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ORIGINAL ARTICLE

# Intermittent PTH(1–34) signals through protein kinase A to regulate osteoprotegerin production in human periodontal ligament cells in vitro

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**Abstract** Periodontal ligament (PDL) cells have been associated with the regulation of periodontal repair processes by the differential expression of osteoprotegerin and RANKL in response to intermittent parathyroid hormone (PTH) resulting in a modified activity of bone-resorbing osteoclasts. Here, we examined the intracellular signaling pathways that PDL cells use to mediate the PTH(1–34) effect on osteoprotegerin production and hypothesized that those would be dependent on the cellular maturation stage. Two stages of confluence served as a model for cellular maturation of 5th passage human PDL cells from six donors. Intermittent PTH(1–34) ($10^{-12}$ M) and PTH(1–31), the latter lacking the protein kinase C (PKC) activating domain, induced a significant decrease of osteoprotegerin production in confluent cultures, whereas the signal-specific fragments PTH(3–34) and PTH(7–34), which both are unable to activate protein kinase A (PKA), had no effect. The addition of the PKA inhibitor H8 antagonized the PTH(1–34) effect, whereas the PKC inhibitor RO-32-0432 did not. In pre-confluent, less mature cultures, intermittent PTH(1–34) resulted in a significant increase of osteoprotegerin. Similar results were obtained when PTH (1–31) substituted for PTH(1–34) as opposed to a lack of an effect of PTH(3–34) and PTH(7–34). Likewise, in confluent cultures, H8 inhibited the PTH(1–34) effect in pre-confluent cultures contrasted by RO-32-0432 which had no effect. These findings indicate that PTH(1–34) signaling targeting osteoprotegerin production in PDL cells involves a PKA-dependent pathway. The PTH(1–34) effect is dependent on cell status, whereas intracellular signal transduction is not. Clinical trials will have to prove whether those in vitro data are of physiological relevance for interference strategies.

**Keywords** Human PDL cells · Parathyroid hormone · Osteoprotegerin · PKA · PKC · In vitro

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## Introduction

Periodontitis is an inflammatory disease characterized by an immune response of the tooth-surrounding tissues to microorganisms leading to an inflammation and subsequent destruction of the periodontium and alveolar bone [1, 2]. Promising strategies for treatment of bone loss following periodontal diseases can be divided into two approaches. The aim of the first one is to improve hard tissue regeneration by enhancing biomineralization [3–5], and the second approach is characterized by a prevention or at least a reduction of hard tissue resorption caused by activation of osteoclastogenesis [6–8]. Recently, it has been shown that the RANK/RANKL/OPG system (receptor

activator of nuclear factor kappaB, RANK; RANK-ligand, RANKL; osteoprotegerin, OPG) plays a crucial role in alveolar bone loss caused by periodontal inflammation [9–12]. Upregulation of RANKL and/or decreased expression of OPG was found to be associated with alveolar bone loss in different fields of periodontal research [13]. Bone resorption is mediated by multinucleated osteoclasts which originate from granulocyte- and macrophage-colony-forming unit hematopoietic stem cells [14]. It has been demonstrated that pre-osteoclasts and other cells of this lineage express RANK [15, 16]. The interaction of RANK with its physiological ligand RANKL, which is located on and secreted by osteoblasts, stimulates the formation and activity of osteoclasts [17–21] by an activation of different transcription factors that regulate osteoclastogenesis [22, 23]. OPG, which is as well secreted by osteoblasts, acts as a decoy receptor that binds and sequesters RANKL and, thereby, limits its biological availability and eventually results in an inhibition of bone resorption [24, 25]. Recently, periodontal ligament (PDL) cells were shown to express osteotropic cytokines [26] including OPG and RANKL [27–29]. The osteoblastic phenotype of PDL cells, as evidenced by an expression of several osteogenic markers such as alkaline phosphatase, osteocalcin, bone morphogenetic proteins, or the parathyroid hormone (PTH) receptor, suggests a role for PDL cells in the regulation of periodontal tissue regeneration [30, 31]. Recently, we have demonstrated that PDL cells respond to an intermittent PTH (1–34) administration, which has been established clinically as an anabolic treatment regimen for the regenerative therapy of bony tissues [32], with an altered expression of OPG and RANKL which proved physiologically relevant for the regulation of the formation and activity of osteoclasts [33]. In these experiments, the response to intermittent PTH(1–34) correlated with the cellular maturation state of the PDL cells. Pre-confluent PDL cells, as a model for less mature cells, exhibited an increased OPG/RANKL ratio in response to PTH(1–34), and, in co-culture with RAW 264.7 cells, an inhibition of osteoclastogenesis was observed. In contrast, in confluent, more mature PDL cells, intermittent PTH(1–34) induced a reduction of the OPG/RANKL ratio leading to an increased osteoclast activity in the co-culture model.

Several studies have demonstrated that the intracellular signal transduction of PTH(1–34) involves both protein kinase A (PKA) and protein kinase C (PKC) [34]. Binding of PTH(1–34) to the G-protein-coupled PTH1-receptor leads to a stimulation of adenylyl cyclase and phospholipase C (PLC). Adenylyl cyclase increases the level of cAMP which in turn activates PKA signaling. Stimulation of PLC leads to an accumulation of inositol trisphosphate and diacylglycerol which increase the intracellular calcium concentration and activate PKC [35, 36]. Activation of both signaling transduction cascades finally results in an upregulated expression of several PTH target genes including OPG [37].

The purpose of our study was to elucidate the intracellular signaling pathways which human PDL cells use to mediate the PTH(1–34) effect on OPG production with special attention directed to the role of PKA and PKC. We hypothesized that the cellular response to PTH(1–34) would be distinct in terms of cell status and the mechanism. To address these hypotheses, signal-specific PTH fragments as well as specific inhibitors to the respective protein kinases were employed in PDL cell culture experiments using cells at different stages of confluence as a model for cellular maturation.

## Materials and methods

### PDL cell culture and characterization

Human PDL cells were explanted from premolars of six different human donors, aged between 12 and 14 years, who showed no clinical signs of periodontitis. The teeth had been extracted for orthodontic reasons, with informed parental consent and following an approved protocol of the ethics committee of the University of Bonn (reference number 029/08). To assess whether PTH acts differently at different stages of cellular developmental age, cultures at distinct states of confluence were used as a model for cell maturation.

Fifth passage cells were plated in 24-well plates ($n$=6) such that, at harvest, they either had reached a pre-confluent (~70%) or confluent state which was verified by light microscopical analysis. The seeding density was 3,000 cells/well for pre-confluent cultures and 10,000 cells/well for confluent cultures. Cells were cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5,000 U/ml penicillin and 5,000 U/ml streptomycin; Biochrom AG, Germany) and cultured at 37°C in an atmosphere of 100% humidity, 95% air, and 5% $CO_2$.

### PTH administration

Cells at both stages of maturation were cultured in the presence of $10^{-12}$ M PTH(1–34) (Sigma–Aldrich, Germany) for 1 and 24 h within a 48-h incubation cycle. For the remaining time, experimental media were replaced by tissue culture media without PTH(1–34). These cycles were carried out three times resulting in a total experimental period of 6 days to mimic the anabolic effects of intermittent PTH. Vehicle (ethanol)-treated cultures for each treatment group

served as controls. The exposure protocol to mimic an intermittent treatment protocol was adopted from the literature [38], and likewise, the PTH concentration was established to be effective in PDL cell cultures in a previous work [39].

### Cell number

At harvest, cells were released from the culture surface by trypsinisation for 10 min at 37°C. This reaction was terminated by the addition of DMEM containing 10% FBS. Thereafter, the cell suspension was centrifuged, and the cell pellet was resuspended in 0.9% NaCl. Finally, the cell number was determined by the use of a cell counter (Moelab, Hilgen, Germany). Cells harvested in this manner exhibited >95% viability based on Trypan blue exclusion.

### Osteoprotegerin production

Osteoprotegerin levels in the conditioned media were assayed by a commercially available ELISA kit according to the manufacturer's instructions (Immundiagnostik AG, Bensheim, Germany). The data were assessed as a function of either cell number or protein content to exclude the possibility that changes in osteoprotegerin production simply result from increased cell numbers due to the culture period and not from an altered production by the individual cell.

### Intracellular signal transduction pathways

The pathways involved in the mediation of the PTH effect in PDL cells were investigated by two different methods. In a first step, signal-specific PTH fragments that were lacking either the PKC activating domain ($10^{-12}$ M PTH(1–31)) or the PKA activating domain ($10^{-12}$ M PTH(3–34)) or additionally the $Ca^{2+}$ activating domain ($10^{-12}$ M PTH(7–34)) were employed as substitutes for the PTH(1–34) fragment with the full biological activity during the intermittent exposure protocol.

In a parallel approach, either the PKC inhibitor RO-32-0432 (1 μM) or the PKA inhibitor H8 (10 μM) (Calbiochem, Germany) was added to the cultures 1 h before treatment with intermittent PTH(1–34) or vehicle and remained in the medium for the entire experimental period to examine whether those inhibitors would antagonize the PTH(1–34) effect on the differentiation parameters. The inhibitor concentrations used were confirmed to be effective in PDL cells in previous experiments [40]. Vehicle-treated cultures for each treatment group and cells cultured in the presence of the respective inhibitors but without PTH(1–34) served as controls. At harvest, osteoprotegerin production was determined as described above.

### Statistical analysis

From all data obtained, the osteoprotegerin production at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Each data point represents the mean ± SEM of six independent cultures. Data were analyzed by analysis of variance, and statistical significance was determined using Bonferroni's modification of Student's $t$ test for multiple comparisons. $P$ values <0.05 were considered to be significant. The data are representative of two replicate experiments which both yielded similar results. Only one set of results from the two sets of experiments are presented.

## Results

Vehicle-treated cultures for each experimental group at a particular maturation state did not differ from each other and from untreated controls significantly, and therefore, only one vehicle-treated control for each maturation state is presented in each figure. At the beginning of the PTH(1–34) exposure, in pre-confluent PDL cells, the cell number was $0.03 \pm 0.006 \times 10^5$ cells/well; in confluent cells, there were $0.13 \pm 0.017 \times 10^5$ cells/well.

### Confluent cultures

In confluent, more mature PDL cell cultures, intermittent PTH(1–34) induced a statistically significant inhibition of the osteoprotegerin production (Fig. 1). When the effects of signal-specific fragments were compared, both PTH(1–34) and PTH(1–31) induced a similar reduction of osteoprotegerin protein levels, whereas both PTH(3–34) and PTH(7–34) failed to do so (Fig. 2). The addition of the specific inhibitor to the protein kinase A pathway, H8, inhibited the PTH(1–34)-induced reduction of the osteoprotegerin production and restored control levels of the cytokine as opposed to the PKC inhibitor RO-32-0432 which had no effect (Fig. 3). The effects for intermittent PTH-challenge were similar for treatment variations of 1 h/cycle or 24 h/cycle.

### Pre-confluent cultures

In less mature cultures, an intermittent administration of $10^{-12}$ M PTH(1–34) resulted in a significant increase of the osteoprotegerin production (Fig. 1). The use of the different PTH fragments revealed similar responses when PTH(1–31) served as a substitute for PTH(1–34). In

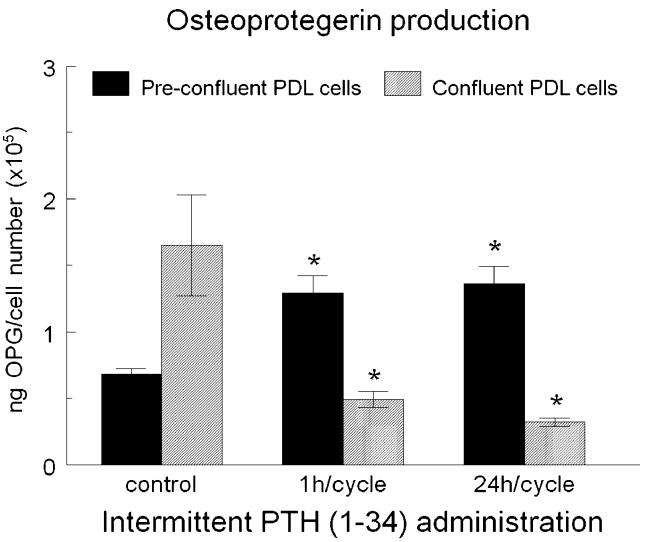


**Fig. 1** Regulation of the osteoprotegerin production by intermittent $10^{-12}$ M PTH(1–34) in fifth passage pre-confluent and confluent human PDL cells. The cells were treated intermittently with $10^{-12}$ M PTH(1–34) for 1 or 24 h during three cycles of 48 h each. Vehicle-treated cultures served as controls. The osteoprotegerin content in the conditioned medium was assayed by ELISA and expressed as a function of the cell number. From all data obtained, the osteoprotegerin level at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. *$P$<0.05, experimental group vs. vehicle control at a particular maturation state

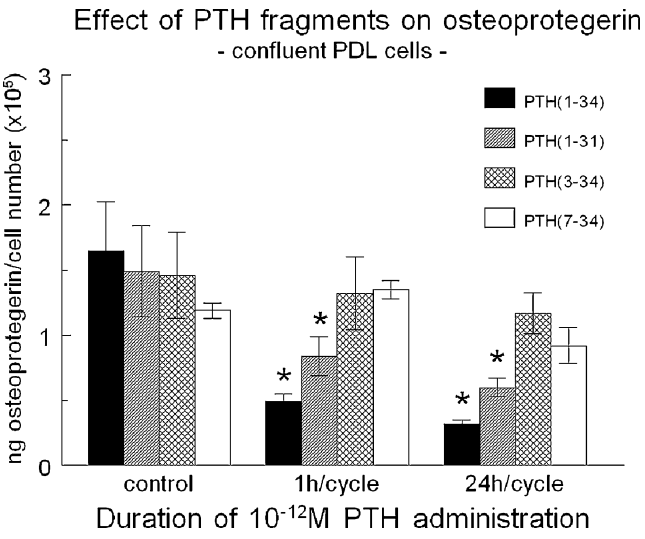


**Fig. 2** Effect of signal-specific PTH fragments on osteoprotegerin production in confluent PDL cells. Fifth passage cells were either exposed intermittently to the fragment with the full biological activity, PTH(1–34), or to pathway-specific fragments which either lack the PKC activating domain (PTH(1–31)) or the PKA-activating terminus (PTH(3–34) and PTH(7–34)). All fragments were used at a concentration of $10^{-12}$ M. The osteoprotegerin content of the conditioned medium was assayed by ELISA and expressed as a function of the cell number. From all data obtained, the osteoprotegerin level at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. *$P$<0.05, experimental group vs. vehicle-treated control

contrast, PTH(3–34) and PTH(7–34) did not alter osteoprotegerin levels significantly (Fig. 4). In the presence of the PKC inhibitor RO-32-0432, enhanced osteoprotegerin production was observed that was comparable to that seen for intermittent PTH(1–34) alone, whereas, when H8 was added to the cultures, the PTH(1–34) effect was inhibited (Fig. 5). The effects for intermittent PTH-challenge were similar for treatment variations of 1 h/cycle or 24 h/cycle.

## Discussion

The results of the present study showed a basal osteoprotegerin expression in human PDL cells and the cellular sensitivity to an intermittent PTH(1–34) administration regarding this parameter. These findings are in line with previous reports [26, 29, 33, 41], further corroborate the osteoblast-like character of PDL cells, and substantiate their regulatory role in periodontal tissue homeostasis under physiological and pathological conditions. The latter has been derived from studies demonstrating the ability of PDL cells to differentiate into a more mature osteoblastic phenotype after hormonal stimulation [33] and to mineralize the extracellular matrix emphasizing their pivotal role in periodontal regeneration [42, 43]. Previously, a promising role for PTH(1–34) in the attempt to prevent hard tissue loss in the course of inflammatory periodontal disease was suggested from experiments in rodents [44–46]. Thus, the intermittent PTH(1–34) effect on osteoprotegerin protein expression provides another piece in the mosaic to explain possible anabolic effects of the hormone on periodontal hard tissues aiming at tissue preservation or regeneration. The increase of osteoprotegerin protein expression we observed in response to intermittent PTH(1–34) in pre-confluent cultures implies an enhanced osteoblastic differentiation of less mature PDL cells which is desirable during the early phase of periodontal regeneration to increase the pool of competent cells for regenerative processes to occur. These benefits are offset by several reports in the literature on an inhibitory effect of PTH on this cytokine [47–49]. However, those experiments followed continuous exposure protocols of the hormone and were conducted in various osteoblastic cell lines and different experimental setups which do not allow for a direct comparison with our data. Nevertheless, in conflu-

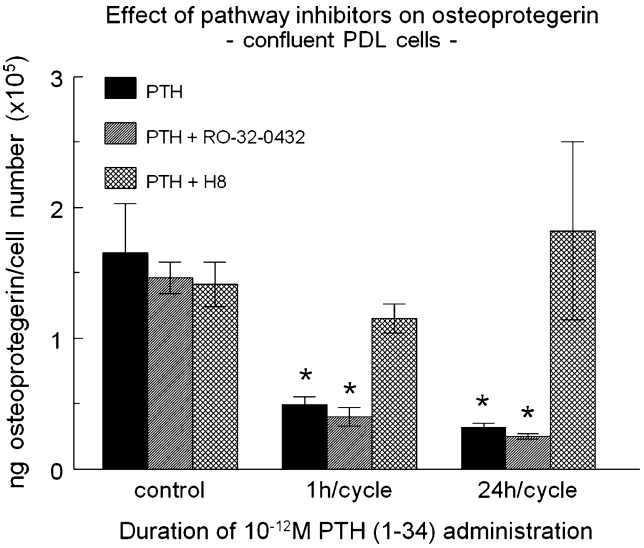


**Fig. 3** Effect of the PKC inhibitor RO-32-0432 and of the PKA inhibitor H8 on the PTH(1–34)-induced regulation of osteoprotegerin in confluent 5th passage PDL cell cultures. Confluent cultures were treated intermittently with $10^{-12}$ M PTH(1–34) for 1 or 24 h during three cycles of 48 h each in the presence of 1 μM RO-32-0432 or 10 μM H8. The inhibitors were added to the cultures 1 h prior to PTH (1–34) administration, and the experimental media were supplemented with the inhibitors for the entire experimental period. Vehicle-treated cultures and cultures challenged with the inhibitor alone served as controls (*control*). From all data obtained, the osteoprotegerin level at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. *$P<0.05$, experimental group vs. respective control

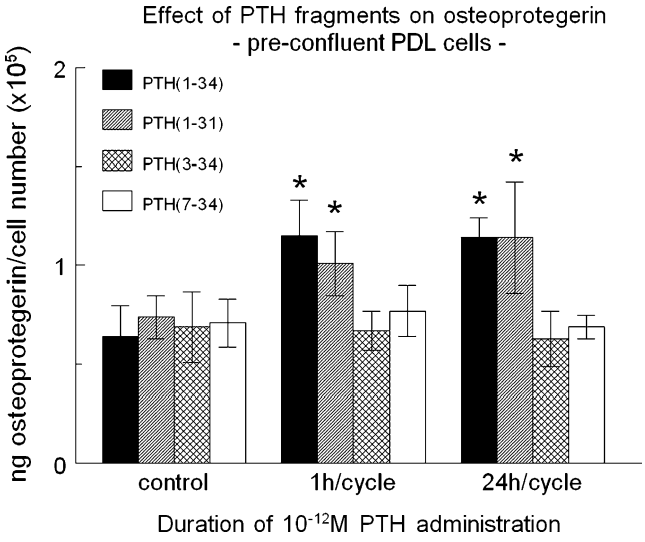


**Fig. 4** Role for PKC in mediating the effect of intermittent PTH(1–34) on the osteoprotegerin production by pre-confluent PDL cells. Fifth passage cells were either exposed intermittently to the fragment with the full biological activity, PTH(1–34), or to pathway-specific fragments which either lack the PKC activating domain (PTH(1–31)) or the PKA-activating terminus (PTH(3–34) and PTH(7–34)). All fragments were used at a concentration of $10^{-12}$ M. The osteoprotegerin content of the conditioned medium was assayed by ELISA and expressed as a function of the cell number. From all data obtained, the osteoprotegerin level at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. *$P<0.05$, experimental group vs. vehicle-treated control

ent PDL cells, a similar inhibition of osteoprotegerin secretion was observed in the present study which is not necessarily incompatible with the supportive role of PTH in hard tissue formation. The anabolic effect of PTH on bone formation may result from an influence on secondary remodeling activities of the bone including increased bone resorption to facilitate subsequent deposition of new matrix [48].

The distinct response of PDL cells at different stages of confluence points to the crucial role of the cell status for the cellular response [50, 51] and strengthens the need for a refined view when comparing results that were obtained under specific experimental conditions. Despite the drawbacks of any cell culture study which cannot portray the complexity of a living tissue, our data that were collected at different stages of confluence allow for a closer relation of the findings and conclusions to an in vivo microenvironment where PDL cells at different developmental ages coexist, ranging from mesenchymal stem cells over precursors of certain phenotypes to committed fibroblasts, osteoblasts, cementoblasts, etc. The validity of the model for cell maturation we used in the present study was established in previous research and is based upon our pre-experimental cell characterization by microarray and real-time PCR. It revealed that the degree of confluence of PDL cells correlates well with the mRNA expression of markers typical of cells of mesenchymal origin (alkaline phosphatase, osteocalcin, PTH-receptor, BMP-2, BMP-4, BMPR-1a, BMPR-1b, BMPR-2, integrinA6, B4, TGF-ß1, and cyclin D1) at different stages of maturation [33]. In another recent study, we demonstrated the differential expression of alkaline phosphatase, and osteocalcin mRNA was associated with significant differences of the respective protein levels as well. Thus, it seems justified to consider pre-confluent cells to be less mature and confluent cells to be more mature.

Another methodological consideration addresses the question on how far culturing the cells under serum-reduced or serum-free conditions rather than in the presence of serum as done in our experiments might have affected the cellular response to PTH. Based upon recent findings in osteoblasts showing that intermittent PTH indeed alters the expression of cell cycle proteins including cyclin D1 [52, 53], we did not want to disregard

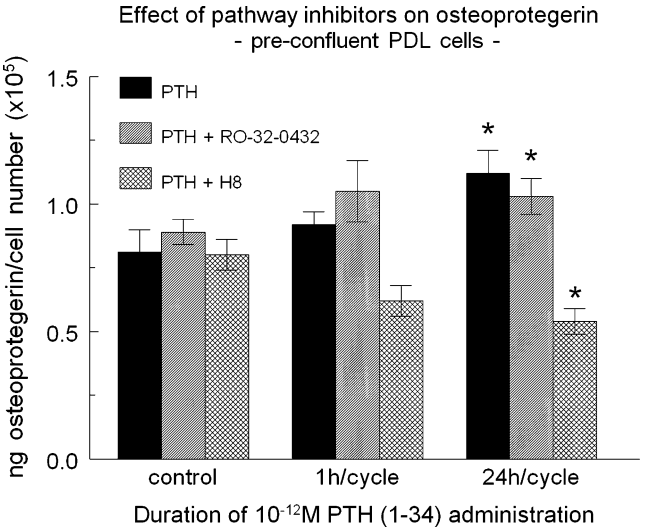


**Fig. 5** Influence of specific inhibitors to the protein kinase A and C dependent pathways on the PTH(1–34)-induced stimulation of osteoprotegerin production in pre-confluent PDL cells. Pre-confluent cultures were treated intermittently with $10^{-12}$ M PTH(1–34) for 1 or 24 h during three cycles of 48 h each in the presence of 1 μM RO-32-0432 or 10 μM H8. The inhibitors were added to the cultures 1 h prior to PTH(1–34) administration, and the experimental media were supplemented with the inhibitors for the entire experimental period. Vehicle-treated cultures and cultures challenged with the inhibitor alone served as controls (*control*). From all data obtained, the osteoprotegerin level at the onset of PTH administration ($T_0$) was subtracted serving as a baseline correction. Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. *$P<0.05$, experimental group vs. vehicle-treated control

this aspect of the PTH effect and, therefore, made an informed choice in favor of serum-containing experimental media. Serum-free conditions would have resulted in an exit of the cells from the cell cycle and would have unified the PDL cell population regarding this parameter, but at the same time, made the interpretation of the data regarding the physiological relevance in an in vivo situation more difficult.

There is considerable congruity amongst our experimental efforts to identify the intracellular pathways involved in the mediation on the PTH(1–34) effect on osteoprotegerin production. In doing so, signal-specific PTH fragments and specific inhibitors to the protein kinases A and C were employed without measuring kinase activity directly. This approach is well established [40, 49, 54, 55] and based on the confirmation that PTH (1–31) activates only PKA with no demonstrable effects on PKC or phospholipase C [56, 57] as opposed to the analogs PTH(3–34) and PTH(7–34) which both activate PKC, but not adenylyl cyclase [58–60]. The lack of an effect of the N-terminally truncated fragments PTH(3–34) and PTH(7–34) together with the ability of the PKA inhibitor H8 to inhibit the PTH(1–34)-induced changes in target protein expression leads to the conclusion that the cAMP/PKA-dependent pathway, but not PKC, is of predominant importance in PTH signal transduction with respect to osteoprotegerin expression. The cellular response to the signal-specific fragment truncated at the PKC-activating terminus, PTH(1–31), which was comparable to PTH(1–34), and the inability of the PKC inhibitor RO-32-0432 to antagonize the action of the stimulus further substantiate this interpretation. These data reinforce previous observations in other cell culture systems that PTH can signal through both PKA and PKC with a predominance of the cAMP/PKA-dependent pathway [55, 61–63]. Conflicting data exist regarding the role of PKA as an activator or inhibitor of PTH-induced target gene regulation [64, 65]. As for osteoprotegerin regulation, Kanzawa et al. showed an involvement of the PKA-dependent pathway in the PTH-induced downregulation of osteoprotegerin mRNA expression in the mouse stromal cell line ST2 [66] resulting in enhanced osteoclastogenesis. In support of those findings, studies in murine bone marrow cultures suggested the inhibition of OPG by PTH to be a selective PKA response as well [47]. Similar to the data on PTH regulation of osteoprotegerin, most of these studies applied a continuous treatment regimen, and consequently, the findings cannot be transferred to our experimental setup using a pulsatile PTH administration. Once again, the need for a cautious analysis of results that were obtained under different experimental conditions becomes obvious.

In the present study, the intracellular signal transduction was independent of the cell status with PKA being predominant in both pre-confluent and confluent cultures. In the light of published material from our group demonstrating a cAMP/PKA-dependent pathway in less mature PDL cells as opposed to a PKC-dependent signal transduction in more mature cultures being responsible for the mediation of the PTH(1–34) effect on the osteoblastic differentiation parameters alkaline phosphatase activity and osteocalcin production [67], this is an interesting finding that highlights the differential activation of specific pathways by PTH(1–34) depending on the target parameter influenced.

In summary, the regulation of osteoprotegerin by PTH (1–34), together with the demonstration of the involvement of the cAMP/PKA-dependent pathway in the intracellular signal transduction in human PDL cells, provides a mechanistic explanation for the mediation of the PTH(1–34) effect on periodontal tissue remodeling. Clinical studies will have to prove whether those in vitro data which widen the basic understanding of cell biological regulatory mechanisms are of physiological relevance for interference strategies.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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