institutional Animal Care and Use Committee. A marrow cell suspension was generated from 6-week-old male C57BL/6 mice essentially as described by Takahashi et al. [1988]. Cells were plated at 1×10^6 nucleated cells/cm^2 in αMEM containing 1% penicillin/streptomycin (Gibco BRL, Gaithersburg, MD; final concentration 100 U/ml penicillin; 100 μg/ml streptomycin) and 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco BRL, Gaithersburg, MD). They were then maintained in culture at 37°C in 5% CO_2 in air for 6 days before beginning 96 h of continuous or intermittent PTH treatment. Cells were grown in 6-well plates (area: 10 cm^2) for RNA extraction and in 24-well plates (area: 2 cm^2) for osteoclast formation and enzyme assays. Medium was changed at 3 days, when the non-adherent cells were removed. Human PTH (1-34) (Bachem, Torrance, CA) was then added to cultures in fresh medium, as indicated, at a concentration of 10 nM (~40 ng/ml). Control vehicle-treated cultures, and those treated with continuous PTH, were fed with fresh medium every 48 h. For intermittent treatment, PTH-containing medium was added for the first 6 h of each 48-h period, after which the medium was removed, the cell layer was rinsed with warm medium, and fresh medium without hormone was added for the remaining 42 h. After two 48-h cycles of treatment, cultures were fixed and stained, or harvested for RNA extraction.

Real Time RT-PCR

Total RNA was isolated from marrow cultures using a Qiagen RNeasy kit. cDNA was synthesized from 1 μg RNA. The resulting cDNA was diluted to 200 μl and a 5 μl aliquot of this was used for each PCR reaction. PCR reactions were carried out on a Light-Cycler™ instrument (Roche Diagnostics, Basel, Switzerland) as described previously [Locklin et al., 2001]. Specific amplification reactions for the following cDNAs were carried out: OPG, RANKL, RANK, calcitonin receptor (CTR), IGF-I, TGFβ_1, and β_2, Runx2 (synonyms for Runx2 are Cbfa1, AML3, and PEBP2aA), procollagen type I (COL1A1) AP, GAPDH. Reaction product was quantified using a simultaneously amplified series of dilutions of a sequence of known concentration (in

this case a vector containing the *RANKL* gene) to generate a standard curve at each run. Primer sequences and amplification profiles are as shown in Table I.

Assessment of Osteoclast Formation

Cultures were fixed for 30 s in citrate-buffered acetone/formaldehyde. Staining for TRAP (tartrate-resistant acid phosphatase) was performed using a commercial leukocyte acid phosphatase kit (Sigma Chemical Co., St. Louis, MO). Osteoclasts were quantitated by counting TRAP-positive multinucleated cells (more than 3 nuclei per cell) at 10-fold magnification in 10 fields in each of three culture wells per condition; results are expressed as number of TRAP-positive cells/10 fields.

Investigation of Signaling Pathways

Using the culture method described above, we compared the effects of PTH(1-34) with that of specific peptides for either the cAMP/PKA or Ca^{2+}/PKC pathways. PTH(1-31) (Peptide Institute Inc, Osaka, Japan) or forskolin (Sigma, St. Louis, MO) were used to stimulate the PKA pathway, whereas PTH(3-34) (Bachem, Torrance, CA) or phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) were used to stimulate PKC. The compounds were administered either intermittently or continuously, as described for PTH(1-34). PTH fragments and PMA were used at a concentration of 10 nM, as for PTH(1-34), while forskolin was used at 10 μM.

cAMP Enzyme Immunoassay

Following two cycles of PTH treatment, cell cultures were rinsed and then treated with fresh, warm medium containing PTH, without or with isobutylmethylxanthine (IBMX) at 500 μM, for 10 min. The cells were then lysed, and intracellular cAMP was measured using a specific enzyme immunoassay (Biotra cellular communication assays, Amersham Pharmacia, Piscataway, NJ).

Statistical Analysis

Differences between all groups were analyzed using ANOVA. Differences between vehicle and intermittent or continuous treatment was assessed by Student's unpaired two-tailed *t*-test. A *P*-value of <0.05 was considered significant.

RESULTS

Effect of PTH Treatment on Markers of Osteoblast Differentiation

There was a large increase ($P < 0.02$) in the expression of the early osteoblastic marker, Runx2, after intermittent, but not after continuous, PTH treatment (Fig. 1a). However, there were smaller increases in expression of AP ($P = 0.05$) and COL1A1 ($P = 0.13$) (Fig. 1b,c). Continuous PTH treatment resulted in decreases in AP and COL1A1 expression in three out of six experiments and no significant change in the others.

Regulation of Osteoclast-Related Gene Expression by Continuous or Intermittent PTH Treatment

In all six experiments carried out, PTH(1-34) (10 nM) consistently increased RANKL mRNA levels, but the increase was only significant with continuous treatment (Fig. 2a). Similarly, we observed a highly significant decrease in OPG expression with continuous, but not with

TABLE I. RT-PCR Primers Used in the Study

Gene	Sequence	Product length (bp)	Amplification profile (temp/time (s))	No. cycles
OPG	TGAAGCTGTGGAAACATCAA TTTGCAGGTCTTTCTCGTTC	163	95/0; 58/7; 72/7	36
RANKL	CATCGGGTTCCCATAAAGTC TTGCCCGACCAGTTTTTC	256	95/0; 58/7; 72/11	40
RANK	AAGATGGTTCCAGAAGACGGT CATAGAGT-CAGTTCTGCTCGGA	350	95/0; 55/7; 72/14	30
CTR	TTTCAAGAACCTTAGCTGCCAGAG CAAGGCACGGA-CAATGTTGAGAAG	565	95/0; 60/7; 72/22	40
IGF-I	TCATTATTCCTGCCCACCAA ATCCACAATGCCCGTCTG	411	95/0; 60/7; 72/16	36
$TGF\beta_1$	CCGCAACAACGCCATCT ACCAAGGTAACGCCAGGAAT	205	95/0; 55/7; 72/10	35
$TGF\beta_2$	AAAACCCCAAAGCCAGAG GAGGGCAACAACATTAGCAG	400	95/0; 56/7; 72/16	36
Runx2	CCGCACGACAACCGCACCAT CGCTCCGGCCCACAAATCTC	287	95/0; 60/7; 72/20	35
Procollagen I	CACCCCAGCCGCAAAGAGT CGGGCAGAAAGCACAGCACT	300	95/0; 55/7; 72/12	30
AP	GGGGACATGCAGTATGAGTT GGCCTGGTAGTTGTTGT-GAG	472	95/0; 55/7; 72/16	30
GAPDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	500	95/0; 55/7; 72/20	35

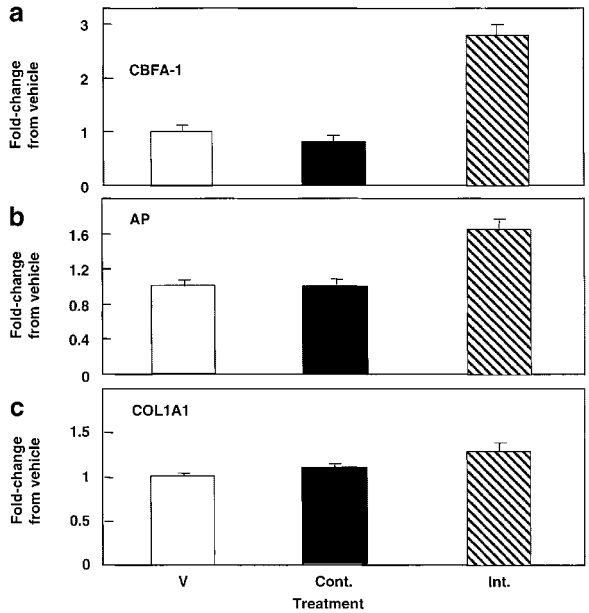

Fig. 1. Effect of continuous or intermittent PTH (1-34) on markers of osteoblast differentiation from a representative experiment of six performed. Mouse marrow cells were treated with PTH (10 nM), either continuously or intermittently, or vehicle, for 4 days. mRNA was extracted and analyzed by real-time RT-PCR, and results were normalized to GAPDH values. Bars represent mean ± SEM from three replicates. **a**: For Runx2 expression, the intermittent treatment group was significantly different ($P = 0.019$) from the controls as well as from the continuous treatment group ($P = 0.005$). **b**: Alkaline phosphatase (AP) was also marginally increased with intermittent treatment compared to controls ($P = 0.056$) and to continuous treatment ($P = 0.058$). **c**: The differences between groups were not significant for gene expression of COL1A1.

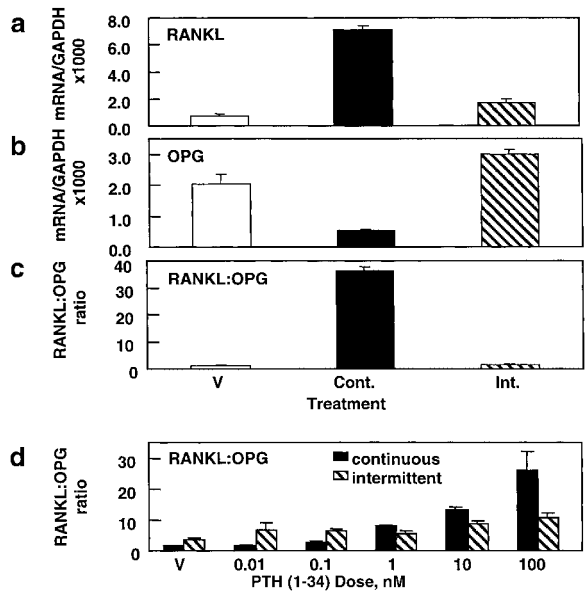

Fig. 2. mRNA expression of (**a**) RANKL and (**b**) OPG from a representative experiment of six. Bars represent mean ± SEM from three replicates samples. Mouse marrow cells were treated with PTH(1-34) [10 nM], either continuously or intermittently, or vehicle, for 4 days. mRNA was extracted and analyzed by real-time RT-PCR; results were normalized to GAPDH values. The ratio of RANKL:OPG expression is shown in panel (**c**), expressed relative to controls. Comparing continuous to intermittent PTH treatment, $P < 0.001$ for each set of data. The increase in RANKL:OPG expression ratio with increasing doses of PTH(1-34) is shown in panel (**d**). Only the ratio in continuously treated cultures at doses of 1 nM and above was significantly increased compared to respective controls. *P*-values for continuous treatment at 1, 10, and 100 nM are 0.0005, 0.007, and 0.005, respectively.

intermittent, PTH treatment (Fig. 2b). Thus, the ratio of RANKL:OPG was increased with continuous PTH treatment by up to 25-fold (Fig. 2c). The magnitude of these effects increasesd as the dose increased. At higher doses intermittent PTH also increased the RANKL:OPG ratio, but only at 100 nM, the highest dose tested, and the ratio remained significantly lower than in cultures treated with continuous PTH (Fig. 2d). No significant change in RANK expression was detected (data not shown). These effects were more marked in cultures of stromal cells, in which non-adherent cells were removed from the marrow cultures 24 h after plating (data not shown).

Effect of PTH Treatment on Osteoclast Formation

Continuous treatment with 10 nM PTH (1-34) for two 48-h cycles resulted in numerous mature osteoclasts in these cultures whereas none developed in intermittently treated cultures or vehicle-treated controls (Fig. 3a).

The induction of osteoclast formation by continuous PTH treatment was dose-dependent (Fig. 3a). At the highest PTH dose of 100 nM, a few osteoclasts were observed with intermittent PTH treatment, but the number formed under these conditions was significantly lower than with continuous treatment. Although TRAP stained cells were evident with intermittent 10 nM PTH treatment, these stained cells were not multinucleated. With continuous PTH treatment, the increase in osteoclast numbers was associated with an increase in CTR expression, as assessed by RT-PCR (data not shown). Addition of exogenous rhOPG (20 ng/ml) to the cultures treated continuously with PTH completely blocked osteoclast formation (Fig. 3b).

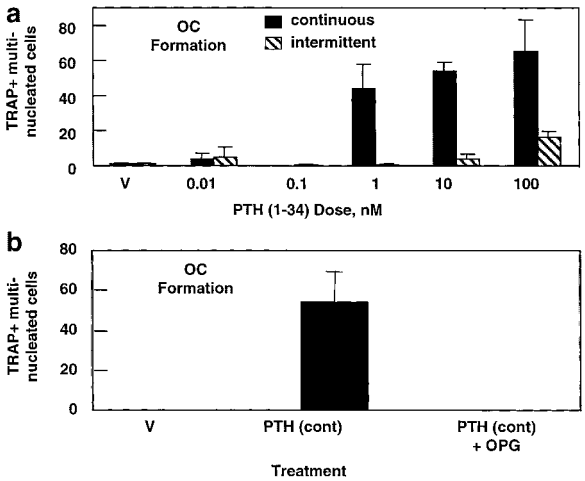

Fig. 3. Effect of increasing doses of PTH on osteoclast formation. **a**: Number of TRAP-positive multinucleated cells counted in 10 fields in each of three culture wells. Bars represent mean ± SEM. Continuous PTH was significantly different from either controls or intermittent treatment, $P < 0.001$. **b**: Effect of rhOPG on induction of osteoclastogenesis by PTH. Mouse marrow cells were treated with PTH, either continuously or intermittently, or vehicle, for 4 days, in the absence or presence of rhOPG (20 ng/ml). Stained TRAP-positive multinucleated cells were counted in 10 fields in each of three culture wells. Bars represent mean ± SEM. rhOPG abolished osteoclast formation induced by continuous PTH; $P < 0.001$.

Signal Transduction of Intermittent PTH on Markers of Osteoblast Formation

Expression of Runx2 and AP were significantly upregulated by intermittent treatment with PTH(1-34); COL1A1 was also upregulated although this was not significant. This effect could be simulated by either PTH(1-31) or PTH(3-34) when administered intermittently (Fig. 4).

Signal Transduction of Continuous PTH on Osteoclast Formation

The effect of continuous PTH(1-34) could be reproduced by PTH(1-31), which activates the protein kinase A (PKA) signaling pathway, but not by PTH(3-34), which activates the protein kinase C (PKC) pathway. PTH(1-31) induced an increase in the RANKL:OPG ratio and thus in osteoclast formation (Fig. 5a,b). Similarly, continuous treatment with 10^{-5} M forskolin, which increases intracellular cAMP levels, increased RANKL expression, decreased OPG, and resulted in osteoclast formation, while 10^{-8} M PMA, which stimulates protein kinase C, did not (Fig. 5c).

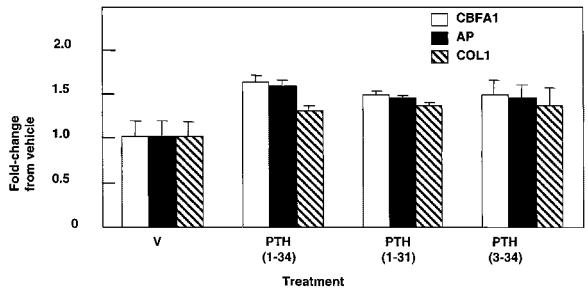

Fig. 4. Expression of Runx2, AP, and procollagen type I (COL1A1), as assessed by real-time RT-PCR, in the cultures treated with PTH fragments. Mouse marrow cells were treated intermittently with PTH(1-34), PTH(1-31), or PTH(3-34) [all at 10 nM], or vehicle, for 4 days, in three independent experiments. Bars represent mean ± SEM from three replicate samples. Expression of Runx2 and AP with intermittent PTH(1-34) were significantly increased ($P = 0.03$ and 0.04, respectively) compared to controls; COL1A1 was also increased although this was not significant. The induction of expression of these genes by intermittent treatment with either PTH(1-31) or PTH(3-34) was not significantly different from the PTH(1-34) group.

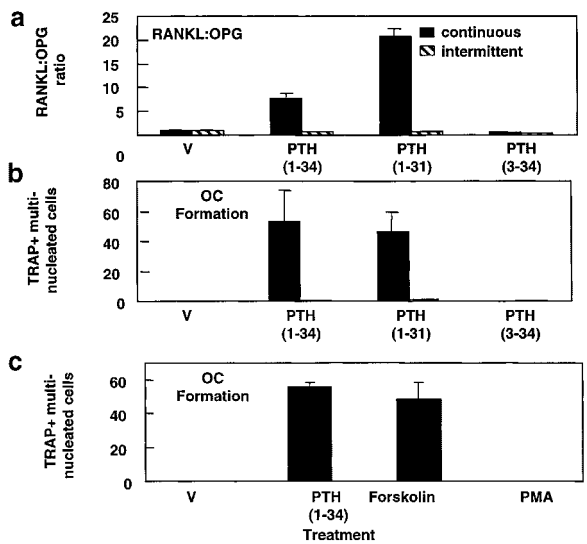

Fig. 5. Effect of PTH fragments, or stimulators of PKA and PKC signaling pathways, on RANKL:OPG ratio and osteoclast formation. Mouse marrow cells were treated with PTH(1-34), PTH(1-31), or PTH(3-34) [all at 10 nM] either continuously or intermittently, or vehicle, for 4 days. **a**: Relative expression ratio of RANKL:OPG, as assessed by real-time RT-PCR, in the cultures treated with PTH fragments. **b**: Number of TRAP-positive multinucleated cells counted in 10 fields in each of three culture wells treated with PTH fragments. In each case, the PTH(1-34) and PTH(1-31) continuous treatment groups were significantly different from controls and PTH(3-34) group, $P < 0.001$. Continuous treatment with PTH(3-34) was not significantly different from controls. **c**: Number of TRAP-positive multinucleated cells counted in 10 fields in each of three culture wells treated with vehicle, PTH(1-34), forskolin, or PMA. Bars represent mean ± SEM. PTH(1-34) and forskolin treatment groups were significantly different from controls, $P < 0.001$. PMA group was not significantly different from controls. Bars represent mean ± SEM from three replicate samples.

cAMP Induction by PTH

At the end of two 48-h treatment cycles, no significant difference in cAMP levels was observed between controls and either of the two treatment groups. To assess whether prior PTH treatment affected the marrow cells' response to a fresh PTH challenge, we examined the levels of intracellular cAMP induced by a 10-min exposure to PTH, following two previous cycles of treatment, and compared this to previously unstimulated cells. When cultures, which had not been treated with PTH, were exposed to fresh PTH at 10 nM, intracellular cAMP levels increased approximately 3-fold compared to unstimulated cultures. The same response was observed in cultures previously treated with intermittent PTH. However, when cultures, which had previously been exposed to continuous PTH, were challenged with a fresh treatment, there was no significant induction of intracellular cAMP (Fig. 6a).

When the same experiment was conducted in the presence of IBMX, which inhibits the breakdown of intracellular cAMP, the response of cultures previously treated with continuous PTH was approximately 2-fold greater than unstimulated cultures, suggesting a low level of cAMP accumulation. However, in the intermittent group, cAMP levels were 10-fold higher than unstimulated controls. Control cultures exposed to PTH for the first time exhibited the greatest response, producing a 20-fold increase in cAMP compared to unstimulated cells (Fig. 6b).

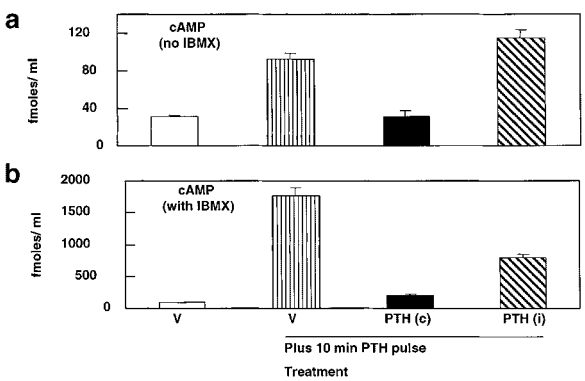

Fig. 6. Induction of cAMP by PTH in the absence (**a**) or presence (**b**) of IBMX (500 μM). Mouse marrow cells were treated with PTH (10 nM), either continuously or intermittently, or vehicle, for 4 days. They were then challenged with a fresh dose of PTH(1-34) [10 nM] (plus 10 min PTH pulse), or fresh medium without PTH (V), for 10 min. cAMP was measured using a commercial assay kit. Bars represent mean ± SEM from six replicate samples.

Effect of PTH Treatment on Growth Factor Expression

We found that IGF-I expression was consistently suppressed by continuous PTH treatment, and that intermittent treatment always resulted in significantly higher levels of IGF-I expression than continuous treatment (Fig. 7a). In addition, we observed significant reductions in PTH-induced AP and COL1A1 expression with intermittent PTH treatment when the experiment was conducted in the presence of anti-IGF-I antibody at 40 μg/ml (Mouse anti-Human IGF-I; Upstate Biotechnology Inc, Lake Placid) (Fig. 7b). Controls treated with anti-IGF-I antibody were not significantly different from untreated controls. A similar effect on AP expression was achieved by treating the cultures intermittently with rhIGF-I at 10ng/ml and neutralizing this effect with the same antibody (data not shown). Although both $TGF\beta_1$ and $TGF\beta_2$ were constitutively expressed in the marrow cultures, no consistent change in expression was observed with either continuous or intermittent PTH treatment (data not shown).

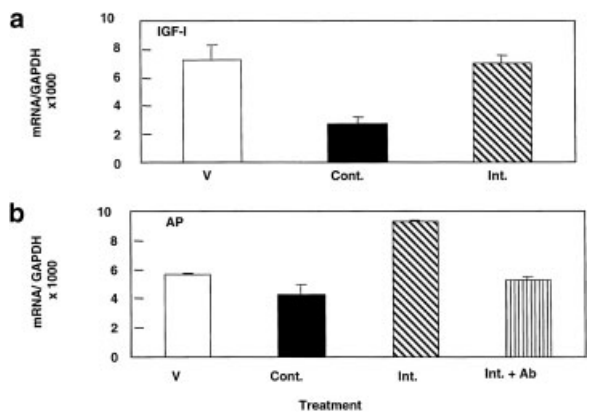

Fig. 7. **a**: Effect of continuous or intermittent PTH(1-34) on expression of IGF-I message from a representative experiment. Continuous treatment resulted in a decrease from control levels ($P = 0.02$). Intermittent treatment group was not significantly different from controls. Bars represent mean ± SEM from three replicates. **b**: Effect of a neutralizing antibody to IGF-I on expression of AP in cultures treated with PTH(1-34). The increase in expression of AP induced by intermittent treatment was effectively blocked by the addition of a neutralizing antibody to IGF-I. Controls treated with antibody were not significantly different to untreated controls. Mouse marrow cells were treated with PTH (10 nM), either continuously or intermittently, or vehicle, for 4 days. In (b), human anti-mouse IGF-I (cross-reacts with mouse IGF-I) was added at a concentration of 40 μg/ml. mRNA was extracted and analyzed by real-time RT-PCR; results were normalized to GAPDH values. Bars represent mean ± SEM from three replicate samples.

DISCUSSION

In our mouse marrow system, we demonstrated that intermittently administered PTH enhanced osteoblast differentiation while continuously administered PTH resulted in osteoclast formation. On the osteoblastic side, Runx2, the putative 'master gene' for osteoblast differentiation, as well as the characteristic osteoblast markers AP and procollagen I, increased after intermittent, but not continuous, PTH treatment. These observations are in agreement with published data [Canalis et al., 1989; Ishizuya et al., 1997], although increased expression of the early osteoblastic transcription factor Runx2 [Komori et al., 1997; Ducy et al., 1999; Gori et al., 1999] after intermittent PTH treatment has not been previously reported. Using the same model, we were able to compare responses to different treatment regimes at the same time points without the addition of other factors. Although the four days of treatment were sufficient for full differentiation of preosteoclasts in bone marrow to mature osteoclasts, it was not sufficient time to allow full differentiation to mature osteoblasts. Thus, there was a large increase in Runx2, but only smaller increases in AP and COL1A1. Assessment of mineralized nodule formation would require an even longer time course and the use of differentiating factors that might have affected the response.

Some studies have implicated IGF-I in mediating anabolic effects of intermittent PTH [Canalis et al., 1989; Ishizuya et al., 1997]. However, we did not observe increased IGF-I expression with intermittent PTH, but, rather, a decrease with continuous PTH. Nonetheless, the increase in AP and procollagen I expression after intermittent PTH treatment was mimicked by intermittent treatment with IGF-I, and was blocked by co-treatment with a neutralizing antibody to IGF-I. The increased IGF-I transcript and polypeptide levels reported by others may be due to PTH effects on transcript stability or the interaction of IGF-I with the IGF binding proteins (IGFBPs). Our data are in agreement with an in vivo study reported by [Watson et al., 1999], in which rats continuously treated with PTH(1-84) showed a decrease in IGF-I positive osteoblasts and a dose-dependent increase in expression of IGFBP-3, -4, and -5. The study of Ishizuya et al. [1997], suggests that intermittent PTH may exert effects on IGF-I levels independently of changes in mRNA expression. Thus, IGF-I may play a permissive, although necessary role, in allowing the effect of PTH on increasing osteoblast differentiation to be expressed.

PTH has been shown to induce expression of both TGFβ_1 and β_2 [Wu and Kumar, 2000], via the PKC and PKA pathways, respectively. TGFβ_1, and possibly also TGFβ_2, may be involved in regulation of bone resorption [Erlebacher and Derynck, 1996; Murakami et al., 1998; Takai et al., 1998; Fuller et al., 2000; Kaneda et al., 2000]. However, in our model system, we found no effect of PTH on TGFβ_1 or TGFβ_2. Thus, TGFβ mediation may occur at some other level, such as by inducing changes in activation of latent TGFβ; although the direct involvement of TGFβ cannot be completely ruled out without further studies.

Continuous PTH decreases expression of OPG and increases expression of RANKL, [Horwood et al., 1998; Lee and Lorenzo, 1999; Kanzawa et al., 2000; Halladay et al., 2002] the major determinants of osteoclast formation (see reviews: Suda et al., 1999; Hofbauer et al., 2000], in a dose-dependent fashion in vitro, and increases bone turnover and the RANKL/OPG ratio in parathyroidectomized rats in vivo [Ma et al., 2001]. In our system, continuous treatment stimulated RANKL expression, down-regulated OPG, and induced osteoclast formation. There was a reproducible correlation between a significant increase in the RANKL:OPG expression ratio over controls, and the generation of numerous large multinucleated TRAP-positive cells.

PTH acts on bone cells via the PTH1 receptor, which activates two pathways—signaling via cAMP-dependent PKA and phospholipase C-dependent PKC [Friedman et al., 1996; reviewed in Morris and Bilezikian, 1996; Swarthout et al., 2002], both of which are active in osteoblastic cells [Fujimori et al., 1992; Boguslawski et al., 2000]. The contribution of these two pathways can be assessed by using the PTH(1-34) fragment, which stimulates both pathways, the carboxy-truncated PTH(1-31), which stimulates only the PKA pathway and the amino-truncated PTH(3-34), which stimulates only PKC. Such studies have demonstrated that PKA may be the crucial pathway for bone formation [Rixon et al., 1994; Whitfield and Morley, 1995; Mohan et al., 2000]. PKA signaling has also been implicated in PTH

regulation of osteoclast formation [Greenfield et al., 1995], and OPG expression [Kanzawa et al., 2000; Halladay et al., 2002], but there is also evidence that the PKC pathway may be involved in osteoclastogenesis [Kaji et al., 1996] and regulation of both OPG and RANKL expression [Takami et al., 2000; Halladay et al., 2002]. Thus, the data are conflicting.

In our study, the effect of PTH(1-34) on osteoclast formation could be reproduced using PTH(1-31) or forskolin, which activate the PKA pathway, but not by PTH(3-34) or PMA, which activate the PKC pathway. The total lack of osteoclasts in cultures treated with the latter compounds strongly support the hypothesis that the cAMP/PKA pathway, but not the calcium/PKC pathway, is involved in stimulation of osteoclastogenesis via RANKL/OPG, at least in our model system. An induction in both RANKL and OPG in response to elevated intracellular calcium levels has been observed in primary osteoblasts but not in bone marrow cells, nor in two of four osteoblastic cell lines tested [Takami et al., 2000]. The reason for this inconsistency is unclear but may be related to differences in the source or differentiation stage of the cells, because cell density and differentiation stage have previously been shown to have a major influence on the response to PTH [Isogai et al., 1996]. However, the findings of Takami et al. showing that murine stromal cells do not support osteoclastogenesis through the PKC pathway are in agreement with our results.

In contrast to the effects of continuous PTH on osteoclast formation, induction of osteoblast markers by intermittent PTH appears to involve both the PKA and PKC pathways in our model system. Although neither PTH(1-31) nor PTH(3-34) induced as large an increase in expression of the osteoblast markers as PTH (1-34), both induced increased expression of Runx2, AP, and procollagen I. These results suggest the involvement of both signaling pathways in the induction of early osteoblast differentiation, and are consistent with the findings of Ishizuya et al. [1997] that both the PKA and PKC pathways may be required for stimulation of bone formation with intermittent PTH treatment.

Differences in cAMP induction by PTH following prior treatment suggest that the cells become desensitized with continuous exposure. Desensitization of bone-derived cells to PTH due both to a loss of specific binding caused by receptor down-regulation, and a reduction in PTH-induced cAMP accumulation, have previously been reported [Abou-Samra et al., 1989; Mitchell and Goltzman, 1990; Ikeda et al., 1991; Fukuyama et al., 1992], as has a rapid recovery of the cAMP response [Pun et al., 1990]. However, these studies did not address differences in the response in cultures previously treated with different PTH regimens. In the presence of IBMX, cAMP accumulates at low levels in cultures previously treated with continuous PTH, whereas levels in those previously treated with intermittent PTH were 5-fold higher. Thus, although cAMP is the sole mediator of PTH-induced osteoclast formation, only low levels seem to be required to induce an effect. In contrast, each pulse of intermittent PTH causes a much larger accumulation of cAMP that may act in concert with PKC, and possibly other signaling pathways, to induce osteoblast differentiation.

Gori et al. [2000] have demonstrated that expression of RANKL and support of osteoclast formation by osteoblastic cells is developmentally regulated, with differentiation rapidly switching the RANKL/OPG ratio away from support of osteoclastogenesis. This raises the possibility that differences in the effects of PTH treatment are due to an increase in osteoclast formation with continuous treatment while intermittent treatment permits osteoblast differentiation in the absence of large increases in bone resorption. This is consistent with the study of Zhou et al. [2001] who found in ovariectomized rats that continuously administered PTH increased bone formation rate but failed to increase trabecular bone volume unless estrogen was administered concurrently to reduce bone resorption. Thus, the major difference between the skeletal effects of intermittently and continuously administered PTH may lie not with major differences in stimulation of bone formation but with the disproportionately large increase in bone resorption in the latter but not in the former. This hypothesis is deserving of further study.

In summary, we have shown for the first time that PTH is able to induce increases in bone formation or resorption indices in the same model system in vitro, depending on its mode of administration. Whereas the effect on increased osteoblast differentiation appears to involve both PKA and PKC pathways and may be mediated by, or be partially dependent on,

the effect of IGF-I, the induction of osteoclast formation may involve only the PKA pathway and be mainly due to large increases in the expression ratio of RANKL:OPG in marrow stromal cells. The intermediate steps between stimulation of the PKA or PKC signaling pathway by the PTH1 receptor, and changes in gene transcription under different conditions, remain unclear. However, Halladay et al. [2002] have identified the promoter sequences involved in the inhibition of OPG expression by PTH. Further studies are clearly necessary to define the complete molecular mechanisms for the biphasic effects of PTH on bone cells.

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