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EFFECTS OF HORMONES AND CYTOKINES ON STIMULATION OF ADENYLATE CYCLASE AND INTRACELLULAR CALCIUM CONCENTRATION IN HUMAN AND CANINE PERIODONTAL-LIGAMENT FIBROBLASTS

R. M. NOHUTCU,[1,2] L. K. MCCAULEY,[1,3] J. E. HORTON,[4] C. C. CAPEN[1] and T. J. ROSOL[1]*

[1]Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210, U.S.A., [2]Department of Periodontology, Faculty of Dentistry, Hacettepe University, Ankara, Turkey, [3]Department of Periodontics, Prevention, and Geriatrics, School of Dentistry, 1011 North University Avenue, University of Michigan, Ann Arbor, MI 48109 and [4]Department of Periodontology, College of Dentistry, The Ohio State University, 305 W. Twelfth Avenue, Columbus, OH 43210, U.S.A.

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Summary—Adenylate cyclase was stimulated by prostaglandin E_2 (PGE_2) and parathyroid hormone-related protein (PTHrP) in both these types of fibroblast and by calcitonin gene-related protein (CGRP) in the human fibroblasts *in vitro*. PGE_2 (1 μM), CGRP (1 μM), and PTHrP (1 μM) stimulated adenylate cyclase up to 50-fold, 10-fold and 9-fold, respectively. Calcitonin (CT), substance P (SP), interleukin-1β (IL-1β), and transforming growth factor-β_1 (TGFβ_1) had no effect on adenylate cyclase in either fibroblast. Intracellular Ca^{2+} (iCa^{2+}) was measured in individual fibroblasts from the periodontal ligament using Indo-1 and an adherent cell analysis and sorting interactive laser cytometer. Ionomycin (3 μM) caused a transient rise of iCa^{2+} in all human and canine fibroblasts tested. The mean percentage increase in iCa^{2+} in response to ionomycin was 820 and 840% for human and canine fibroblasts, respectively. The human fibroblasts responded to PGE_2 (1 μM) by an increased iCa^{2+} concentration; the mean percentage increase in iCa^{2+} was 187%. SP caused a less pronounced increase in iCa^{2+} in the human fibroblasts (56%). CGRP and SP caused a similar response in the canine fibroblasts. The mean percentage increase in iCa^{2+} in response to SP and CGRP was 95 and 78%, respectively. PTH, PTHrP, platelet-activating factor, CT, and IL-1β had no effect on iCa^{2+} in either type of fibroblast. The data indicate that cAMP and calcium have roles as intracellular secondary messengers in the action of PGE_2, SP, CGRP, and PTHrP in fibroblasts of human and canine periodontal ligament.

Key words: cyclic AMP, intracellular calcium, prostaglandin E_2, calcitonin gene-related peptide, substance P, parathyroid hormone-related protein, parathyroid hormone, dog, human, periodontal-ligament, fibroblast.

INTRODUCTION

The specialized cells of the periodontal ligament play an important part in periodontal regeneration and alveolar bone homeostasis. Regeneration of periodontal connective-tissue attachment requires the formation of new cementum and functionally oriented collagen fibres that are inserted into the cementum matrix. Studies on periodontal wound healing show that cells of the periodontal ligament are possibly the only cells that can regenerate a new attachment to the root surface (Melcher, 1976; Boyko, Melcher and Brunette, 1981; Isidor *et al.*, 1986; van Dijk *et al.*, 1991). Such cells must migrate and attach to the treated root surface before connective tissue regeneration and then proliferate, differentiate and synthesize connective tissue components. It is known that the microenvironment of the periodontal ligament contains numerous growth factors and neuropeptides (Meikle, Heath and Reynolds, 1986; Davidovitch *et al.*, 1992); however, little is known of their mechanisms of action on the ligament cells. As a current focus of clinical research is to stimulate proliferation and attachment of the periodontal ligament, it is important to determine what intracellular mechanisms are activated after stimulation of its cells by biological agonists.

Numerous laboratories have cultured cells of the periodontal ligament and determined their appearance and biological properties *in vitro* (Ragnarson, Carr and Daniel, 1985; Rose *et al.*, 1987; Davidovitch *et al.*, 1988; Somerman *et al.*, 1988; Piche, Carnes and Graves, 1989; Mariotti and Chochran, 1990). Some of these cells reportedly express osteoblast-like characteristics including high amounts of alkaline phosphatase (Yamashita, Sato and Noguchi, 1987; Somerman *et al.*, 1988; Nojima *et al.*, 1990), PTH-dependent cAMP synthesis (Piche *et al.*, 1989; Nojima

*To whom all correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; iCa^{2+}, intracellular calcium; IL-1, interleukin-1; PAF, platelet-activating factor; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PGE_2, prostaglandin E_2; TGFβ_1, transforming growth factor-β_1.

et al., 1990), production of osteonectin (Wasi *et al.*, 1984; Somerman *et al.*, 1990) and bone gla protein regulated by 1,25-dihydroxyvitamin D (Nojima *et al.*, 1990). Histological and biochemical studies have shown that some of these cells have fibroblastic characteristics (McCullock and Bordin, 1991), including expression of vimentin intermediate filaments (Ngan *et al.*, 1992) and production of predominantly type I collagen *in vitro* (Narayanan and Page, 1983). Cells of the periodontal ligament are also capable of producing mineralized nodules *in vitro* (Arceo *et al.*, 1991).

At least two intracellular secondary messengers, cAMP and calcium, are involved in the transduction of extracellular signals to regulate cellular function. Cyclic AMP mediates the effects of various chemical and physical stimuli on target cells and plays a major part in the regulation of cellular metabolism and functional activity (Kuehl, 1974). Binding of PTH stimulates adenylate cyclase in osteoblasts. Human fibroblasts also have adenylate cyclase-linked PTH, tumour necrosis factor, and IL-1 receptors (Pun, Arnaud and Nissenson, 1988; Zhang *et al.*, 1988). Although there are reports of increased cytoplasmic cAMP production in periodontal-ligament cells in response to cytokines, hormones and neurotransmitters (Davidovitch *et al.*, 1988; Piche *et al.*, 1989; Nojima *et al.*, 1990; Saito *et al.*, 1990a), there are few data on the role of iCa^{2+} as a secondary messenger in these cells. Receptor-mediated increases in iCa^{2+} play an important part in signal transduction in many cell types with a variety of hormones, neurotransmitters and growth factors (Rasmussen and Barret, 1984). Changes in the iCa^{2+} are important in the regulation of a variety of cellular functions including secretion, proliferation, growth and differentiation (Olashaw and Pledger, 1988; Agarwal, Reynolds and Suzuki, 1989; Barnea, Levy and Shany, 1990). Knowledge of the intracellular mechanisms that regulate the function of periodontal-ligament cells is vital to understanding the pathophysiology of the periodontium.

Our purpose was to investigate the response of adenylate cyclase and iCa^{2+} in periodontal-ligament fibroblasts from man and dog to various hormones and peptides. We chose agonists that have been localized to the ligament and may play an important part in the maintenance and regeneration of the periodontium.

MATERIALS AND METHODS

Supplies

DMEM/F-12 was purchased from Sigma Chemical Co., St Louis, MO, U.S.A. FBS was supplied by Hyclone Laboratories Inc., Logan, UT, U.S.A. CGRP and substance P were purchased from Peninsula Laboratories Inc., Belmont, CA, U.S.A. Calcitonin, synthetic bovine PTH 1-34 and human PTHrP 1-34 were purchased from Bachem California, Torrance, CA, U.S.A. IL-1β was supplied by Immunex Research Laboratories, Seattle, WA, U.S.A. Indo-1/AM was supplied from Molecular Probes, Eugene, OR, U.S.A. Porcine TGFβ_1 was obtained from R & D Systems, Minneapolis, MN, U.S.A. PGE_2, PAF and ionomycin were purchased from Calbiochem. Corp., La Jolla, CA, U.S.A.

Cell culture

Fibroblasts were obtained from the periodontal ligaments of extracted, erupted third molar or premolar teeth of young people (under the age of 20 yr). Similar fibroblasts were obtained from the incisors of dogs at The Ohio State University Veterinary Teaching Hospital. After extraction, the teeth were placed in a carrier solution (DMEM supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin) and washed several times. Only periodontal ligament attached to the middle third of the root was removed by curettage to avoid contamination with gingival and apical tissue. Small pieces of tissue were incubated in the same medium supplemented with 10% FBS and 200 units/ml collagenase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) on a rocker platform in a humidified atmosphere of 95% air and 5% CO_2 at 37°C overnight. The following day, medium containing the cells was centrifuged and the cell pellet was suspended in DMEM supplemented with 10% FBS and transferred to 25-cm^2 tissue-culture flasks. After reaching confluency the cells were passaged with 0.25% trypsin–0.1% EDTA. Cells cultured from the periodontal ligament in this manner are fibroblast-like. The cells grow with a spindle shape, are negative for keratin and endothelial-cell antigen (HPCA-1), express type I collagen and are positive for vimentin intermediate filaments (Ngan *et al.*, 1992). Human and dog fibroblasts were used between the second and fifth passages for assay of intracellular cAMP and calcium.

Adenylate cyclase stimulation assay

Adenylate cyclase stimulation was evaluated by measuring cAMP production by periodontal-ligament fibroblasts (McCauley *et al.*, 1992). The fibroblasts were grown in 24-well (2 cm^2) tissue-culture plates (100,000 cells/well) with DMEM/F12 medium supplemented with 10% FBS and 50 mg/l gentamicin sulphate until confluent. The medium was then removed and the cells incubated for 10 min at 37°C in calcium and magnesium-free Hank's balanced salt solution containing 0.1% BSA and 1 mM isobutylmethylxanthine to inhibit phosphodiesterase activity. Agonists were used at the following final concentrations—PGE_2, 1–1000 nM; CGRP, 1–1000 nM; calcitonin, 1 μM; substance P, 1 μM; PTHrP, 1 μM; PTH, 1 μM; IL-1β, 1 ng/ml; and TGFβ_1, 1 ng/ml—were added directly to the medium and the plates incubated for 10–60 min at 37°C (most assays were incubated for 10 min). The reaction was stopped by removing the medium and adding ice-cold 5% perchloric acid (250 μl) to each well. The plates were incubated at −20°C for 2 h to extract the cAMP. After thawing, the pH of the extract was adjusted to 7.5 with 4M KOH, and centrifuged to remove the precipitate. The neutralized extract was then tested for cAMP content using a cAMP-binding protein assay. Each group was evaluated in triplicate.

The cAMP-binding protein assay was as described by Miyamoto, Kuo and Greengard (1969) with minor modifications (Shanfeld, Jones and Davidovitch, 1981), using bovine skeletal muscle as the source of

binding protein. The assay was made as follows: to each tube was added assay buffer (50 mM tris, 5 mM EDTA, 0.2% BSA; pH 7.4), 100 μl standards or unknowns, [^{3}H]-cAMP (10,000 cpm/tube) (ICN Biomedicals, Costa Mesa, CA, U.S.A.), and cAMP-binding protein sufficient to bind 40–60% of the added radioactivity in a final volume of 250 μl. The tubes were incubated for 90 min on ice. Dextran-coated charcoal (0.5 mg/ml dextran and 5 mg/ml charcoal) was added to each tube (600 μl), incubated for a further 30 min on ice, then centrifuged to remove the unbound from the bound cAMP-binding protein complexes. The supernatant was decanted directly into scintillation vials and counted in a liquid scintillation counter (2000CA Packard Tri-Carb Liquid Scintillation Analyzer). Samples were run in duplicate and the concentration of cAMP was calculated by log–logit transformation (Securia 1.0, Packard, Downers Grove, IL, U.S.A.). Data were expressed as pmol cAMP/well.

Measurement of intracellular calcium

Intracellular calcium was measured in individual human and dog fibroblasts using an ACAS-570 (adherent cell analysis and sorting interactive laser cytometer) (Meridian Instruments Inc., Okemos, MI, U.S.A.) equipped with a dual detector system to measure free and bound calcium within subcellular regions (Burnatowska-Hiedin and Spielman, 1989; Wirtz and Dobbs, 1990; McCauley *et al.*, 1992). Confluent cultures of periodontal-ligament cells were harvested by trypsinization and passaged at 2.5×10^4 cells into modified Petri dishes 24 h before each experiment. A 1-cm dia hole was made in a 35-mm culture dish (Corning), and the hole was covered with a glass coverslip attached with rubber cement. Culture of cells on the glass coverslip provided a low background autofluorescence, a short optical working distance with a 100 × oil immersion objective and good transmittance of u.v. light. On the day of the experiment, fibroblasts in a final volume of 400 μl assay buffer (Dulbecco's phosphate-buffered saline with 4 mM dextrose, 0.9 mM $CaCl_2$ and 1.3 mM $MgSO_4$) were loaded with Indo-1/AM (1 μM) for 1 h in the dark at 37°C. When excited with 361–363 nm light, the unbound Indo-1 within the cell emits light at 485 nm while the calcium-bound Indo-1 emits light at 405 nm (Grynkiewicz, Poenie and Tsien, 1985; Wahl, Lucherini and Gruenstein, 1990). After the incubation period for loading, fibroblasts were rinsed twice with assay buffer and 200 μl of assay buffer was placed over the cells. Individual cells were positioned to allow a linear optical scan across one or two cells. A line scan was made, monitoring the changes in the ratio of free to bound calcium. The following test solutions were added in a volume of 200 μl shortly after the initiation of the line scan: ionomycin (6 μM), PGE_2 (2 μM), substance P (2 μM), CGRP (2 μM), PTH (2 μM), PTHrP (2 μM), IL-1β (2 ng/ml) and PAF (2 μM). The ratio of F_{405}/F_{485} was calculated for each cell and the iCa^{+2} concentration was derived from a standard curve that was generated using free Indo-1,3-[N-morpholino] propane sulphonic acid (10 mM), KCl (115 mM), NaCl (20 mM), $MgSO_4$ (1 mM), EGTA (1 mM) and 20% ethanol (Burnatowska-Hiedin and Spielman, 1989; Wirtz and Dobbs, 1990).

Data analysis

Data for cAMP were analysed by one-way analysis of variance using Dunnett's mean separation test and unpaired Student's *t*-test (SAS 6.03 and Instat 1.0, GraphPAD Software, San Diego, CA, U.S.A.). Data are presented as mean ± SD of triplicate wells with statistical significance reported at $p < 0.05$ or $p < 0.01$.

Line scans of the ratio of free to bound iCa^{2+} were converted to time plots of iCa^{2+} concentration using free-Ca^{2+} working curves (Burnatowska-Hiedin and Spielman, 1989; Wirtz and Dobbs, 1990). The iCa^{2+} concentrations were expressed as numerical values or as percentage increase over the initial baseline. Two periods were measured from time plots, (1) the time from agonist addition to the peak calcium value (rise time) and (2) the time from agonist addition to return to baseline (peak duration). The numerical data were analysed using SAS 6.03, one-way analysis of variance, and Tukey's mean separation test. Significance was reported at $p < 0.05$.

RESULTS

Adenylate cyclase stimulation

PGE_2 was a potent stimulator of adenylate cyclase in the fibroblasts (Figs 1 and 2); significant stimulation occurred at concentrations of 30–1000 and 300–1000 nM in human and canine fibroblasts, respectively. Stimulation of intracellular cAMP accumulation was routinely 40–50-fold or more compared to controls when the cells were stimulated with PGE_2 (1 μM) for 10 min. The stimulation of adenylate cyclase was maximal at 10 min and decreased at 30 and 60 min, but remained markedly increased, probably due to inhibition of phosphodiesterase by isobutylmethylxanthine (Table 1).

CGRP (30–1000 nM) significantly stimulated adenylate cyclase in human but not canine fibroblasts

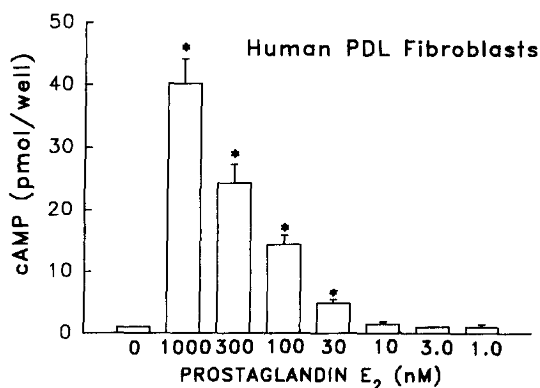

Fig. 1. Effect of prostaglandin E_2 on adenylate cyclase stimulation in human periodontal ligament (PDL) fibroblasts. Adenylate cyclase stimulation was measured by determining the accumulation of intracellular cAMP in PDL fibroblasts during 10-min incubations with PGE_2. Data are the mean ± SD of triplicate wells. *$p < 0.5$, significantly different from control (0 nM).

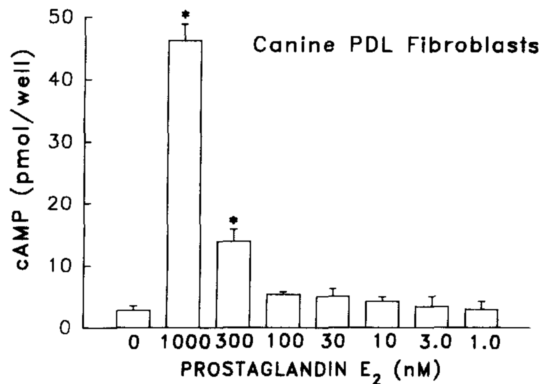

Fig. 2. Effect of prostaglandin E_2 on adenylate cyclase stimulation in canine periodontal ligament (PDL) fibroblasts. Adenylate cyclase stimulation was measured by determining the accumulation of intracellular cAMP in PDL fibroblasts during 10 min incubations with PGE_2. Data are the mean ± SD of triplicate wells. *$p < 0.05$, significantly different from control (0 nM).

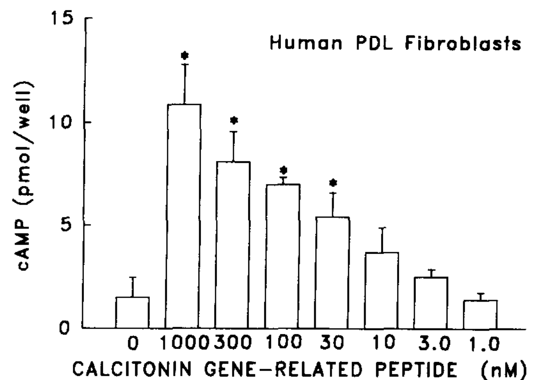

Fig. 3. Effect of calcitonin gene-related peptide on adenylate cyclase stimulation in human periodontal ligament (PDL) fibroblasts. Adenylate cyclase stimulation was measured by determining the accumulation of intracellular cAMP in PDL fibroblasts during 10 min incubations with calcitonin gene-related peptide. Data are the mean ± SD of triplicate wells. *$p < 0.05$, significantly different from control (0 nM).

(Fig. 3). The stimulation was not as great as PGE_2, but was as high as 10-fold greater than controls at a concentration of 1 μM. PTHrP and PTH (1 μM) were weak agonists of adenylate cyclase in human fibroblasts; however, canine cells only responded to PTHrP (Table 2). Calcitonin (CT), substance P, IL-1β, and $TGF\beta_1$ had no effect on adenylate cyclase in the human or canine fibroblasts.

Intracellular calcium concentration

Resting iCa^{2+} concentration was between 0.060–0.250 μM ($n = 80$) and 0.069–0.240 μM ($n = 49$) in the human and canine fibroblasts, respectively. In human fibroblasts, ionomycin (3 μM), PGE_2 (1 μM) and substance P (1 μM) caused a transient rise in iCa^{2+} (Figs 4 and 5 and Table 3). Upon addition of ionomycin to human fibroblasts, iCa^{2+} increased by approx. 820 ± 550% within 14 s and returned to near baseline within 81 ± 12 s ($n = 10$) (Fig. 4). All the cells tested responded to ionomycin. Most of the human fibroblasts (15 out of 19) responded to PGE_2 by increasing iCa^{2+}. The percentage increase in iCa^{2+} in response to PGE_2 varied greatly between cells, ranging from 27 to 470%. The mean percentage increase in iCa^{2+} in response to PGE_2 was 190 ± 130% ($n = 19$) (Fig. 5). Peak iCa^{2+} concentrations were reached in 30 ± 12 s and returned to baseline within 74 ± 17 s. Addition of substance P to the human fibroblasts caused a less pronounced increase in iCa^{2+}. The mean percentage increase in iCa^{2+} concentration was 56 ± 24% ($n = 15$), was at its maximum at 25 ± 9 s after addition of substance P, and decreased to baseline wtihin 84 ± 18 s (Fig. 6). Only half of the cells responded to substance P (eight out of 15) by increasing iCa^{2+} concentrations.

In the canine fibroblasts, stimulation of the cells with 3 μM ionomycin induced a rapid 840 ± 230% increase in iCa^{2+} within 18.5 ± 8.0 s ($n = 7$) (Fig. 7). The iCa^{2+} fell to baseline within 95 ± 5 s. CGRP (1 μM) and substance P (1 μM) caused a similar pattern of response in canine fibroblasts; the mean percentage increase in iCa^{2+} concentrations were 95 ± 37% ($n = 7$) and 78 ± 37% ($n = 7$), respectively.

PTH (1 μM, $n = 6$), PTHrP (1 μM, $n = 6$), PAF (1 μM, $n = 6$), calcitonin (1 μM, $n = 6$), and IL-1β (1 ng/ml, $n = 6$) in human and PTH (1 μM, $n = 6$), PTHrP (1 μM, $n = 6$), calcitonin (1 μM, $n = 5$), and IL-1β (1 ng/ml, $n = 5$) in canine fibroblasts had no effect on iCa^{2+}. In contrast to the canine fibroblasts, CGRP (1 μM, $n = 6$) did not increase iCa^{2+} in the human fibroblasts. In addition, PGE_2 (1 μM, $n = 6$) did not increase iCa^{2+} in the canine fibroblasts. Table 3 summarizes the positive responses of the fibroblasts from human and dog periodontal ligament to the various agents tested.

The range of resting iCa^{2+} of from 0.060 to 0.250 μM in the human fibroblasts was not

Table 1. Effect of incubation time of PGE_2 (1.0 μM) on stimulation of adenylate cyclase in human periodontal-ligament fibroblasts

Group	cAMP/well (pmol)
Control	0.4 ± 0.2
PGE_2 (10 min)	104 ± 7*
PGE_2 (30 min)	64 ± 5*
PGE_2 (60 min)	56 ± 2*

Data are mean ± SD of triplicate wells.
*$p < 0.01$, significantly different from control.

Table 2. Effect of PTHrP and PTH on adenylate cyclase stimulation in human and canine periodontal-ligament (PDL) fibroblasts

Group	Human PDL fibroblasts [cAMP/well (pmol)]	Canine PDL fibroblasts [cAMP/well (pmol)]
Control	2.3 ± 1.0	1.0 ± 0.7
PTHrP (1 μM)	7.6 ± 3.8*	9.6 ± 5.0**
Control	1.2 ± 0.2	1.0 ± 0.7
PTH (1 μM)	3.0 ± 0.4*	1.3 ± 0.8

Data are mean ± SD of triplicate wells.
*$p < 0.01$ significantly different from control.
**$p < 0.05$ significantly different from control.

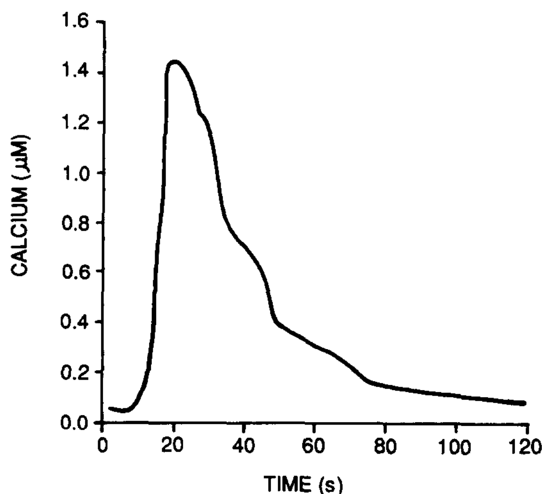

Fig. 4. Intracellular calcium concentration in a human periodontal-ligament fibroblast in response to ionomycin (3 μM), added at 10 s.

significantly different from the resting iCa^{2+} concentration of from 0.069 to 0.240 μM in the canine fibroblasts.

DISCUSSION

We investigated the intracellular cAMP and Ca^{2+} concentrations in fibroblasts of human and canine periodontal ligament in response to stimulation by various hormones and peptides. The human fibroblasts responded to PGE_2, CGRP, PTHrP and PTH *in vitro* by increasing cAMP production. The canine fibroblasts responded to PGE_2 and PTHrP, but not PTH and CGRP. PGE_2 was found to be the most potent stimulator of adenylate cyclase in these human and canine fibroblasts. Prostaglandins have a broad spectrum of biological activity for many cell types; particularly those of the E series are associated with the inflammatory response, stimulate bone resorption *in vitro* (Dietrich, Goodson and Raisz, 1975), enhance alveolar bone resorption during orthodontic tooth movement (Chao *et al.*, 1988), and stimulate bone formation *in vivo* (High, 1988). Their action is mediated by effects on the concentrations of intracellular secondary messengers. There is abundant evidence that PGE_2 increases cAMP production in fibroblasts (Piche *et al.*, 1989; Somerman *et al.*, 1990) and osteoblasts (Boland, Fried and Tashjian, 1986). Our findings for PGE_2 are in agreement with those of Somerman *et al.* (1990), who reported that cells of human periodontal ligament exhibit increased cAMP production in response to PGE_2. Rao, Moe and Heersche (1978) and Nojima *et al.* (1990) have also reported increased levels of cAMP in response to PGE for porcine and bovine periodontal-ligament cells, respectively.

We found small but significant increases in cAMP production in response to PTH and PTHrP in human periodontal-ligament fibroblasts. Normal human dermal fibroblasts also have PTH receptors and respond to PTH by increasing intracellular cAMP. Human dermal fibroblasts are more sensitive to the effects of PTHrP than PTH (Orloff, Wu and Stewart, 1989). Similarly, we observed that PTHrP was more potent than PTH at stimulating adenylate cyclase in fibroblasts of the periodontal ligament. Rao *et al.* (1978) and Somerman *et al.* (1990) reported that cells of that ligament did not have increased cAMP in response to PTH. In contrast, Nojima *et al.* (1990) demonstrated PTH-dependent cAMP production for freshly isolated periodontal ligament and its cultured cells. Piche *et al.* (1989) isolated a cell line from the periodontal ligament that produced cAMP in response to PTH.

CGRP caused a marked and dose-dependent increase in intracellular cAMP in the human but not the canine fibroblasts. Stimulation of cAMP production by CGRP has also been reported in mixed bone cell cultures obtained from newborn chick, rat and mouse calvaria (Michelangeli *et al.*, 1989) and osteoblastic cell lines and primary bone cells (Bjurholm *et al.*, 1992). It is likely that CGRP functions in fibroblasts of the periodontal ligament by interacting with specific CGRP receptors and not calcitonin receptors, as calcitonin did not stimulate adenylate cyclase. The role of CGRP in the periodontal ligament is unknown, but the presence of CGRP responsiveness may be a feature of osteoblast-like differentiation among its fibroblasts (Bjurholm *et al.*, 1992); however, the adenylate cyclase response of osteoblasts to CGRP is much less than to PTH. This is in contrast to the ligament fibroblasts, which had a greater response to CGRP than PTH.

There was no stimulation of the fibroblasts' adenylate cyclase by IL-1β. Saito *et al.* (1990a) demonstrated that IL-1α or IL-1β stimulated increased cAMP production in fibroblasts of human periodontal ligament. The positive response to IL-1β was maximal at 60 min of incubation and was inhibited by indomethacin, indicating that the cAMP production was secondary to prostaglandin synthesis and not a primary result of IL-1 stimulation.

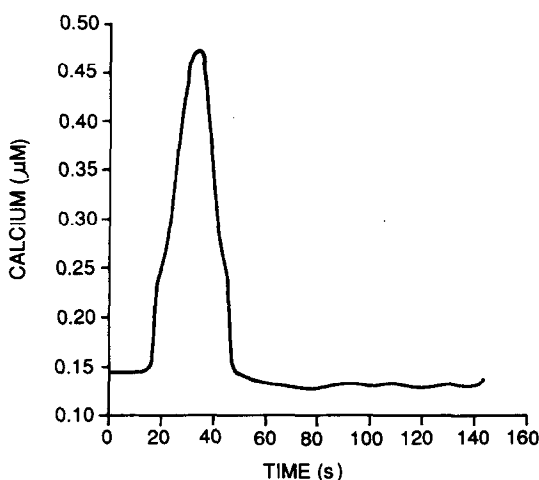

Fig. 5. Intracellular calcium concentration in a human periodontal-ligament fibroblast in response to prostaglandin E_2 (1 μM), added at 10 s.

Table 3. Effects of ionomycin, PGE_2, substance P and CGRP on intracellular calcium in human and dog periodontal-ligament fibroblasts

Substance		Cells responding (%)	Increase in $[Ca^{2+}]$ (%)	Rise time (s)	Peak duration (s)
Ionomycin	Human	100 (10/10)	820 ± 550	14.3 ± 4.4	81 ± 12
(3 μM)	Dog	100 (7/7)	840 ± 230	18.5 ± 8.0	95 ± 5
PGE_2 (1 μM)	Human	79 (15/19)	190 ± 130	30 ± 12	74 ± 17
Substance P	Human	53 (8/15)	56 ± 24	25 ± 9	84 ± 18
(1 μM)	Dog	57 (4/7)	95 ± 37	27 ± 18	103 ± 9
CGRP (1 μM)	Dog	71 (5/7)	78 ± 37	27 ± 20	101 ± 11

Results are expressed as mean ± SD.

The Ca^{2+}-sensitive fluorescence dye, Indo-1, and the use of the ACAS 570 permitted the measurement of iCa^{2+} in individual fibroblasts. The resting iCa^{2+} concentration of the human and canine periodontal-ligament fibroblasts was similar to that measured for most other cell types including fibroblasts. Resting concentrations of iCa^{2+} were 266 ± 39 nM in primary cultures of bone cells from mouse calvaria and 363 ± 42 nM for ROS 17/2.8 cells (Boland *et al.*, 1986), 246 ± 10 nM for normal human osteoblasts (Schöfl *et al.*, 1991), 147 ± 19 nM for UMR-106 cells (Yamaguchi *et al.*, 1987), 89 ± 15 nM for mouse osteoblastic cell line, MCT3-E1 (Toriyama *et al.*, 1990), 265 ± 47 nM for human keratinocytes (Bittiner, Bleehan and MacNeil, 1991) and 78 ± 7 nM for human skin fibroblasts (Corkey *et al.*, 1991). As expected, ionomycin (a calcium ionophore) resulted in a similar increase in iCa^{2+} in all the fibroblasts from human and canine periodontal ligament tested.

Most of our human fibroblasts responded to PGE_2 by increasing iCa^{2+} concentrations. Yamashita and Takai (1987) and Nakada, Stadel and Crooke (1990) reported a transient mobilization of iCa^{2+} in response to PGE_1 in Swiss 3T3 fibroblasts and $PGF_{2\alpha}$ in 3T3-L1 fibroblasts. There are two reports that PGE_2 caused an increase in iCa^{2+} in the mouse osteoblast-like cell line, MC3T3-E1 cells (Yamaguchi *et al.*, 1988; Toriyama *et al.*, 1990). Hakeda *et al.* (1986) reported that PGE_2 stimulated DNA synthesis by a mechanism other than adenylate cyclase stimulation in the same osteoblastic cell line. We found that PGE_2 was able to increase iCa^{2+} in most fibroblasts from human periodontal ligament, but with great variation in response. We anticipated this variety as a varied iCa^{2+} response to agonists which is characteristic of other cells studied (McCauley *et al.*, 1992). For example, individual osteoblast-like osteosarcoma cells (UMR 106-01) had transient increases in iCa^{2+} in only 11% of the cells stimulated by PTH (Ljunggren *et al.*, 1992).

We found that intracellular calcium acts as a secondary messenger in the action of PGE_2 in periodontal-ligament fibroblasts. It is of interest that PGE_2 can activate two intracellular signalling systems, iCa^{2+} and cAMP, in fibroblasts of human periodontal ligament, which may be important in physiological and inflammatory conditions. Concentrations of PGE in periodontal tissues of patients with chronic periodontitis are elevated 20-fold when compared to healthy tissues (Elattar, 1976). Saito *et al.* (1990b) have reported that unstimulated cells of the periodontal ligament produced PGE and these cells showed increased production of PGE after treatment with inflammatory cytokines. The same investigators demonstrated that intermittent positive and negative pressures affected PGE synthesis in cells of

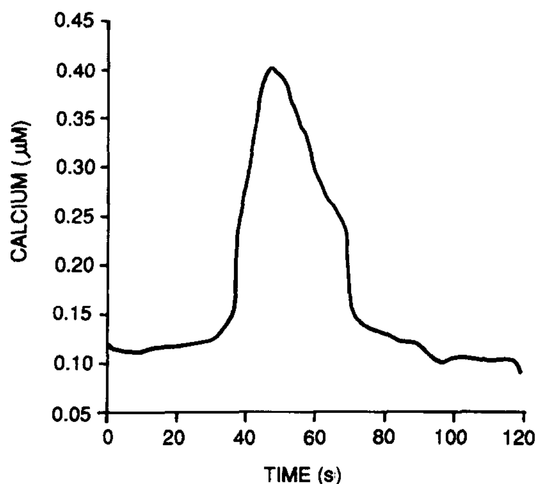

Fig. 6. Intracellular calcium concentration in a human periodontal-ligament fibroblast in response to substance P (1 μM), added at 10 s.

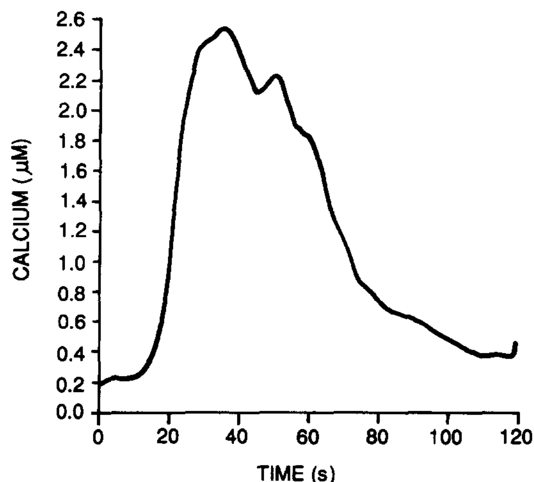

Fig. 7. Intracellular calcium concentration in a canine periodontal-ligament fibroblast in response to ionomycin (3 μM), added at 10 s.

the periodontal ligament (Saito *et al.*, 1991). Thus PGE, produced by fibroblasts of that ligament during mechanical and chemical stimuli, may be important during physiological and orthodontic tooth movement by using the cAMP and iCa^{2+} second-messenger pathways. Lack of increased iCa^{2+} response in some cells may be due to the heterogeneity of such fibroblasts *in vitro* (McCulloch and Bordin, 1991) or variations in response due to differences in the stage of the cell cycle. Increased iCa^{2+} was not observed in any canine fibroblasts tested after stimulation with PGE_2; this may be a species-related difference or may suggest that the populations of fibroblasts isolated from canine incisors differed from those isolated from human premolars. Rapid phenotypic drift or senescence of the canine fibroblasts in culture might also cause unresponsiveness of these cells to PGE_2; the canine fibroblasts were much less prolific *in vitro* than the human and often reached senescence after 2–3 passages.

In contrast to PGE_2, CGRP caused an increase in iCa^{2+} in the canine but not in the human fibroblasts. However, substance P caused a similar increase in iCa^{2+} in both groups of fibroblasts. CGRP and substance P affect bone cells directly and indirectly. Substance P has been found in dental tissues and in fibres around blood vessels in dental pulp and periodontal ligament. Release of this substance is possibly increased by the application of force during orthodontic tooth movement (Nicolay *et al.*, 1990). Our findings that fibroblasts from human and canine periodontal ligament respond to substance P or CGRP by increasing their iCa^{2+} and/or intracellular cAMP concentrations suggest that these cells have receptors for these peptides. Thus these fibroblasts may have an important role as intermediaries between the signals and the cells responsible for alveolar bone remodelling during orthodontic tooth movement.

Although there are some reports of an increase in iCa^{2+} concentration in skin fibroblasts in response to IL-1 (Corkey *et al.*, 1991) and in osteoblastic cells in response to PAF (Tatakis and Dziak, 1991), we did not observe any increase in fibroblasts of the periodontal ligament after stimulation with IL-1 or PAF.

In conclusion it is apparent that cAMP and/or calcium have a role as intracellular secondary messengers in the action of PGE_2, substance P, CGRP, and PTHrP in fibroblasts of canine and human periodontal ligament. Certain of these agents are capable of activating both of the intracellular signalling systems in these fibroblasts but may function differently in the human and canine cells. The data are consistent with the expression of both a fibroblastic and osteoblastic phenotype by the fibroblasts of periodontal ligament. This is further evidence of the diverse nature of cells isolated from the ligament. Investigations on the role of iCa^{2+} and cAMP in the function of periodontal-ligament fibroblasts and the regulation of their production in response to various cytokines and hormones will be necessary to understand the physiology, pathophysiology, and regenerative potential of the ligament.

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