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Intermittent administration of PTH(1–34) regulates the osteoblastic differentiation of human periodontal ligament cells via protein kinase C- and protein kinase A-dependent pathways *in vitro*

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Background and Objective: Intermittent parathyroid hormone (PTH) is recognized as an anabolic agent in regenerative treatment strategies for bony tissues. Periodontal ligament (PDL) cells share features that are typical of osteoblasts, including an osteoblast-like response to stimulation with PTH, which implies a role for these cells in the regulation of repair processes following inflammatory periodontal disease. In the present study we explored the effect of intermittent administration of a PTH fragment [PTH(1–34)] on the osteoblastic differentiation of human PDL cells *in vitro*, and we investigated the signaling pathways used by the cells to mediate this effect.

Material and Methods: PDL cells at two stages of confluence were characterized and used as a model for the role of cell maturation in the cellular response.

Results: In preconfluent, less mature cultures, intermittent administration of PTH(1–34) and PTH(1–31) fragments increased alkaline phosphatase (ALP) activity and osteocalcin production, whereas intermittent administration of PTH(3–34) and PTH(7–34) had no effect. RO-32-0432, a specific protein kinase C inhibitor, did not inhibit the PTH(1–34) effect, whereas the protein kinase A inhibitor, H8, antagonized the PTH(1–34)-induced increase in ALP activity and osteocalcin. In contrast, in confluent, more mature cultures, intermittent administration of PTH(1–34), PTH(3–34) and PTH(7–34) fragments, but not of the PTH(1–31) fragment, decreased ALP activity, and osteocalcin and RO-32-0432, but not H8, inhibited the effect.

Conclusions: This study showed that the PTH(1–34) effect on ALP activity and osteocalcin production in human PDL cells is maturation state-dependent and specific in terms of the pathways involved. Whereas in less mature cells the PTH effect is associated with cyclic AMP/protein kinase A-dependent signaling, more mature cells seem to mediate the PTH signal primarily via protein kinase C-dependent pathways.

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Contemporary anabolic treatment approaches for bony tissues focus on the anabolic properties of parathyroid hormone (PTH). A once-daily administration of the hormone was demonstrated to be anabolic and to enhance bone mineral density and bone mass in osteoporotic women (1) by stimulating osteoblastic activity and prolonging cell survival as a result of inhibited apoptosis (2). Studies using osteoblasts support the concept that multiple mechanisms are involved in the cellular response to PTH, as evidenced by an involvement of both cyclic AMP (cAMP) and protein kinase C (PKC) in mediating signal transduction (3). The intracellular signaling pathways that the cells use to mediate the PTH effect after binding of PTH to the G-protein-coupled PTH1 receptor include the stimulation of adenylyl cyclase and phospholipase C, resulting in an increase in cAMP levels and an activation of protein kinase A (PKA), or activation of the phospholipase C signaling cascade, which leads to an accumulation of inositol trisphosphate and diacylglycerol, which increase the intracellular calcium concentration and activate PKC (4,5). The activation of either pathway finally results in the phosphorylation of transcription factors, such as Cbfa-1 and cAMP response element-binding proteins (6), which control the transcription of PTH target genes, including alkaline phosphatase (ALP) and osteocalcin (6–10). The selective activation of PKA- and/or PKC-dependent pathways, with the subsequent stimulation of various transcription factors, establishes the diversity of actions by which PTH may regulate different genes in a tissue-specific manner (5).

Although the regulatory function of PTH within the skeletal system has been well established, little is known about the influence of PTH on the metabolic functions of other mineralized tissues, such as dental tissues. Although there are several clinical procedures that may be employed successfully to, at least in part, re-establish the periodontal architecture after breakdown of inflammatory tissue, the underlying cellular regulatory mechanisms that mediate the periodontal repair processes remain to be elucidated. In the light of an increasing number of patients, even within the comparatively younger age group of 30–40 years, who suffer from inflammatory periodontal disease, it is desirable and elementary to widen the basic knowledge of the periodontal ligament (PDL) cell characteristics and of the PTH effect on the formation and maintenance of those dental tissues.

The PDL connects the tooth root to the alveolar bone, consists of elastic and collagenous fibers, and hosts a mixed population of PDL cells, which comprise fibroblastic subpopulations, osteoblasts, cementoblasts, endothelial cells, perivascular cells and epithelial cells. In addition, the PDL contains progenitor cells that can differentiate into the above-mentioned specialized cell types (11). Amongst those different cell types, fibroblasts resemble the major cell population within the PDL. It remains to be elucidated whether those cells represent a terminally differentiated phenotype or an intermediate state of maturation that can be further directed towards an osteoblastic phenotype. This unresolved issue holds out the prospect of modifying the phenotype and consequently the reparative capacities of those cells. Accumulating evidence points to a role for PDL cells in the regulation of periodontal repair processes (12) based on the phenotypic characteristics that a certain percentage share with osteoblasts (13,14) and based on the response of this subpopulation to intermittent stimulation with PTH fragment 1–34 [PTH(1–34)] in an osteoblast-like manner (15–19). In support of this, we previously demonstrated PTH(1–34)-induced changes in PDL cell proliferation and survival, as well as in the production of key regulatory molecules of bone remodeling (17–19).

The purpose of this study was to provide further insight into the PTH(1–34)-stimulated regulation of the osteoblastic differentiation of human PDL cells. As both ALP and osteocalcin, as marker genes for early (ALP) and late [osteocalcin; (20)] osteoblastic developmental stages, are expressed by PDL cells (21), the present investigation focused on determining the molecular mechanisms underlying ALP and osteocalcin regulation by PTH(1–34) in PDL cells. We hypothesized the following: (i) that intermittent exposure of human PDL cells to PTH would affect the cellular ALP-specific activity and osteocalcin production; (ii) that the intracellular signal transduction would involve PKA- and PKC-dependent pathways; and (iii) that the cellular response would be distinct in terms of the cell status.

Material and methods

PDL cell culture and characterization

Human PDL cells were scraped from the middle third of the tooth root (to avoid contamination with gingival or apical tissue) from the premolars of six different human donors, 12–14 years of age, 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 cell development, cultures at distinct states of confluence were used as a model for cell maturation. This model was based upon our pre-experimental cell characterization by microarray (SABiosciences, Frederick, MD, USA) and real-time PCR analyses, as described below, which revealed that the degree of confluence of PDL cells correlates well with the expression of markers typical of cells of mesenchymal origin [e.g. ALP, osteocalcin, PTH receptor, bone morphogenetic proteins 2 and 4, bone morphogenetic protein receptors 1a, 1b and 2, integrins A6 and B4, transforming growth factor-β1 and cyclin D1) at different stages of maturation (Fig. 1). Therefore, it seems justified to consider preconfluent cells to be less mature and confluent cells to be more mature.

Fifth-passage cells were plated in 24-well plates ($n = 6$) such that, at harvest, they had reached either a preconfluent (approximately 70%) or a confluent state. Cells were cultured in

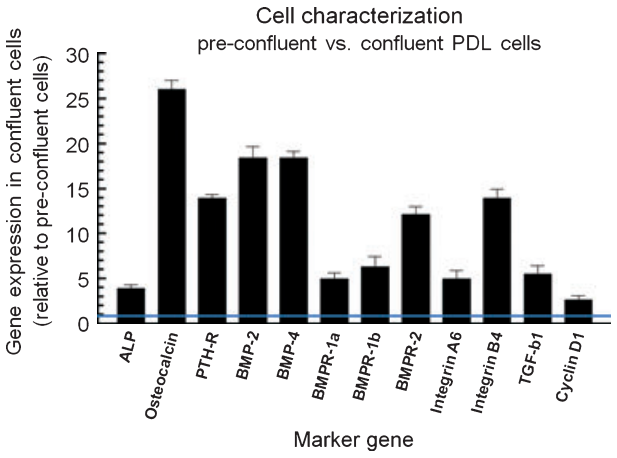

Fig. 1. Characterization of preconfluent vs. confluent periodontal ligament (PDL) cell cultures. Fifth-passage human PDL cells from six donors were cultured to either 70% confluence (preconfluent cells) or 100% confluence and were then characterized for the expression of mesenchymal marker genes [alkaline phosphatase (ALP), osteocalcin, parathyroid hormone receptor (PTH-R), bone morphogenetic protein (BMP)-2 and -4, bone morphogenetic protein receptor (BMPR)-1a, -1b and -2, integrin A6, integrin B4, transforming growth factor-β1 (TGF-β1) and cyclin D1] by the use of a microarray. Striking differences in osteoblastic gene expression at both stages of confluence were further verified and quantified by real-time PCR. For comparison, the expression level of the investigated genes in preconfluent cells was set to 1 and served as a reference for the expression at the confluent state. Each value represents the mean ± standard error of the mean for six independent cultures.

Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/mL of penicillin and 5000 U/mL of streptomycin; Biochrom AG, Berlin, Germany) and cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2.

Cell characterization

For cell characterization, total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Afterwards, 1 μg of RNA was reverse transcribed with 200 ng of the respective antisense primers in a 15 μL volume using the Amersham-Pharmacia-Biotech RT kit (Amersham Biosciences, Freiburg, Germany). The primers used to amplify human complementary DNA (cDNA) were purchased from Invitrogen (Karlsruhe, Germany). For the microarray procedure, cDNA templates were mixed with a ready-to-use master mix provided with the kit and aliquoted into each well of the plates containing predispensed gene-specific primer sets. Striking differences in gene expression at both stages of confluence were further confirmed and quantified by real-time PCR on a light-cycler (Roche, Mannheim, Germany) using the LightCycler Software version 3.5.3. PCR amplifications were carried out in a total volume of 20 μL in PCR master mix containing 10 μL of SYBR® Green, 2 μL of 10 × QuantiTect primer assay and 2 μL of the reverse transcription product made up to 20 μL with RNase-free H_2O. The amplifications were performed in duplicate for each sample, and the optimal annealing temperature for all primers was 55°C for 40 cycles. To normalize the content of cDNA samples, the comparative threshold (Ct) cycle method, consisting of the normalization of the number of target gene copies vs. an endogenous reference gene, such as glyceraldehyde-3-phosphate dehydrogenase, was used. For comparative analysis of gene expression, data were obtained using the $\Delta\Delta$Ct method.

PTH administration

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

Cell number

At harvest, cells were released from the culture surface by trypsinization for 10 min at 37°C. This reaction was terminated by the addition of DMEM containing 10% fetal bovine serum. Thereafter, the cell suspension was centrifuged and the cell pellet 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.

ALP specific activity

The ALP specific activity was measured at the protein level in lysates of isolated cells as a function of release of paranitrophenol from paranitrophenylphosphate at pH 10.2, as described previously (22). ALP activity was expressed as a function of cell number or protein content to exclude that changes in ALP activity simply result from changes of the proliferation or apoptosis, rather than from a direct PTH(1–34) effect on ALP.

Osteocalcin production

Osteocalcin levels in the conditioned media were assayed using a commercially available ELISA kit, according to the manufacturer's instructions (Immundiagnostik AG, Bensheim, Germany).

Dissection of the signal transduction pathways

Two different approaches were taken to analyze the pathways involved in the transduction of the PTH signal in PDL

cells. In a first step, different 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, which has 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, Darmstadt, Germany) were 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 had been confirmed to be effective in PDL cells in previous experiments (19). 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, ALP activity and osteocalcin production were determined as described above.

PTH(1–34) effect on biomineralization

To assess whether intermittent PTH (1–34) would regulate PDL cell activities, the effect of intermittent PTH(1–34) on the PDL cell-mediated biomineralization was determined. PDL cells were cultured in the presence of osteogenic medium containing 10^{-8} M dexamethasone and 10 nM β-glycerophosphate for 3 wk and treated intermittently for 24 h/cycle with 10^{-12} M PTH(1–34) during this period. At harvest, the cultures were stained according to the von Kossa protocol to visualize the formation of mineralized nodules.

Statistical analysis

From all data obtained, the ALP activity and osteocalcin production at the onset of PTH administration (T_0) was subtracted, serving as a baseline correction. Each data point represents the mean ± standard error of the mean of six independent cultures. Data were analyzed using analysis of variance and statistical significance was determined using Bonferroni's modification of the Student's *t*-test for multiple comparisons; *p*-values of < 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

Cell characterization

Comparison of expression of the mesenchymal marker gene by preconfluent vs. confluent PDL cells revealed an up-regulated expression, of at least four-fold (up to 26-fold), of mRNA for most genes that indicate an osteoblastic phenotype in confluent cells compared with preconfluent cells (Fig. 1).

Vehicle-treated cultures for each experimental group at a particular maturation state did not differ significantly from each other and from untreated controls, and therefore only one vehicle-treated control for each maturation state is presented in each figure. Likewise, the control cultures treated with the respective inhibitors alone did not differ significantly from the vehicle controls, and thus only the vehicle controls are presented.

At the start of treatment with PTH(1–34), there were $0.03 \pm 0.006 \times 10^5$ preconfluent PDL cells/well and $0.13 \pm 0.017 \times 10^5$ confluent cells/well.

Preconfluent cultures

An intermittent exposure of preconfluent cultures to PTH(1–34) for 24 h/cycle enhanced the ALP-specific activity significantly at the protein level (Fig. 2).

When the effect of different PTH fragments was investigated, intermittent administration of both PTH(1–34) and PTH(1–31), the latter lacking the PKC-activating domain and therefore permitting investigation of the role of the PKC-dependent pathway in the mediation of the PTH(1–34) effect, resulted in a significant increase of ALP-specific activity after 24 h/cycle. The role of the cAMP/PKA-dependent pathway was determined by the use of signal-specific PTH fragments lacking the PKA-activating domain. Those fragments, namely PTH(3–34) and PTH(7–34), failed to enhance ALP-specific activity (Fig. 3A). Inhibition of the PKC pathway with RO-32-0432 did not affect the PTH(1–34)-induced inhibition of ALP-specific activity (Fig. 4A). When preconfluent cultures were challenged with intermittent administration of PTH(1–34), the induced stimulation of ALP activity was inhibited in the presence of the PKA inhibitor H8 (Fig. 4B).

As found for ALP, osteocalcin protein expression was also enhanced significantly by intermittent administration of PTH(1–34) in preconfluent cultures (Fig. 5). Intermittent exposure

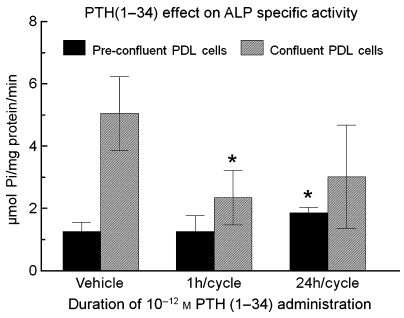

Fig. 2. Effect of intermittent administration of 10^{-12} M of a parathyroid hormone (PTH) fragment (1–34) on the regulation of alkaline phosphatase (ALP)-specific activity in preconfluent and confluent human periodontal ligament (PDL) cells. Fifth-passage cells were treated intermittently with 10^{-12} M of the PTH(1–34) fragment for 1 or 24 h during three cycles of 48 h each. Vehicle-treated cultures served as controls (vehicle). ALP activity was determined by a biochemical assay. From all data obtained, the ALP activity at the onset of PTH(1–34) 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 ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle control at a particular maturation state.

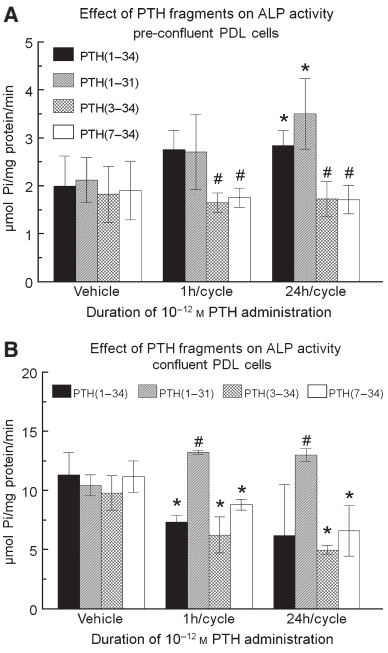

Fig. 3. Effect of different parathyroid hormone (PTH) fragments on alkaline phosphatase (ALP)-specific activity. Different PTH fragments, at a concentration of 10^{-12} M, that were lacking either the protein kinase C (PKC)-activating domain [PTH (1–31)] or the protein kinase A (PKA)-activating domain [PTH(3–34)], or additionally the Ca^{2+}-activating domain [PTH(7–34)], were employed as substitutes for the PTH(1–34) fragment, which has full biological activity, during the intermittent exposure of the cultures. At harvest, the ALP activity was quantified by a biochemical assay and expressed as a function of the cell number or protein content. From all data obtained, the ALP activity at the onset of PTH administration (T_0) was subtracted, serving as a baseline correction. In preconfluent cells, the PTH(1–31) fragment elicited a similar increase of ALP activity as the PTH(1–34) fragment, whereas PTH(3–34) and PTH(7–34) fragments failed to induce a comparable cellular response (A). In contrast, PTH(1–34), PTH(3–34) and PTH(7–34) fragments induced an inhibition of ALP activity in confluent cultures, whereas the PTH(1–31) fragment failed to do so (B). The data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle-treated control; $p < 0.05$, experimental group vs. PTH (1–34) fragment-treated group at the same time-point.

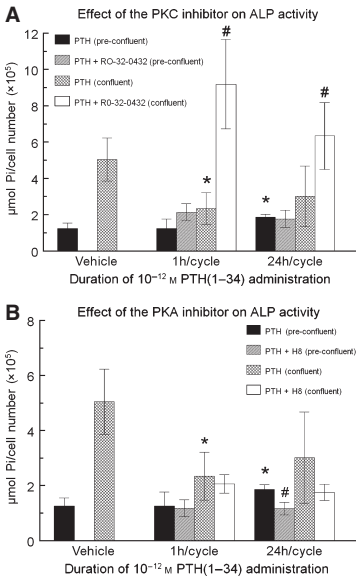

Fig. 4. Effect of the protein kinase C (PKC) inhibitor, RO-32-0432 (A), and of the protein kinase A (PKA) inhibitor, H8 (B), on the parathyroid hormone (PTH) fragment (1–34)-induced regulation of alkaline phosphatase (ALP) activity in preconfluent and confluent periodontal ligament (PDL) cell cultures. Fifth-passage cells were treated intermittently with 10^{-12} M of the PTH (1–34) fragment 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 before administration of the PTH(1–34) fragment and the experimental media were supplemented with the inhibitors for the entire experimental period. Vehicle-treated cultures served as controls (vehicle). From all data obtained, the ALP activity at the onset of PTH administration (T_0) was subtracted, serving as a baseline correction. RO-32-0432 did not modify the increase in ALP activity induced by intermittent administration of the PTH(1–34) fragment in preconfluent cells, but inhibited the PTH (1–34)-induced decrease in ALP in confluent cultures (A). In the presence of the PKA inhibitor, H8, the PTH(1–34) fragment-induced increase of ALP activity was inhibited in preconfluent cells as opposed to a lack of an effect of H8 on the PTH(1–34) fragment-induced decrease of ALP in confluent cells (B). Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle-treated control; $p < 0.05$, experimental group vs. PTH (1–34)-treated group at the same time-point.

of preconfluent cultures to different fragments of the hormone resulted in a significant increase in the production of osteocalcin when PTH(1–34) and PTH(1–31) were used, whereas PTH (3–34) and PTH(7–34) did not stimulate osteocalcin (Fig. 6A). The addition of the PKC inhibitor RO-32-0432 did not modify the PTH(1–34)-induced increase in osteocalcin production (Fig. 7A), whereas the PKA inhibitor, H8, inhibited it (Fig. 7B).

Confluent cultures

In confluent cultures, intermittent PTH(1–34) induced a significant reduction of ALP activity (Fig. 2). An intermittent challenge of confluent PDL cell cultures with the fragments PTH(3–34) and PTH(7–34) resulted in a comparable and significant reduction of ALP-specific activity, whereas a pulsatile exposure of the cells to PTH(1–31) failed to induce a cellular response different from that of the vehicle-treated control (Fig. 3B). The PTH(1–34)-induced decrease of ALP activity was restored by the inhibition of PKC (Fig. 4A) but not by the PKA pathway inhibitor, H8 (Fig. 4B).

Similar results were obtained regarding the PTH effect on osteocalcin production in confluent PDL cell cultures. Intermittent PTH(1–34), PTH(3–34) and PTH(7–34), but not intermittent PTH(1–31), led to a reduction of osteocalcin protein expression (Figs 5 and 6B). Inhibition of the PKC pathway with RO-32-0432 restored the osteocalcin production to control levels (Fig. 7A). Conversely, the PKA inhibitor H8 failed to reverse the PTH(1–34)-induced reduction of osteocalcin in confluent cultures (Fig. 7B).

Biomineralization

After 3 wk in culture, mineral deposition in the extracellular matrix of the PDL cell cultures was detectable when cells were cultured in osteogenic medium. Intermittent administration of PTH(1–34) for 24 h/cycle enhanced the number and staining intensity of the mineralization nodules (Fig. 8).

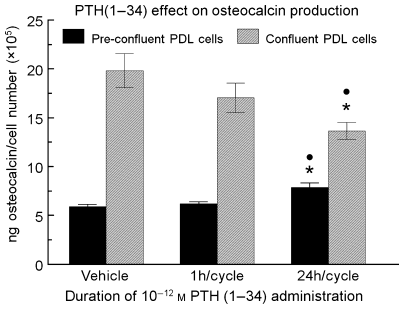

Fig. 5. Effect of intermittent administration of 10^{-12} M of a parathyroid hormone (PTH) fragment (1–34) on the regulation of osteocalcin protein production in preconfluent and confluent human periodontal ligament (PDL) cells. Fifth-passage cells were treated intermittently with 10^{-12} M of the PTH (1–34) fragment for 1 or 24 h, during three cycles of 48 h each. Vehicle-treated cultures served as controls (vehicle). The osteocalcin content of the conditioned medium was determined by ELISA and is expressed as a function of the cell number. From all data obtained, the osteocalcin production 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 ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle control at a particular maturation state; •$p < 0.05$, experimental group vs. same treatment for 1 h/cycle at a particular maturation state.

Discussion

There is considerable agreement amongst all our experimental approaches, allowing for the conclusion that intermittent administration of PTH(1–34) regulates the osteoblastic differentiation of human PDL cells and that the cellular response is distinct in terms of the signaling pathways involved and also reflects differences in cell maturation. In less-mature preconfluent cultures, only the N-terminally intact PTH fragments containing the PKA-activating domain enhanced ALP activity and osteocalcin production and those effects were antagonized by the PKA inhibitor, H8, but not by the PKC inhibitor, RO-32-0432, allowing for the conclusion that PTH(1–34) mediates its effects via a

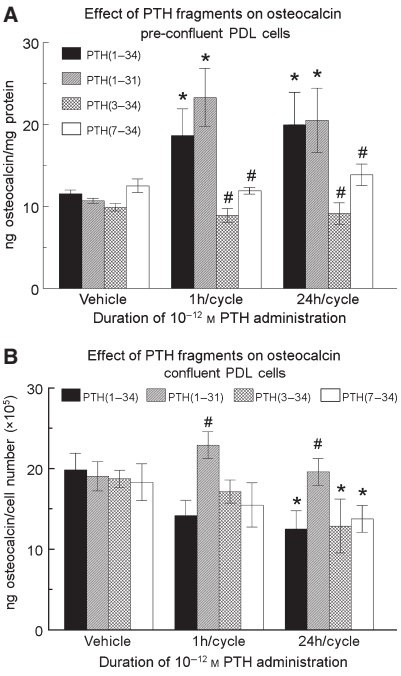

Fig. 6. Effect of different parathyroid hormone (PTH) fragments on osteocalcin production. Different PTH fragments at a concentration of 10^{-12} M that were lacking either the protein kinase C (PKC)-activating domain [PTH(1–31)] or the protein kinase A (PKA)-activating domain [PTH(3–34)] or additionally the Ca^{2+}-activating domain [PTH(7–34)] were employed as substitutes for the PTH(1–34) fragment with the full biological activity during the intermittent exposure of the cultures. At harvest, the osteocalcin content of the conditioned medium was quantified by ELISA and expressed as a function of the cell number or protein content. From all data obtained, the osteocalcin produced at the onset of PTH administration (T_0) was subtracted, serving as a baseline correction. In preconfluent cells, both PTH(1–31) and PTH(1–34), fragments elicited a similar increase of osteocalcin production whereas PTH(3–34) and PTH(7–34) fragments failed to induce a comparable cellular response (A). In contrast, PTH(1–34), PTH(3–34) and PTH (7–34) fragments induced an inhibition of osteocalcin production in confluent cultures, whereas the PTH(1–31) fragment failed to do so (B). Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle-treated control; $p < 0.05$, experimental group vs. PTH (1–34)-treated group at the same time-point.

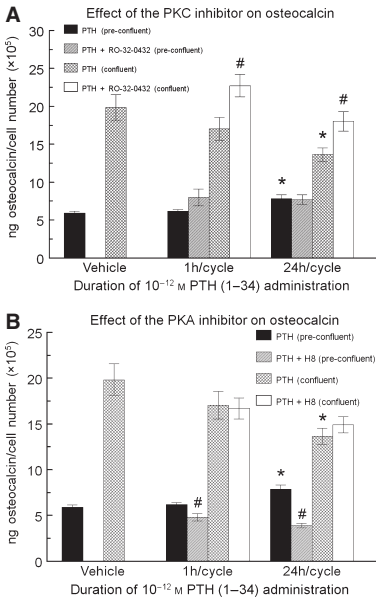

Fig. 7. Effect of the protein kinase C (PKC) inhibitor, RO-32-0432 (A), and of the protein kinase A (PKA) inhibitor, H8 (B), on the parathyroid hormone (PTH) fragment (1–34)-induced regulation of osteocalcin production in preconfluent and confluent periodontal ligament (PDL) cell cultures. Fifth-passage cells were treated intermittently with 10^{-12} M of the PTH(1–34) fragment 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 before the administration of the PTH(1–34) fragment and the experimental media were supplemented with the inhibitors for the entire experimental period. Vehicle-treated cultures served as controls (vehicle). From all data obtained, the osteocalcin production at the onset of PTH administration (T_0) was subtracted, serving as a baseline correction. RO-32-0432 did not modify the increase in osteocalcin induced by intermittent PTH(1–34) in preconfluent cells, but inhibited the PTH(1–34)-induced decrease in osteocalcin in confluent cultures (A). The PKA inhibitor, H8, exerted an inhibitory effect on the PTH(1–34) fragment-induced increase of osteocalcin production in preconfluent cells, as opposed to a lack of an effect of H8 on the PTH(1–34)-induced decrease of osteocalcin in confluent cells (B). Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, experimental group vs. vehicle-treated control; $p < 0.05$, experimental group vs. PTH(1–34)-treated group at the same time-point.

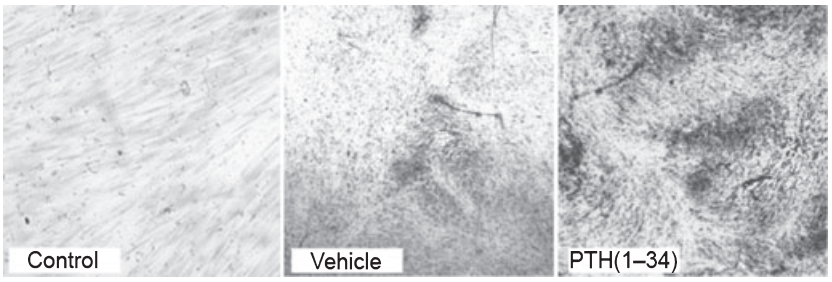

Fig. 8. Stimulatory effect of intermittent administration of a parathyroid hormone (PTH) fragment (1–34) on the periodontal ligament (PDL) cell-mediated *in vitro* biomineralization of the extracellular matrix. Fifth-passage PDL cells were cultured in the absence (control) or presence (vehicle) of osteogenic medium and treated intermittently for 24 h/48 h-cycle with 10^{-12} M of the PTH(1–34) fragment during this period. At harvest, the cells were stained according to the von Kossa protocol.

cAMP-dependent mechanism at this maturation stage. These results strengthen the findings of previous reports on the predominant role of the cAMP/PKA-dependent pathway in PTH signaling for the regulation of osteoblastic differentiation parameters, including ALP specific activity and bone sialoprotein (23–25). Conflicting evidence regarding the role of PKA as an activator or inhibitor of PTH-induced target gene transcription (10,24,26,27) exists with the majority of the data obtained in osteoblast studies, supporting the concept that multiple mechanisms are involved in the PTH response by demonstrating the involvement of both cAMP and PKC in PTH signal transduction with a predominance of PKA as an activator of ALP activity (3,10,24). In contrast to this view, Fukayama *et al.* (26) reported on a PKA-dependent inhibition of ALP release by SaOS-2 human osteoblastic cells. These apparent discrepancies stress the need for a refined view when interpreting data relative to previously published material that was obtained in different cell culture systems or following different experimental protocols.

Contrasting the role of PKA as a mediator of the PTH effect in preconfluent cells, more mature, confluent PDL cultures utilized the PKC-dependent pathway to mediate the inhibitory effect of PTH on ALP activity and osteocalcin production, as evidenced by the failure of PTH(1–31) to modify the cellular response and by an inhibition of the PTH(1–34) effect by RO-32-0432 but not by H8. These data clearly indicate that the PTH effect in confluent cells is mediated by a PKC-dependent pathway and reinforce observations in other cell culture systems demonstrating the ability of PTH to signal through PKC (28–31). Experiments in rat and human osteoblast cell lines indicated that PTH regulation of osteocalcin expression is mediated via both PKA- and PKC-dependent pathways (7). From our experiments, it cannot be concluded whether both PKA and PKC pathways act independently of, or interconnected with, each other. The association of these activation steps in PDL cells remains to be elucidated by further research.

Data obtained in chondrocyte cultures corroborate the crucial role of cell status regarding the pathways involved in PTH signaling in PDL cells. In resting zone chondrocytes, the PTH effect on ALP activity correlated with changes in PKC signaling, whereas, in growth zone chondrocytes, PTH-induced changes of this parameter were mediated via a cAMP-dependent pathway (32). Likewise in chondrocytes, the role of cell maturation was highlighted in experiments using osteoblasts (9). The authors demonstrated that PTH enhances cellular differentiation in immature cells but inhibits it in mature cells, as shown by reduced ALP activity in the latter. The similarity in PDL cell and osteoblast responses to PTH(1–34) regarding ALP activity corroborates the osteoblast-like character of PDL cells and supports the idea that those cells might be involved in the regulation of dental hard tissue repair (e.g. in the course of inflammatory periodontal disease or orthodontic tooth movement). Further support for this assumption comes from our functional assays demonstrating that PDL cells are capable of mineralizing their extracellular matrix, as shown by von Kossa staining. This PDL cell activity was enhanced by intermittent administration of PTH(1–34) in our study, indicating the anabolic potential of such a treatment regimen in periodontal regeneration. Our data do not allow for a direct interpretation of the relationship between the PTH-induced increase in biomineralization and the PTH effect on ALP activity and osteocalcin production, because changes in ALP and osteocalcin were examined after intermittent administration of PTH for a total of 6 d, as opposed to the mineralization experiments, which were conducted for 3 wk. However, it might be speculated that osteocalcin, which represents a major component of the extracellular matrix of bone and is widely accepted as a marker for late osteoblastic differentiation events and the initiation of mineralization (20), may inhibit mineralization based on the observation that osteocalcin-knockout animals exhibit a higher bone density (33). Osteocalcin was hypothesized to serve as a termination signal for bone formation once the bone matrix is completely matured (7). ALP activity is required for the cleavage of inorganic pyrophosphate, to generate phosphate, which then aggregates with calcium to form calcium phosphate, the basic component of bone. Changes in ALP activity will result in an altered embedment of calcium phosphate in the collagen matrix of bone. In the event of a reduction in ALP activity, inorganic pyrophosphate accumulates in the blood serum and inhibits bone mineralization. Therefore, the PTH-induced changes of ALP activity and osteocalcin production observed in our PDL cell cultures might contribute to the regulation of the initiation and

termination of PDL cell matrix mineralization as well as to the quality of the mineralized tissue.

Cementoblasts, another component of the PDL, were also shown to be targets of PTH and PTHrP (16,27). In these studies, the continuous administration of PTHrP activated cAMP/PKA- and PKC-dependent pathways with an inhibitory role for the former and anabolic activities being mediated by the latter. However, in those experiments, cementoblasts were exposed continuously to the stimulus, which does not allow a direct comparison of the data with our findings in PDL cells.

In the present investigation, two different PTH exposure regimens were used to mimic an intermittent application of the hormone, namely 1 h/cycle and 24 h/cycle. Although one might argue that a PTH-induced stimulation of cAMP and downstream regulation of PKA occurs within minutes and consequently question the rationale for the 24 h/cycle challenge, this regimen was chosen to examine the role of exposure time for the cellular response. Indeed, the two intermittent regimens elicited similar changes in target protein production, but differed in that the administration of PTH(1–34) for 24 h/cycle induced a stronger response than a challenge for 1 h/cycle, supporting the concept of an exposure time-dependence of the PTH effect.

The role of cell maturation was examined using fifth-passage cells at two states of confluence as an experimental model in our experiments. Based on the characterization of the cultures at different degrees of confluence, which demonstrated that the degree of confluence correlated strongly with the expression pattern of markers typical of mesenchymal cells, it seems justified to regard preconfluent PDL cells as being less mature and confluent PDL cells as being more mature.

As a drawback of any cell culture study, it has to be discussed that data obtained in fifth-passage PDL cells do not necessarily relate directly to the *in vivo* situation because the microenvironment *in vivo* is more complex and cultured PDL cells differ from freshly isolated tissue in that they display a less differentiated phenotype, as evidenced by a reduced expression of osteoblastic marker genes, including ALP, bone sialoprotein, osteopontin and osteocalcin (34,35). Previously, our own group demonstrated a reduction of ALP expression by PDL cells with increasing cell passage (35). These two studies indicate that PDL cells in culture express fewer of the molecules associated with mature mineralized tissue and therefore seem to represent a less-mature differentiation state than living tissue. However, likewise in our model of cellular maturation using preconfluent and confluent cells, it is likely that *in vivo* there are PDL cells at different stages of maturation and therefore our experimental approach provides a valuable piece of information extending current knowledge on the action and intracellular signaling of PTH by the aspect of dental tissues. The present data clearly indicate that the phenotype of human PDL cells can be modified by PTH, which might prove beneficial in the attempt to enhance the reparative capabilities of those cells.

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