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. Capen1 and T. J. Rosol1

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

(Accepted 25 May 1993)

Summary—Adenylate cyclase was stimulated by prostaglandin E\textsubscript{2} (PGE\textsubscript{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\textsubscript{2} (1 \mu M), CGRP (1 \mu M), and PTHrP (1 \mu M) stimulated adenylate cyclase up to 50-fold, 10-fold and 9-fold, respectively. Calcitonin (CT), substance P (SP), interleukin-1\beta (IL-1\beta), and transforming growth factor-\beta\textsubscript{1} (TGF\beta\textsubscript{1}) had no effect on adenylate cyclase in either fibroblast. Intracellular Ca\textsuperscript{2+} (iCa\textsuperscript{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 \mu M) caused a transient rise of iCa\textsuperscript{2+} in all human and canine fibroblasts tested. The mean percentage increase in iCa\textsuperscript{2+} in response to ionomycin was 820 and 840% for human and canine fibroblasts, respectively. The human fibroblasts responded to PGE\textsubscript{2} (1 \mu M) by an increased iCa\textsuperscript{2+} concentration; the mean percentage increase in iCa\textsuperscript{2+} was 187%. SP caused a less pronounced increase in iCa\textsuperscript{2+} in the human fibroblasts (56%). CGRP and SP caused a similar response in the canine fibroblasts. The mean percentage increase in iCa\textsuperscript{2+} in response to SP and CGRP was 95 and 78%, respectively. PTH, PTHrP, platelet-activating factor, CT, and IL-1\beta had no effect on iCa\textsuperscript{2+} in either type of fibroblast. The data indicate that CAMP and calcium have roles as intracellular secondary messengers in the action of PGE\textsubscript{2}, SP, CGRP, and PTHrP in fibroblasts of human and canine periodontal ligament.

Key words: cyclic AMP, intracellular calcium, prostaglandin E\textsubscript{2}, calcitonin gene-related peptides, 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 neuro-peptides (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...
et al., 1990), production of osteoectin (Wass 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, tumor 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 iCa2+ as a secondary messenger in these cells. Receptor-mediated increases in iCa2+ 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 iCa2+ 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 iCa2+ 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 Peninsular 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 TGFβ1 was obtained from R & D Systems, Minneapolis, MN, U.S.A. PGE2, 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% CO2 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-cm2 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 cm2) tissue-culture plates (100,000 cells/well) with DMEM/F12 medium supplemented with 10% FBS and 50 μg/ml 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—PGE2, 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 TGFβ1, 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 μM standards or unknowns, [3H]-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 cytomter) (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 passed twice with assay buffer and 200 μl of assay buffer was placed over the cells. Individual cells were positioned in the dark at 37°C. When excited with 361-363 nm light, the unbound Indo-1 within the cell emits light at 405 nm (Grynkiewicz, Poenie and Tsien, 1985; McCauley et al., 1992). After the incubation period for loading, fibroblasts were rinsed twice with assay buffer and 200 μl of assay buffer was 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.

RESULTS

Adenylate cyclase stimulation

PGE₂ 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 PGE₂ (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.
Canine PDL Fibroblasts

Fig. 2. Effect of prostaglandin E₂ 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 PGE₂. Data are the mean ± SD of triplicate wells. *p < 0.05, significantly different from control (0 nM).

The stimulation was not as great as PGE₁, 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 TGFB₁, had no effect on adenylate cyclase in the human or canine fibroblasts.

Intracellular calcium concentration

Resting iCa²⁺ 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 (5 μM), PGE₂ (1 μM) and substance P (1 μM) caused a transient rise in iCa²⁺ (Figs 4 and 5 and Table 3). Upon addition of ionomycin to human fibroblasts, iCa²⁺ 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 PGE₂ by increasing iCa²⁺. The percentage increase in iCa²⁺ in response to PGE₂ varied greatly between cells, ranging from 27 to 470%. The mean percentage increase in iCa²⁺ in response to PGE₂ was 190 ± 130% (n = 19) (Fig. 5). Peak iCa²⁺ 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 iCa²⁺. The mean percentage increase in iCa²⁺ concentration was 56 ± 24% (n = 15), was at its maximum at 25 ± 9 s after addition of substance P, and decreased to baseline within 84 ± 18 s (Fig. 6).

Only half of the cells responded to substance P (eight out of 15) by increasing iCa²⁺ concentrations.

In the canine fibroblasts, stimulation of the cells with 3 μM ionomycin induced a rapid 840 ± 230% increase in iCa²⁺ within 18.5 ± 8.0 s (n = 7) (Fig. 7). The iCa²⁺ 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 iCa²⁺ 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 = 6), and IL-1β (1 ng/ml, n = 6) in canine fibroblasts had no effect on iCa²⁺. In contrast to the canine fibroblasts, CGRP (1 μM, n = 6) did not increase iCa²⁺ in the human fibroblasts. In addition, PGE₂ (1 μM, n = 6) did not increase iCa²⁺ 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 iCa²⁺ of from 0.060 to 0.250 μM in the human fibroblasts was not

Table 1. Effect of incubation time of PGE₂ (1.0 μM) on stimulation of adenylate cyclase in human periodontal ligament fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>cAMP/well (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>PGE₂ (10 min)</td>
<td>104 ± 7*</td>
</tr>
<tr>
<td>PGE₂ (30 min)</td>
<td>64 ± 5*</td>
</tr>
<tr>
<td>PGE₂ (60 min)</td>
<td>56 ± 2*</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Human PDL fibroblasts</th>
<th>Canine PDL fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.3 ± 1.0</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>PTHrP (1 μM)</td>
<td>7.6 ± 3.8*</td>
<td>9.6 ± 5.0**</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>PTH (1 μM)</td>
<td>3.0 ± 0.4*</td>
<td>1.3 ± 0.8</td>
</tr>
</tbody>
</table>

Data are mean ± SD of triplicate wells. *p < 0.01 significantly different from control. **p < 0.05 significantly different from control.
Periodontal-ligament fibroblasts

1.6 - 1.2 - 1.0. 2 0.8 - 0.6 - 0.4 -

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, PTH\(_rP\) and PTH in vitro by increasing cAMP production. The canine fibroblasts responded to PGE\(_2\) and PTH\(_rP\), 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 vitro (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 PTH\(_rP\) 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 PTH\(_rP\) than PTH (Orloff, Wu and Stewart, 1989). Similarly, we observed that PTH\(_rP\) 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, PGE₂, substance P and CGRP on intracellular calcium in human and dog periodontal-ligament fibroblasts

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cells responding (%)</th>
<th>Increase in [Ca²⁺] (%)</th>
<th>Rise time (s)</th>
<th>Peak duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionomycin</td>
<td>Human 100 (10/10)</td>
<td>820 ± 550</td>
<td>14.3 ± 4.4</td>
<td>81 ± 12</td>
</tr>
<tr>
<td>(3 μM) Dog</td>
<td>100 (7/7)</td>
<td>840 ± 230</td>
<td>18.5 ± 8.0</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>PGE₂ (1 μM)</td>
<td>Human 79 (15/19)</td>
<td>190 ± 130</td>
<td>30 ± 12</td>
<td>74 ± 17</td>
</tr>
<tr>
<td>Substance P</td>
<td>Human 53 (8/15)</td>
<td>56 ± 24</td>
<td>25 ± 9</td>
<td>84 ± 18</td>
</tr>
<tr>
<td>(1 μM) Dog</td>
<td>57 (4/7)</td>
<td>95 ± 37</td>
<td>27 ± 18</td>
<td>103 ± 9</td>
</tr>
<tr>
<td>CGRP (1 μM)</td>
<td>Dog 71 (5/7)</td>
<td>78 ± 37</td>
<td>27 ± 20</td>
<td>101 ± 11</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.

The Ca²⁺-sensitive fluorescence dye, Indo-1, and the use of the ACAS 570 permitted the measurement of iCa²⁺ in individual fibroblasts. The resting iCa²⁺ concentration of the human and canine periodontal-ligament fibroblasts was similar to that measured for most other cell types including fibroblasts. Resting concentrations of iCa²⁺ 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 (Schöll et al., 1991), 147 ± 19 nM for UMR-106 cells (Yamaguchi et al., 1987), 89 ± 15 nM for mouse osteoblastic cell line, MC3T3-E1 (Toriyama et al., 1990), 265 ± 47 nM for human keratinocytes (Bit- tiner, Reehan 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 iCa²⁺ in all the fibroblasts from human and canine periodontal ligament tested.

Most of our human fibroblasts responded to PGE₂ by increasing iCa²⁺ concentrations. Yamashita and Takai (1987) and Nakada, Stadel and Crooke (1990) reported a transient mobilization of iCa²⁺ in response to PGE₂ in Swiss 3T3 fibroblasts and PGE₁ in 3T3-L1 fibroblasts. There are two reports that PGE₂ caused an increase in iCa²⁺ in the mouse osteoblast-like cell line, MC3T3-E1 cells (Yamaguchi et al., 1988; Toriyama et al., 1990). Hakeda et al. (1986) reported that PGE₂ stimulated DNA synthesis by a mechanism other than adenylate cyclase stimulation in the same osteoblastic cell line. We found that PGE₂ was able to increase iCa²⁺ in most fibroblasts from human periodontal ligament, but with great variation in response. We anticipated this variety as a varied iCa²⁺ response to agonists which is characteristic of other cells studied (McCausley et al., 1992). For example, individual osteoblast-like osteosarcoma cells (UMR 106-01) had transient increases in iCa²⁺ 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 PGE₂ in periodontal-ligament fibroblasts. It is of interest that PGE₂ can activate two intracellular signalling systems, iCa²⁺ 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 human and canine periodontal-ligament fibroblasts.
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 move-
ment by using the cAMP and iCa2+ second-messen-
ger pathways. Lack of increased iCa2+ 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 iCa2+ was not observed in
y any canine fibroblasts tested after stimulation with
PGE2; 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 senes-
cence of the canine fibroblasts in culture might also
cause unresponsiveness of these cells to PGE2; the
canine fibroblasts were much less prolific in vitro than
the human and often reached senescence after 2–3
passages.

In contrast to PGE2, CGRP caused an increase in
iCa2+ in the canine but not in the human fibroblasts.
However, substance P caused a similar increase in
iCa2+ 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 peri-
odontal ligament. Release of this substance is poss-
ibly 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 iCa2+ 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
iCa2+ 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 messen-
gers in the action of PGE2, substance P, CGRP, and
PTHrP in fibroblasts of canine and human periodon-
tal 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 iCa2+ and cAMP in the function of periodontal-
ligament fibroblasts and the regulation of their pro-
duction in response to various cytokines and
hormones will be necessary to understand the physi-
ology, pathophysiology, and regenerative potential of
the ligament.

Acknowledgements—This work was supported by the State
of Ohio Canine Research Fund (611335) and by a research
fellowship grant (RMN) from NATO through the Scientific
and Technical Research Council of Turkey (TÜBİTAK).

REFERENCES
free cytosolic calcium changes and neutrophil chemotaxis
24, 149–154.
Arceo N., Saul J. J., Moehring J., Foster R. A. and
mineral-like nodules in vitro. J. Periodont. 62,
499–503.
Barnea E., Levy R. and Shany S. (1990) 1,25-Dihydroxy-
vitamin D3 enhances cytosolic free calcium in HL-60 cells.
Exp. Hemat. 18, 1147–1151.
Bittiner B., Bleehan S. S. and MacNeil S. (1991) 1α,25(OH),
Vitamin D3 increases intracellular calcium in human keratinocytes.
Bjurholm A., Kreierberes A., Schultzberg M. and
formation in osteoblastic cell lines (UMR106-01, ROS
17/2,8, MC3T3-E1, and Saos-2) and primary bone cells.
Measurement of cytosolic free Ca2+ concentrations in
human and rat osteosarcoma cells: action of bone resorp-
Formation of new periodontal ligament by periodontal
ligament cells implanted in vivo after culture in vitro. A
preliminary study of transplanted roots in the dog.
Vasopressin V1 receptors on the principal cells of the
rabbit cortical collecting tubule: stimulation of cytosolic
free calcium and inositol phosphate production via coup-
lung to a pertussis toxin substrate. J. clin. Invest. 83,
84–89.
Effects of prostaglandin E2 on alveolar bone resorption
during orthodontic tooth movement. Acta Anat. 132,
304–309.
Corkey B. E., Geschwind J. F., Deeney J. T., Hale D. E.,
to interleukin 1 and tumor necrosis factor in cultured
human skin fibroblasts. Possible implication for Reye
Davidovich Z., Nicolay 0. F., Nean P. W. and Shanfeld
J. L. (1992) Neurotransmitters, cytokines and the control
of alveolar bone remodeling in orthodontics. Dent.
Davidovich Z., Gogen M. H., Okamoto Y., Slivka M. W.
and Shanfeld J. L. (1992) Inflammatory mediators in the
mechanically stressed periodontal ligament in vivo. In
Biological Mechanisms of Tooth Movement and Craniofa-
cial Adaption (Ed. Davidovich Z.), pp. 391–400. EBSCO
Media, Birmingham, AL.
Dietrich J. W., Goodson J. M. and Raiiz L. G. (1975)
Stimulation of bone resorption by various prostaglandins
in organ culture. Prostaglandins 10, 231–240.
van Dijk I. J., Schakenraad J. M., van der Voort H. M.,
of periodontal ligament fibroblasts. A novel technique to
18, 196–199.
in health and disease and its stimulation by female sex
steroids. Prostaglandins 11, 331–335.
generation of Ca2+ indicators with greatly improved


Nakada M. T., Stadel J. M. and Crooke S. T. (1990) Mobilization of extracellular \( \text{Ca}^{2+} \) by prostaglandin \( \text{F}_{2\alpha} \) can be modulated by fluoride in 3T3-L1 fibroblasts. Biochem. J. 272, 167-174.


