The regulation of intracellular calcium ion concentration and mitochondrial function by cyclosporin A: a putative mechanism for the pathogenesis of gingival overgrowth

by

Livia Silvestri

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Dentistry
University of Toronto

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Abstract

The regulation of intracellular calcium ion concentration and mitochondrial function by cyclosporin A: a putative mechanism for the pathogenesis of gingival overgrowth, by Livia Silvestri. Degree: Master of Science, Department of Periodontology, Faculty of Dentistry, University of Toronto, 2000

Gingival overgrowth is associated with loss of homeostasis of collagen metabolism subsequent to the administration of several drugs, including the immunosuppressant cyclosporin A. Currently, the mechanisms by which CsA induces gingival overgrowth have not been resolved. My hypothesis is that the induction of gingival overgrowth by cyclosporin A (CsA) involves the deregulation of collagen turnover due to a disturbance of calcium utilization in the mitochondria of gingival fibroblasts. CsA (10nM) inhibited collagen bead phagocytosis (2-fold; p< 0.05) in cultured human gingival fibroblasts (HGF) and Rat2 cells. Thapsigargin (Tg; 1 μM; p<0.01) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 10 μM; p< 0.02) also inhibited phagocytosis of collagen beads, indicating the importance of mitochondrial function in phagocytosis. Collagen-coated beads (CCB) induced equivalent increases of intracellular calcium [Ca^{2+}] in HGF and Rat2 cells. Tg inhibited the collagen-bead induced [Ca^{2+}] response 3.7-fold. Pre-treatment with CsA inhibited collagen-induced [Ca^{2+}] and [Ca^{2+}]_{SERCA} responses. Collagen bead-induced phagocytosis induced a time-dependent decrease in [Ca^{2+}]_{mito} in both Rat2 cells and Rat2 cells pre-treated with CsA. CsA prevented the loss of mitochondrial membrane potential of cells in suspension indicating that CsA was indeed acting upon the mitochondrial permeability transition pore (MPTP). Phagocytosis was decreased in mitochondria-depleted Rat2 cells compared to Rat2 cells by 2-fold (p<0.05) and by at least 2-fold when they were pre-treated with CsA (p< 0.05). Collectively, these data indicate that CsA acts at an intracellular locus where it: (i) inhibits calcium flux through the MPTP; (ii) inhibits calcium exchange between the SERCA and mitochondria; and (iii) inhibits collagen phagocytosis through a mitochondrial-dependent, calcium-regulated pathway. Future work could focus on how the interaction of mitochondria and SERCA calcium stores contributes to the anti-phagocytic activity of CsA.
Acknowledgements

This is truly a project that could not have been accomplished without the intellectual leadership, technical insight and friendship of my supervisor, Dr. Christopher McCulloch. The exceptional generosity that he demonstrated with his time and knowledge enriched my experience as a graduate student. I am also grateful for the technical assistance of the laboratory staff, including Pam Arora for fluorescence microscopy, Wilson Lee for flow cytometry, Cheung Lo for the culture of mitochondria-depleted cells and Elisabeth Lukse for immunoblotting.

I would like to thank my family for all their support, encouragement, concern and love over the years. The comfort and security that stems from a caring family has allowed me to achieve my goals. For his unfailing motivation and understanding, I thank my fiancé, Frank Nicolais.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Psi_m$</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>BAPTA</td>
<td>dibromo-1,2-bis-(o-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_i$</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{mito}$</td>
<td>mitochondrial calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{SERCA}$</td>
<td>SERCA calcium concentration</td>
</tr>
<tr>
<td>CCB</td>
<td>collagen-coated beads</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide $m$-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>COX -I</td>
<td>cytochrome oxidase I</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>HGF</td>
<td>human gingival fibroblasts</td>
</tr>
<tr>
<td>JC-1</td>
<td>using 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolecarbocyanine iodide</td>
</tr>
<tr>
<td>MPTP</td>
<td>membrane permeability transition pore</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>Rat2EtBr</td>
<td>Rat2 cells treated with ethidium bromide</td>
</tr>
<tr>
<td>SERCA</td>
<td>smooth endoplasmic reticulum Ca$^{2+}$ ATPase pump</td>
</tr>
</tbody>
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I. Literature Review

The specific problem addressed in this thesis is the mechanism of action of cyclosporin A (CsA) on mitochondrial calcium signalling and collagen phagocytosis in the context of CsA-induced gingival overgrowth. As any one of these specific topics entails a large literature which cannot be surveyed in detail due to space restrictions, I will focus this review on those studies which are specific to the central problem. I will begin with an introduction on the main forms of drug-induced gingival overgrowth and a discussion of the specific characteristics of CsA as an immunosuppressant and a modulator of gingival overgrowth. A brief overview of the mechanisms for fibrosis will lead into a discussion of collagen phagocytosis and its regulation. As the ionic regulation of phagocytosis is a lengthy topic to review, this discussion will be restricted to regulation of phagocytosis by calcium. This last topic will be expanded to include calcium responses to collagen phagocytosis within different organelles, mitochondrial calcium metabolism, and calcium response to CsA. I will conclude the review with a discussion of collagen phagocytic model systems and their rationale.

A. Periodontal Diseases

1. Inflammatory periodontal diseases

Periodontal diseases encompass a group of conditions which affect the supporting structures of the teeth including alveolar bone, root cementum, periodontal ligament, and gingiva. The clinical signs associated with periodontal diseases may include gingival inflammation, gingival fibrosis, epithelial ulceration and resorption of alveolar bone. A recent classification of these diseases indicates that 'gingival
diseases' are separate from 'periodontitis'. This view is based primarily on the absence of bone resorption with gingival diseases. As the gingiva can exhibit many altered features with disease, gingival diseases are segmented into smaller groups based on their etiology. Gingival diseases modified by medications include the drug-influenced gingival enlargements (Armitage, 1999).

Although the most common cause of gingival enlargement is an inflammatory response to bacterial plaque (Löe, 1965), there are systemic factors which predispose to enlargement. Specifically, the administration of certain drugs produces gingival overgrowth (Carranza, 1996). Kimball (1939) first described drug-induced gingival overgrowth due to phenytoin and until 1981 this continued to be the only drug associated with enlargement. Subsequently, other drugs, specifically anticonvulsants, calcium channel blockers and the immunosuppressant cyclosporin A, have been shown to produce gingival overgrowth as a side effect. Notably, gingival overgrowth was referred to as 'gingival hyperplasia' before histological examination revealed normal numbers of fibroblasts surrounded by an abundance of collagen (Bonnaure-Mallet et al., 1995).

2. Drug-induced lesions

a) Drugs which cause gingival overgrowth

The categories of drugs which are associated with gingival overgrowth are anticonvulsants, calcium channel blockers, and immunosuppressants (which only includes CsA). Table I provides a list of common drugs within these categories. I will provide a brief overview of one drug from each category to introduce a possible common link with calcium metabolism in their mode of action.
### Table I. Drugs that induce gingival overgrowth

<table>
<thead>
<tr>
<th>Drug Category</th>
<th>Agent</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anticonvulsant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydantoins</td>
<td>Ethotoin</td>
<td>Paganone®</td>
</tr>
<tr>
<td></td>
<td>Mephenytoin</td>
<td>Mesantoin®</td>
</tr>
<tr>
<td></td>
<td>Phenytoin</td>
<td>Dilantin®</td>
</tr>
<tr>
<td>Succinimides</td>
<td>Ethosuximide</td>
<td>Zerontin®</td>
</tr>
<tr>
<td></td>
<td>Methsuximide</td>
<td>Celontin®</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Phensuximide</td>
<td>Not reported®</td>
</tr>
<tr>
<td></td>
<td>Valproic acid</td>
<td>Depakene®</td>
</tr>
<tr>
<td><strong>Immunosuppressant</strong></td>
<td>Cyclosporine A</td>
<td>Sandimmune®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neoral®</td>
</tr>
<tr>
<td><strong>Calcium channel blockers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydropyridine derivatives</td>
<td>Amlodipine</td>
<td>Lotrel®</td>
</tr>
<tr>
<td></td>
<td>Felodipine</td>
<td>Norvasc®</td>
</tr>
<tr>
<td></td>
<td>Nicardipine</td>
<td>Plendil®</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>Cardene®</td>
</tr>
<tr>
<td></td>
<td>Nimodipine</td>
<td>Adalat®</td>
</tr>
<tr>
<td></td>
<td>Nisoldipine</td>
<td>Procardia®</td>
</tr>
<tr>
<td></td>
<td>Nitrendipine</td>
<td>Nimotop®</td>
</tr>
<tr>
<td></td>
<td>Diltiazem</td>
<td>Sular®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not reported</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cardizem®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dilacor XR®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tiazac®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calan®</td>
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<td></td>
<td></td>
<td>Isoptin®</td>
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<td></td>
<td></td>
<td>Verelan®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Covera HS®</td>
</tr>
<tr>
<td>Benzothiazine derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalkylamine derivatives</td>
<td>Verapamil HCl</td>
<td></td>
</tr>
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</table>
Phenytoin is an anticonvulsant which blocks or interferes with calcium influx across cell membranes. This in turn stabilizes neuronal discharge and limits the progression of neuronal excitation, thus depressing the motor cortex of the central nervous system (Wilson and Kornman, 1996). Nifedipine is a calcium channel blocker used primarily to treat acute and chronic coronary insufficiency, including angina pectoris and refractory hypertension (Hancock and Swan, 1992). CsA is a potent immunosuppressive drug used primarily to prevent rejection after organ transplantation and for treatment of autoimmune diseases (Calne et al., 1979). While the exact immunosuppressive mechanism of action of CsA is not completely understood, it appears selectively and reversibly to inhibit T helper cells by interfering with the response of these cells to an elevation of intracellular calcium ([Ca^{2+}]_i) (Granelli-Piperno et al., 1984).

The characteristic clinical picture of drug-induced gingival overgrowth usually includes combined enlargement and inflammation. This overgrowth consists of a primary expansion of connective tissue and epithelium which is related to the effects of the drug and a secondary inflammatory component. Due to the enlargement, the gingival sulcus is deepened which impedes effective hygienic measures. Consequently the entire lesion takes on an inflammatory appearance because of increased colonization by sub-gingival plaque. Consequently, the pockets (or pseudopockets) become deeper, the inflammatory response is more severe, and the area becomes more difficult to debride (Carranza, 1996). CsA-specific gingival overgrowth is also characterized by initial enlargement of the interproximal papillae which is frequently restricted to the anterior facial areas and may include partial coverage of the crowns.
The tissue is usually pink, dense, and resilient, with a stippled or granular surface. There is little bleeding on probing (Raeste et al., 1978). Table II summarizes the epidemiological and clinical properties of phenytoin, nifedipine, and CsA.

Clinical management of gingival fibrosis is difficult as the treatment often employs surgical removal of excess tissue. This approach only addresses the signs and does not control the source of the problem. Recurrence is common as long as drug administration persists and treatment often consists of repeated surgical excision. If the mechanism of fibrosis associated with gingival overgrowth were to be unraveled, treatment outcomes could likely be improved.

b) Cyclosporin A

CsA is an immunosuppressant used after bone marrow and organ transplantation that causes gingival overgrowth (Kahan, 1984). There is controversy with respect to the role of plaque in CsA-induced gingival overgrowth. McGaw et al. (1987) reported that plaque-induced gingival inflammation exacerbated the expression of CsA-induced gingival overgrowth. However, this notion was contested by Seymour et al. (1987) who concluded that the magnitude of CsA-induced gingival enlargement is related more to the plasma concentration of CsA than to the patient’s periodontal status. CsA was first studied as an immunosuppressant by Borel et al. (1976). Subsequent to successful clinical trials in the 1970s, CsA became the drug of choice to prevent rejection after organ transplantation. The trade name of CsA was originally Sandimmune®. A new oral formulation (Neoral®) has been developed which is better absorbed from the digestive system and can be monitored by blood drug
Table II. Properties of the most common drugs which induce gingival overgrowth

<table>
<thead>
<tr>
<th>Drug</th>
<th>Incidence</th>
<th>Gender or Age Predilection</th>
<th>Reversibility</th>
<th>Present in edentulous sites</th>
<th>Dose which causes overgrowth</th>
<th>Rate of recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>17% if CsA blood levels &lt; 400 ng/mL, 59% if CsA blood levels &gt; 400 ng/mL (Helti et al., 1994) 70% over 2.5 years (Daley et al., 1996) 81% (Friskopp and Klintmalm, 1986)</td>
<td>Greater risk for development in adolescents and young females (Daley et al., 1996; Helti et al., 1994; Seymour and Heasman, 1988)</td>
<td>no recurrence after extraction of teeth (Raiszelschak-Pluss et al., 1983)</td>
<td>not present in edentulous patients (Friskopp and Klintmalm, 1986)</td>
<td>CsA blood levels &gt; 400 ng/mL (Helti et al., 1994; Seymour and Heasman, 1988)</td>
<td>combined drug therapy (concomitant administration of nifedipine) is a significant risk factor for recurrence (Rossmann et al., 1994)</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>varies from 3% to 84.5% (Angelopoulos and Goaz, 1972; Glickman and Lewitus, 1941; Panuska et al., 1981)</td>
<td>more common among younger patients and institutionalized people (Babcock, 1965; Dahlflo and Modèr, 1986)</td>
<td>spontaneous disappearance within few months after discontinuation of phenytoin</td>
<td>not present in edentulous spaces and the overgrowth disappears when teeth are extracted</td>
<td>enlargement in edentulous mouths is rare (Dreyer et al., 1978)</td>
<td>some reports show a direct relationship between the degree of gingival enlargement and dose of the phenytoin (Kapur et al., 1973)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>15% to 83% (Barak et al., 1987; Barclay et al., 1992; Fattore et al., 1991; Slavin and Taylor, 1987)</td>
<td>mild improvement within 1 week of discontinuation of nifedipine, reinstitution of drug following withdrawal results in recurrence within 4 weeks (Lederman et al., 1984)</td>
<td>marked reduction of gingival enlargement require much longer (Lainson, 1986)</td>
<td>does not affect edentulous areas (Lucas et al., 1985; Wilson and Kornman, 1996)</td>
<td>enlargement seen about dental implants (Silverstein et al., 1995)</td>
<td>no recurrence after surgical removal of gingival enlargement can be attenuated with diligent plaque control (Hall, 1985)</td>
</tr>
</tbody>
</table>

*Table II.* Table II. Properties of the most common drugs which induce gingival overgrowth.

- **Incidence:**
  - CsA: 17% if CsA blood levels < 400 ng/mL, 59% if CsA blood levels > 400 ng/mL (Helti et al., 1994).
  - Phenytoin: varies from 3% to 84.5% (Angelopoulos and Goaz, 1972; Glickman and Lewitus, 1941; Panuska et al., 1981).
  - Nifedipine: 15% to 83% (Barak et al., 1987; Barclay et al., 1992; Fattore et al., 1991; Slavin and Taylor, 1987).

- **Gender or Age Predilection:**
  - CsA: Greater risk for development in adolescents and young females (Daley et al., 1996; Helti et al., 1994; Seymour and Heasman, 1988).
  - Phenytoin: more common among younger patients and institutionalized people (Babcock, 1965; Dahlflo and Modèr, 1986).
  - Nifedipine: mild improvement within 1 week of discontinuation of nifedipine, reinstitution of drug following withdrawal results in recurrence within 4 weeks (Lederman et al., 1984).

- **Reversibility:**
  - CsA: no recurrence after extraction of teeth (Raiszelschak-Pluss et al., 1983).
  - Phenytoin: spontaneous disappearance within few months after discontinuation of phenytoin.
  - Nifedipine: marked reduction of gingival enlargement require much longer (Lainson, 1986).

- **Present in edentulous sites:**
  - CsA: not present in edentulous patients (Friskopp and Klintmalm, 1986).
  - Phenytoin: not present in edentulous spaces and the overgrowth disappears when teeth are extracted.
  - Nifedipine: does not affect edentulous areas (Lucas et al., 1985; Wilson and Kornman, 1996).

- **Dose which causes overgrowth:**
  - CsA: blood levels > 400 ng/mL (Helti et al., 1994; Seymour and Heasman, 1988).
  - Phenytoin: > 500 mg/day (Daley et al., 1986).
  - Nifedipine: dose which causes overgrowth.

- **Rate of recurrence:**
  - CsA: combined drug therapy (concomitant administration of nifedipine) is a significant risk factor for recurrence (Rossmann et al., 1994).
  - Phenytoin: some reports show a direct relationship between the degree of gingival enlargement and dose of the phenytoin (Kapur et al., 1973).
  - Nifedipine: recurrence after surgical removal of gingival enlargement can be attenuated with diligent plaque control (Hall, 1985), but not entirely eliminated (Staple et al., 1978).
levels more accurately. Lindholm and Kahan (1993) showed that a 25% increase in the bioavailability of CsA resulted in a significant increase in the one-year graft survival rate and the number of patients who were rejection-free. As such, Neoral®, which is absorbed more predictably and is not degraded by bile acids, is the preferred form of CsA (Sells, 1994).

CsA is a highly hydrophobic cyclic endecapeptide which binds to many types of cells with low affinity (Kₐ = 1-5 x 10⁻⁷ M). Since lymphocytes do not possess specific receptors for CsA, and as its binding affinity is similar to that of phospholipid vesicles, LeGrue et al. (1983) suggested that the hydrophobic CsA molecule intercalates into the phospholipid bilayer of the cell membrane and is split up into intracytoplasmic lipid droplets to facilitate crossing of the plasma membrane.

Histopathological analysis of CsA-induced gingival overgrowth reveals excessive collagen covered by a parakeratinized, multilayered epithelium with elongated rete pegs (Carranza, 1996). The connective tissue may appear highly vascularized with foci of chronic inflammatory cells (Rateitschak-Plüss, 1983), especially plasma cells (Deliliers et al., 1986) that are presumably a response to sub-gingival plaque.

The mode of action of CsA as an immunosuppressant has been investigated and relies on its capacity to inhibit proliferation of activated B and T lymphocytes. However, the mechanism of CsA-induced gingival overgrowth has not been clarified and there is speculation on the role of CsA as an inhibitor of the membrane permeability transition pore in mitochondria (see below).

Both arms of the lymphocytic immune response are suppressed by CsA. T-lymphocyte-dependent immunity (cell-mediated) and B lymphocyte-dependent
immunoglobulin synthesis (humoral immunity) are inhibited by CsA (Dongworth and Klaus, 1982), i.e., upon stimulation, these cells respond less robustly in mounting an immune response. Lymphocytes must be activated to respond appropriately to stimuli and two important cytokines responsible for their activation are IL-2 and IL-4, for T and B lymphocytes, respectively. After IL-2 or IL-4 bind to their cell surface receptors, phosphatidylinositol bisphosphate is cleaved into diacylglycerol and inositol-1,4,5-triphosphate (Isakov et al., 1987). Diacylglycerol can contribute to the downstream activation of protein kinase C which then induces lymphocyte activation. Inositol-1,4,5-triphosphate induces lymphocyte activation by elevation of cytoplasmic calcium through release of calcium from internal cellular stores (King, 1988) and the maintenance of calcium stores by leaking extracellular calcium through the cell membrane (Zilberman et al., 1987). In view of these findings, many investigators have tried to establish a locus of action for CsA as an immunosuppressant based on its role as a modulator of [Ca$^{2+}$]$_i$.

CsA has been reported to not inhibit the generation of calcium signals from intracellular (Kay et al., 1983) or extracellular sources (Gelfand et al., 1987). Based on in vitro studies, its immunosuppressive behavior may be dependent on the availability of calcium in the culture medium and/or on its ability to enter the cell (Kay, 1987). Further, lymphocyte proliferation does not generally occur without generation of a calcium signal. Therefore it was suggested that CsA inhibits proliferation of activated T and B lymphocytes by interfering with calcium permeability of the cell membrane (Figure 1). Since IL-2 is an important activator of T lymphocytes, and as this process is dependent on a rise of [Ca$^{2+}$]$_i$, the finding of highly selective inhibition of IL-2 synthesis by CsA in activated T lymphocytes was significant (Granelli-Piperno et al., 1984).
Figure 1. Examples of possible activation mechanisms of T and B lymphocytes

- Phosphatidyl inositol bisphosphate
- Diacylglycerol
- Inositol-1,4,5-trisphosphate
  - Activation of protein kinase C
  - Elevated cytoplasmic Ca$^{2+}$
  - Production of IL-2 and IL-4
- Lymphocyte activation
Emmel et al. (1989) characterized the CsA-induced IL-2 suppression further and demonstrated that transcription of IL-2 was arrested due to the inhibition of dephosphorylation of cytoplasmic nuclear factors, (e.g. NF-AT). These factors are necessary for transcription of IL-2. CsA binds cyclophilin, a low molecular weight protein. Cyclophilin and CsA inhibit activation of calcineurin, a calcium- and calmodulin-dependent phosphatase which inhibits dephosphorylation of nuclear factors (Hanschumaker et al., 1984). If this process is the route by which CsA causes immunosuppression, then addition of exogenous IL-2 should reverse the immunosuppression as shown by Bunjes et al. (1981). However others have shown only a partial recovery of T lymphocyte activation after addition of IL-2 (Kay and Benzie, 1986). Therefore other signalling intermediates may be sensitive to CsA.

Aside from its main use as an immunosuppressant, CsA unfortunately exhibits many adverse effects in vivo (Table III). While one of the more innocuous side effects is gingival overgrowth, these lesions provide a biological system which can be analyzed in vitro and which suggests alternative modes of action. Apart from its action on lymphocytes, other possible cellular functions of CsA in cells have been investigated and an important locus of cell function seems to be centered on mitochondria. Accordingly, in cells with increased calcium and phosphate ion concentrations, mitochondria may undergo a permeability transition in the conductance of mitochondrial membrane channels which results in the loss of small molecules such as glutathione and calcium. This loss can lead to mitochondrial dysfunction. CsA inhibits these processes (Savage et al., 1991). Crompton et al. (1988) showed that CsA binds to cyclophilin D, a mitochondrial protein that mediates opening of the permeability
**Table III. Adverse effects of CsA therapy**

<table>
<thead>
<tr>
<th>Side Effect</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>1. Nephrotoxicity</td>
<td>-dose-dependent</td>
</tr>
<tr>
<td></td>
<td>-exact mechanism is unclear</td>
</tr>
<tr>
<td></td>
<td>-reversible with dose reduction</td>
</tr>
<tr>
<td>2. Hypertension</td>
<td>-may be secondary to nephrotoxicity</td>
</tr>
<tr>
<td>3. Hepatotoxicity</td>
<td>-usually dose-dependent</td>
</tr>
<tr>
<td></td>
<td>-usually reversible</td>
</tr>
<tr>
<td></td>
<td>-related to toxicity of graft rejection</td>
</tr>
<tr>
<td>4. Infection</td>
<td>-due to immunosupression</td>
</tr>
<tr>
<td>5. Malignant neoplasms</td>
<td>-CsA is not carcinogenic</td>
</tr>
<tr>
<td></td>
<td>-due to immunosuppressive EBV proliferation</td>
</tr>
<tr>
<td></td>
<td>-CsA enhances production of TGF-β which promotes cells to become cancerous</td>
</tr>
<tr>
<td></td>
<td>(Nabel, 1999)</td>
</tr>
<tr>
<td>6. Dermatological</td>
<td>-dose-dependent hypertrichosis on the face and upper trunk in 80% of patients</td>
</tr>
<tr>
<td>7. Neurological toxicity</td>
<td>-tremor which resolves with dose reduction</td>
</tr>
<tr>
<td></td>
<td>-convulsions associated with concomitant use of steroids in children</td>
</tr>
<tr>
<td>8. Gingival overgrowth</td>
<td>-dose-dependent</td>
</tr>
</tbody>
</table>

Adapted from Thiru, S., 1989, pgs 324-359.
transition pore in the mitochondrial membrane (MPTP). This interaction inhibits opening of the MPTP thus preventing transit of small molecules and in consequence preventing the loss of the mitochondrial membrane potential as well. If CsA regulates the traffic of small molecules within the mitochondria, and if mitochondrial ion homeostasis is important in the regulation of collagen metabolism, CsA may play a role in the deregulation of collagen secretion or degradation seen with gingival overgrowth.

An imbalance between collagen synthesis and collagen degradation may lead to drug-induced gingival overgrowth (Jones, 1986). Many investigators have studied the effects of overgrowth-inducing drugs on collagen synthesis and the results have been inconclusive (Newell and Irwin, 1997; Bartold, 1989; McGaw and Porter, 1988; Tipton et al., 1991; McGaw and Ten-Cate, 1983). Drug-induced effects on collagen degradation have also been investigated. Tipton et al. (1991) demonstrated a dose-dependent decrease in collagenase activity after CsA administration, but conflicting studies from Ingman et al. (1994) reported that matrix metalloproteinase-1 (MMP-1) and MMP-3 cannot be detected in inflamed marginal gingival tissue of patients with periodontal disease. Thomason et al. (1998) demonstrated that MMPs are not detected within fibroblasts from overgrown gingiva. As collagen degradation is mediated by both a collagenase-dependent extracellular pathway and a collagenase-independent intracellular pathway (i.e. phagocytosis), CsA-induced deregulation of collagen phagocytosis may be important in the etiology of gingival overgrowth. Accordingly, the mechanisms of fibrosis and phagocytosis will be reviewed below and the possible deregulation of these processes by CsA will be introduced.
3. Common mechanisms for fibrosis

a) The development of fibrosis

Fibrosis is defined as the excessive deposition of matrix components that results in distortion or disruption of normal tissue architecture and compromised tissue function. The development of fibrosis follows a similar pathway to that of normal wound healing but resolution (or scarring) is impaired (Mutsaers et al., 1997). Fibrosis can be induced by several growth factors, including insulin-like growth factor-1 (Goldstein et al., 1989) and transforming growth factor β1 (Border et al., 1992). After the administration of CsA, the expression of both insulin-like growth factor-1 and transforming growth factor β1 is increased, thus promoting fibrosis (Johnson et al., 1999). As mentioned above, gingival fibrosis may be the result of an increased rate of collagen synthesis and/or decreased rate of collagen degradation. One aspect of degradation which may be significant in the context of CsA-induced gingival overgrowth is intracellular degradation by phagocytosis. Phagocytosis is particularly important in tissue remodelling under physiological conditions such as the final stages of wound healing or during scar resolution. In both examples, the degradation of wound collagen and other matrix proteins by phagocytic cells is mediated by phagocytosis (Miganatti et al., 1996). In pathological conditions, such as fibrosis, the rate of collagen phagocytosis may be decreased, thereby resulting in net collagen overgrowth.

When phenytoin- or CsA- induced fibrosis were examined at the ultrastructural level with stereologic methods, both Hall and Squier (1982) and McGaw and Porter (1988) observed a decreased amount of phagocytosed collagen within the gingival cells. McCulloch and Knowles (1993) provided further in vitro support for these
observations when phagocytosis of collagen-coated beads was inhibited in fibroblasts treated with phenytoin or nifedipine.

**B. Mechanisms of phagocytosis in fibroblasts**

1. **How is phagocytosis mediated?**

   Gingival overgrowth is a term which describes the excessive accumulation of collagen in affected tissues (Figure 2). As such it is useful to examine the mechanisms of collagen turnover in physiological conditions.

   The fibroblast is the cell responsible for the synthesis and secretion of collagen in gingival overgrowth, however, the fibroblast can also act as a resorptive cell when it degrades collagen by phagocytosis. Indeed phagocytosis by gingival fibroblasts is the main mechanism of physiological collagen degradation *in vivo* (Melcher and Chan, 1981) and due to the ubiquitous occurrence of collagen phagocytosis by fibroblasts, they may be considered as professional phagocytes (Rabinovitch, 1995).

   Collagen degradation may be mediated by matrix metalloproteinases (MMPs; e.g. collagenase, gelatinase, and stromelysin), cysteine proteinases (e.g. cathepsin B and L), or serine proteinases (e.g. plasmin and plasminogen activator). As collagen degradation is a common process in many physiological and pathological events, it is not surprising that the remodeling of collagen exhibits wide variations of rate. For example, MMPs have been implicated in rapid remodeling or digestion of large amounts of collagen during the loss of tissue architecture in acute inflammatory processes (Birkedal-Hansen *et al.*, 1993), tumor cell invasion and metastasis (Aznavoorian *et al.*, 1993), and uterine involution (Brandes and Anton, 1969). This
Figure 2. Maintenance of collagen homeostasis

Collagen Metabolism in the Balance

MMPs
phagocytosis

Resorption

Steady State

Synthesis
pathway is also called the collagenase-dependent extracellular pathway (Everts and Beertsen, 1988; Sodek and Overall, 1988).

MMPs are not usually associated with equilibrium conditions in which collagen synthesis is balanced by collagen degradation. More commonly, physiological turnover of soft connective tissues involves phagocytosis of collagen fibrils and their final intracellular lysosome-associated degradation by cysteine proteinases. This pathway can be regulated by growth factors and cytokines and is called the collagenase-independent intracellular pathway (Everts and Beertsen, 1988; Sodek and Overall, 1988).

Although gingival collagen does undergo rapid turnover, this does not lead to the net loss of tissue because the collagen turnover is in a steady-state (Figure 2). Several studies have demonstrated that collagen degradation in physiological conditions employs the intracellular route. Svoboda et al. (1981) showed that cross-banded collagen fibrils were present in fibroblast lysosomes, especially in gingiva and periodontal ligament. They also demonstrated a positive correlation between the amount of intracellular collagen and the rate of collagen turnover. Further, calculation of the digestion time of internalized collagen (30 minutes); (Everts et al, 1989) is consistent with an expected half-life of collagen corresponding to the turnover time as assessed biochemically (Everts and Beertsen, 1988; Everts et al., 1989). Finally, Liu et al. (1995) used mutant mice with collagenase-resistant type I collagen to show that the collagenase-dependent extracellular pathway played an insignificant role except during periods of extensive connective tissue turnover. As mice aged, signs such as fibrosis of the dermis and impaired postpartum involution of the uterus were evident. Therefore,
when steady state collagen turnover predominates, as is the case during growth and development, the intracellular collagenase-independent pathway prevailed, but when rapid collagen degradation is necessary, the extracellular collagenase-dependent pathway prevailed (Figure 3).

The collagenase-independent intracellular pathway of collagen phagocytosis involves an initial recognition of the fibril by membrane-bound receptors/integrins (McKeown et al., 1990). This binding step initiates actin filament remodeling (Isberg and Tran Van Nhieu, 1995) and an increase in the expression and release of gelatinase A (Larjava et al., 1993). This step is followed in turn by segregation and partial digestion of the fibril and/or its surrounding non-collagenous proteins by matrix metalloproteinases and finally lysosomal fusion with the phagosome containing partially digested collagen fibrils and eventual degradation by cysteine proteinases within the lysosomes.

2. Physiological Regulation

a) Growth Factors and Cytokines

Van der Zee et al. (1995) examined the effect of several growth factors and cytokines on phagocytosis in vitro. They concluded that only two substances had an effect on phagocytosis: (i) interleukin-1α inhibited phagocytosis and (ii) transforming growth factor-β enhanced phagocytosis. When combined, they were antagonistic. Chou et al. (1996) investigated the role of tumor necrosis factor-α and showed inhibition of collagen adherence and phagocytosis.
Figure 3. Regulation of collagen degradation

MMPs
↑ interleukin-1
↑ tumor necrosis factor-α
↑ concanavalin A
↓ transforming growth factor-β

Phagocytosis
↑ concanavalin A
↑ transforming growth factor-β
↑ tumor necrosis factor-α
↓ interleukin-1α

Collagen fibres
Hormones may also influence phagocytosis: (i) cortisol decreases collagen phagocytosis in pig synovial tissue (Fell et al., 1986) and (ii) increased collagen phagocytosis was associated with post-partum uterine involution (Brandes and Anton, 1969), a process which is associated with increased progesterone.

b) Integrins

Integrins are cell surface heterodimeric adhesion receptors which bind to extracellular matrix proteins. The integrins on fibroblasts which are involved in recognition, adhesion, and signalling in response to collagen are the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ (Kirchhofer et al., 1990). However, in gingival fibroblasts the $\alpha_2\beta_1$ is considered to be particularly important for the process of collagen phagocytosis (Lee et al., 1996). Using flow cytometry, they initially showed that $\alpha_2$ expression was ubiquitous among phagocytic cells and collagen phagocytosis was inhibited by blocking access to the $\alpha_2\beta_1$. The blockade of other integrins did not alter phagocytosis. To investigate the role of $\alpha_2\beta_1$, after binding of collagen, fibroblasts were incubated with a large number of collagen-coated beads to occupy as many $\alpha_2\beta_1$ binding sites as possible. The levels of $\alpha_2$ and phagocytic efficiency were decreased. Evidently, collagen must bind to the $\alpha_2$ subunit before being internalized. Evidence was also presented to suggest that the $\alpha_2$ subunit was internalized in the process of collagen phagocytosis as bead internalization led to a decrease in surface levels of $\alpha_2$, which were restored within 3 hours. Further, Chou et al. (1996) showed that tumor necrosis factor-$\alpha$ inactivated $\alpha_2\beta_1$, thereby inhibiting collagen binding and phagocytosis. Despite these data, the presence of $\alpha_2\beta_1$ alone is not sufficient to mediate phagocytosis. This is not surprising as other collagen
receptors may regulate different activities. Lee et al. (1996) suggested that the $\alpha_1$ subunit may regulate the initial contact of $\alpha_2$ with collagen since the level of collagen-coated beads was shown to be twice as high in cells with high $\alpha_1$ staining than with high $\alpha_2$ staining. The efficiency of phagocytosis was also dependent on the number of available collagen-coated beads and $\alpha_2$. With low bead:cell ratios, the presence of $\alpha_2\beta_1$ did not necessarily promote phagocytosis, suggesting an effect due to collagen-bead availability. Further, phagocytosis seemed to be inhibited unless the $\alpha_2\beta_1$ integrin was present in at least 60% of cells.

C. Calcium Regulation of Phagocytosis

1. Calcium ion regulation

Calcium levels are very tightly controlled within the cell since intracellular calcium concentrations are approximately 100 nM and yet extracellular calcium concentration is $\sim$ 1-2 mM. To maintain calcium homeostasis the cell possesses many pathways of regulated entry and exit for calcium but there are fundamentally two sources of calcium. Calcium can be released from internal stores within the cell or can be allowed to enter the cell from the extracellular medium when channels in the plasma membrane are more permeable. Although the studies on calcium signalling reviewed below refer mainly to "professional" phagocytes, namely macrophages and neutrophils, Rabinovitch (1995) notes that due to the ubiquitous occurrence of collagen phagocytosis by fibroblasts, they may also be considered as professional phagocytes. In the absence of a substantial data set on calcium signalling in fibroblasts, I review data on macrophages and neutrophils to provide some background on calcium signalling in phagocytosis.
Murine macrophages were used by Young et al. (1984) to illustrate the need for tight control of \([\text{Ca}^{2+}]_i\) in the execution of phagocytosis. The clamping of both intracellular and extracellular calcium to low levels led to the inhibition of phagocytosis. When only extracellular calcium was chelated, there was only a partial decrease of phagocytosis. This underlines the need for calcium fluxes from intracellular stores as well. An excessive increase in \([\text{Ca}^{2+}]_i\) by ionomycin treatment also led to inhibition of phagocytosis. After binding of ligand to receptors, \([\text{Ca}^{2+}]_i\) was increased and the amplitude of this increase was directly proportional to the extent of receptor aggregation. Kobayashi et al. (1995) obtained similar results in human neutrophils. To induce maximum phagocytosis, both calcium entry from the extracellular medium and release from intracellular stores were necessary.

From both of these reports it is evident that the same controls that are used to maintain calcium homeostasis in the absence of a phagocytic target must be maintained to execute phagocytosis. Although individual steps of phagocytosis may seem to promote separate rises or falls of calcium in different cell compartments, many types of cells contain signalling networks that involve a great deal of intracellular communication (Berridge et al., 1999)

Kwiatkowska and Sobota (1999) have described the signalling events for phagocytosis specific to Fcγ receptors. Once the receptor binds ligand, a phagocytic signal is generated and the cytoskeleton is remodelled to form a phagosome. Receptor binding induces the activation of tyrosine kinase and protein kinase C. Phosphorylation of cytoplasmic residues on Fcγ serves to promote further binding of more ligand and the phagocytic signal is amplified. In macrophages, protein kinase C accumulates around
phagosomes (Allen et al., 1996) and isoenzymes of the kinase are activated by calcium (Mochly-Rosen and Gordon, 1998). Consequently, calcium can amplify the phagocytic signal further, but these observations are in the context of Fcγ signalling and not integrins.

After formation of the phagosome (which contains the internalized particle, the integrin and parts of the plasma membrane), the phagosome fuses with a lysosome which will provide the acidic environment necessary for degradation by proteases. Fusion is regulated by calcium. For example in human neutrophils, Jaconi et al. (1990) showed a requirement for an increase in [Ca²⁺]i to promote fusion of phagosomes and lysosomes. Initially, Bengtsson et al. (1993) suggested that [Ca²⁺]i does not control the initial actin polymerization seen during receptor-ligand adhesion and subsequent phagocytosis, but [Ca²⁺]i does control the depolymerization of the actin network so as to promote fusion of the phagosome and lysosome. Definitive evidence on this issue has not been obtained because experimental-specific and cell-type variations of fusion control preclude direct comparisons.

2. Calcium response to collagen phagocytosis

In the previous section, I have examined the regulatory effect of calcium on phagocytosis. In this section the effect of calcium on phagocytosis in different cellular compartments will be reviewed. Within the cytosol, the formation of receptor-ligand complexes and pseudopods generates rises in [Ca²⁺]i within seconds. In ongoing phagocytic processes, [Ca²⁺]i remains elevated especially around phagosomes. As expected there is no rise in [Ca²⁺]i when both intracellular and extracellular calcium was
chelated (Bengtsson et al., 1993). Thus \([\text{Ca}^{2+}]_i\) rises do not originate solely from intracellular stores. It is important to consider also the uptake of extracellular calcium within endosomes. In fact, Gerasimenko et al. (1998) showed that endosomes delivered a substantial amount of calcium into the cell within a few minutes.

Direct evidence to illustrate the behaviour of calcium within the endoplasmic reticulum during phagocytosis is not available but several groups have shown a physical movement of the receptors involved in calcium regulation during phagocytosis (Favre et al., 1996). Calcium release from intracellular stores is mediated through inositol-1,4,5-triphosphate (InsP₃) and ryanodine receptors. Calcium accumulation in stores is mediated by sarcoplasmic/endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPases, some of which are inhibited by thapsigargin. During phagocytosis in HL-60 cells, the SERCA2 receptor, calreticulin (a calcium-buffering protein)(Stendahl et al., 1994) and the InsP₃ receptor were translocated to the periphagosomal space (Favre et al., 1996). This suggests that calcium within the ER may play a regulatory role in phagocytosis since the receptors which regulate calcium levels have translocated in order to remain proximal to the phagosome and can thereby act upon it.

Putney (1986) first developed the notion that the process of emptying and replenishing calcium stores must be linked. He proposed that empty calcium stores must somehow trigger the opening of store-activated channels (SOCs) in the plasma membrane and allow calcium into the cell. He thus coined the term 'capacitative calcium entry'. The question of how the signal of empty calcium stores is transmitted to the SOCs is still unanswered although Irvine et al. (1990) and Berridge et al. (1995) suggested a mechanism called 'conformational coupling'. When InsP₃ binds to the
InsP₃ receptor on the endoplasmic reticulum, calcium is released from the internal stores. The InsP₃ receptor then sustains a conformational change. It is this change that is detected by the SOCs which are located physically close to InsP₃ receptors. Indeed van Rossum et al. (2000) have shown that the interaction of InsP₃ receptors and SOCs control calcium entry through the plasma membrane is possibly due to their close approximation.

a) Calcium response within the mitochondria

There is a paucity of literature on the mitochondrial calcium response due to phagocytic stimuli. However, there is indirect evidence describing the interaction and interdependence between calcium in the mitochondria and the endoplasmic reticulum. Subsequent to depletion of calcium in internal stores, ATP is needed to drive the refilling of stores by the SERCA (Gamberucci et al., 1994). This energy is derived from the energy-producing mitochondria (Gamberucci et al., 1994). An additional mitochondria-endoplasmic reticulum interaction exists in the context of the InsP₃ and ryanodine receptors. These receptors indirectly influence [Ca²⁺], due to their capacity to release calcium from internal stores when bound. The extent to which they cause calcium release depends on the calcium buffering capacity of mitochondria (Bezprozvanny et al., 1991). A lower [Ca²⁺] will lead to binding of the InsP₃ and ryanodine receptors and thus cause release of calcium into the cytosol whereas a higher [Ca²⁺] leaves the receptors unbound (Landolfi et al., 1998). These results demonstrate that mitochondrial function is important for appropriate calcium responses within the cell and is needed to amplify this response.
3. Mitochondrial calcium metabolism

Mitochondria have piqued the interest of researchers as these are the only organelles (apart from the nucleus) that possess their own genetic material and are also the essential "powerhouses" of the cell. They are excitable organelles capable of generating calcium signals and actively participate in cellular calcium signalling (Ichas et al., 1997; Babcock et al., 1997). There are several pathways for calcium influx and efflux as shown in figure 4. The predominant pathways for calcium uptake consist of the calcium uniporter and the rapid mode of uptake. Calcium transport by the calcium uniporter is dependent on the mitochondrial membrane potential that can be generated by redox-linked H\(^+\) pumps. Consequently the more that H\(^+\) is pumped out of the mitochondria, the greater is the negative charge inside the mitochondria. To preserve the potential across the mitochondrial membrane, more Ca\(^{2+}\) can be pumped into the mitochondria and removed from the cytosol. The calcium uniporter is thus driven by an electrochemical gradient comprised of a negative mitochondrial inner membrane potential and a low concentration of calcium in the matrix. The rapid mode uptake also transports calcium into the mitochondria but seems to show more specificity when dealing with brief physiologic stimuli (Bernardi, 1999).

There are three pathways for calcium efflux, the Na\(^+\)-independent pathway for Ca\(^{2+}\) efflux (NICE), the Na\(^+\)-dependent pathway for Ca\(^{2+}\) efflux (NCE), and the membrane permeability transition pore (MPTP). The NICE is possibly a H\(^+\)/ Ca\(^{2+}\) antiporter which requires a transmembrane potential as part of its driving force. It conducts very slow movement of ions and mediates steady-state calcium cycling in
Adapted from Bernardi, 1999.

**Figure 4. Pathways for calcium transport in mitochondria.** H+ pump, redox-linked H+ pumps; UN, Ca2+ uniporter; RaM, rapid uptake mode; NICE, Na+ -independent pathway for Ca2+ efflux; NCE, Na+ -dependent pathway for Ca2+ efflux; NHE, H+/Na+ exchanger; MPTP, membrane permeability transition pore, CsA, cyclosporin A; cyp, cyclophilin; [Ca2+]m; mitochondrial Ca2+ concentration; [Ca2+]c; cytosolic Ca2+ concentration.
mitochondria. The NCE is a Na\(^+\)/Ca\(^{2+}\) exchanger that mediates physiological calcium cycling through interplay with the NHE (H\(^+\)/Na\(^+\) exchanger). The MPTP is a large channel with a pore diameter of \(-3\) nM (Haworth and Hunter, 1979). When the pore is open, this is described as a permeability transition and solutes with molecular mass \(\leq 1500\) Da are allowed to pass into and out of the mitochondria. The pore opening depends on transmembrane potential difference, matrix pH, and mitochondrial calcium concentration ([Ca\(^{2+}\)]\(_m\)). Prolonged opening of the pore leads to dissipation of the mitochondrial membrane potential, leakage of cytochrome c and ultimately cell death. This can be simulated \textit{in vitro} with the addition of carbonyl cyanide \(m\)-chlorophenyl hydrazone (CCCP), a protonophore which causes a short circuit of the electrochemical potential for protons. As a result, the ion concentration gradient is dissipated and the electrical potential can no longer be maintained. The pore favours opening with increases of [Ca\(^{2+}\)]\(_m\) and closing with CsA. As mentioned above, CsA induces cyclophilin D to detach from the MPTP (Nicolli \textit{et al.}, 1996) and thus facilitates closure of the MPTP. In view of their involvement in the maintenance of calcium levels and their interaction with the endoplasmic reticulum, mitochondria evidently play an important role in calcium homeostasis and intracellular communication. Indeed using aequorin to target and report mitochondrial calcium ([Ca\(^{2+}\)]\(_{mito}\) specifically, mitochondria can sense microdomains of high [Ca\(^{2+}\)]\(_c\) which result when IP3-sensitive channels release calcium from the ER (Rizzutto \textit{et al.}, 1993).

As described above, CsA inhibits the MPTP and by doing so inhibits the passage of cations via the MPTP. As a result, calcium either cannot exit the mitochondria using this pathway or the rate of efflux is slowed down. Since dissipation of the mitochondrial
membrane potential can lead to cell death, CsA can prevent this event and this has been shown in vitro in gingival fibroblasts (Kulkarni et al., 1998). Sullivan et al. (1999) showed that the addition of CsA to cells subjected to traumatic brain injury is neuroprotective by preventing mitochondrial dysfunction. When they looked at individual synaptosomes, there was a lower [Ca$^{2+}$]$_{mito}$ in CsA-treated cells than controls. They proposed that because CsA inhibits the MPTP, less calcium is allowed to leave the mitochondria and this prevents the cycling of calcium across the mitochondrial membrane using other channels. As the effect of CsA on [Ca$^{2+}$]$_{mito}$ does not involve inhibition of calcineurin (which is the case with immunosuppression; Nicolli et al., 1996), the immunosuppressive effects of CsA and its ability to inhibit the MPTP are likely separate. To date, no other mitochondrial functions other than inhibition of the MPTP appear to be affected by CsA (Bernardi et al., 1994; Zoratti and Szabó, 1995).

4. Calcium responses to CsA

The response of [Ca$^{2+}$]$_i$ to CsA has received little attention although rat cardiac cells show an upregulation of angiotensin II receptors and an increased [Ca$^{2+}$]$_c$ that is independent of the immunosuppressive action of CsA, (i.e., not dependent on calcineurin; Avdonin et al., 1999). They proposed that these data could explain CsA-induced hypertension and nephrotoxicity due to the vasoconstriction induced by an augmentation of angiotensin II receptors. In addition, the same group examined the effect of CsA treatment on human vascular smooth muscle cells but did not detect any change in [Ca$^{2+}$]$_i$. 
Whereas the effect of CsA on the mitochondria was determined to be separate from its action as an immunosuppressant in that it binds calcineurin, this is not the case in the sarcoplasmic reticulum. In rat hearts, CsA mediates the upregulation of calcineurin. By binding more calcineurin, there should be reduced phosphorylation. Park et al. (1999) used this finding to explain a decrease in the maximal ryanodine binding and an increase in the dissociation constant ($K_d$) of ryanodine binding. Since calcium-release channels in the sarcoplasmic reticulum need to be phosphorylated in order to function, decreased phosphorylation due to higher levels of calcineurin leads to less ryanodine binding and consequently less calcium release from the internal stores.

D. Model systems and rationale

An in vivo model to quantify collagen phagocytosis using electron microscopic stereology was used by Svoboda et al., (1981). After obtaining a magnified version of a stained micrograph of the area of interest, two grids were overlayed on this micrograph. With the coarse grid, the point intersections was used to estimate the number of areas falling on cytoplasm and extracellular collagen and the fine grid was used to count the number of areas falling on individual collagen profiles. This analytical system provides a good approach to assess collagen phagocytosis in vivo, but it is both labour-intensive and carries many assumptions on uniformity of intracellular transport, intracellular degradation rates and cellular homogeneity. In contrast, the collagen-coated bead model is an in vitro system used in conjunction with flow cytometry. A cell which has engulfed a collagen-coated fluorescent bead will report a larger fluorescence intensity per cell. The advantages of using this model are; (i) the ability to analyze a large
number of samples in a very short time; (ii) the lack of bias as to whether phagocytosis has occurred and; (iii) the ability to report how many beads are internalized per cell. (McKeown et al., 1990).

As mentioned above, in vivo models are very labour-intensive and cannot provide kinetic analysis of the data. As well, investigators do not agree on whether incomplete phagocytosis (i.e. just cytosegregation) should be considered a positive finding. Svoboda et al., (1981) argued that cytosegregation should be included as this was a necessary step for phagocytosis to occur. An important limitation of the bead model is that it is an in vitro reductionist system and, consequently some component(s) of the more complex in vivo setting may be omitted.

McCulloch and Knowles (1993) used the collagen-coated bead model to show that cells from fibrotic lesions have decreased phagocytic efficiency. They also demonstrated a reduction in phagocytosis when control cells were treated with fibrosis-inducing drugs, including phenytoin. These findings provide the basis for my studies.
II. Statement of the problem

Literature on the cellular effects of CsA suggests a defined mechanism for its main action as an immunosuppressant. However the modes of action which mediate many of the adverse effects resulting from CsA administration have not been described. Gingival overgrowth is an adverse effect that can be studied in vitro using cultured gingival fibroblasts. Notably, fibroblasts are central to the processes of collagen synthesis and degradation and study of collagen metabolism and its deregulation by CsA is a logical approach to understanding the pathobiology of this disorder.

CsA acts on the mitochondrial permeability transition pore and consequently affects calcium transport. This is relevant as two other groups of drugs which cause gingival overgrowth also affect calcium metabolism (i.e. anticonvulsants and calcium channel blockers). As a result, I have considered a role for calcium in the mechanism of CsA-induced gingival overgrowth. My hypothesis is that the induction of gingival overgrowth by cyclosporin A involves deregulation of collagen turnover due to a disturbance of calcium utilization in the mitochondria of gingival fibroblasts. The principal rationale for this study is that the mechanism by which CsA induces gingival overgrowth is not known and consequently, rational approaches to treatment are not possible. If CsA regulates [Ca^{2+}] and/or mitochondrial functions, this may suggest a possible locus of action at which gingival overgrowth is initiated.
To test my hypothesis, I investigated the effect of CsA on the phagocytic capacity of human gingival and Rat2 fibroblasts. Next, I studied the effect of CsA on calcium regulation in different cellular compartments including the cytosol, mitochondria, and SERCA stores. I used mitochondria-depleted cells to explore the functional relationship between mitochondrial calcium regulation in other cell compartments and phagocytosis. My specific objectives were to:

1. Determine if CsA can affect the phagocytic capacity of human gingival fibroblasts and Rat2 fibroblasts and if mitochondria are important for phagocytosis.

2. Establish an *in vitro* system to measure CsA regulation of \([\text{Ca}^{2+}]_i\) in gingival fibroblasts and Rat2 fibroblasts responding to collagen-coated beads.

3. Determine if perturbation of calcium signalling in the cytosol and in mitochondria regulates collagen bead internalization.

4. Evaluate \([\text{Ca}^{2+}]_{\text{mito}}\) and \([\text{Ca}^{2+}]_{\text{SERCA}}\) and their responses to collagen bead stimulation and CsA deregulation and determine if integrin-induced calcium fluxes require mitochondrial function and \([\text{Ca}^{2+}]_{\text{mito}}\) fluxes.
III. Materials and Methods

Materials

Carbonyl cyanide m-chlorophenyl hydrazone and cyclosporin A were obtained from Sigma (St. Louis, MO). Anti-human cytochrome oxidase subunit I mouse monoclonal antibodies (clone # 6403), BAPTA/AM, fura 2/AM, JC-1, mag-fura 2/AM, MitoTracker Green, Pluronic® F-127 and rhod 2/AM were obtained from Molecular Probes (Eugene, OR). Ionomycin and thapsigargin were obtained from Calbiochem (LaJolla, CA).

Fluorescent and non-fluorescent 2 μm diameter polystyrene beads were obtained from Polysciences (Warrington, PA) or from Molecular Probes (Eugene, OR). Bovine collagen (>95% type I) was obtained from Celtrix (Palo Alto, CA).

Methods

Cell Cultures

Human gingival fibroblasts (HGF; 8-15th passage) were derived from primary explant cultures as described (Pender and McCulloch, 1991). Cells from passages 8-15 were grown as monolayer cultures in T-75 flasks (Costar, Mississauga, ON) containing alpha minimal essential medium, 15% heat-inactivated fetal bovine serum (v/v; Flow Laboratories, Maclean, VA), and a 1:10 dilution of an antibiotic solution (0.17% wt/vol; penicillin V, 0.1% (wt/vol) gentamicin sulfate, 0.01 μg/mL amphotericin). The cells were maintained at 37°C in a humidified incubator containing 5% CO2 and were passaged with 0.01% trypsin (Gibco BRL, Burlington, Ontario, Canada). Twenty-four hours before each experiment, cells were harvested with 0.01% trypsin and ~50,000 cells were plated onto 0.1-mm-thick, 31-mm-diameter, round glass coverslips (VWR, Toronto, ON) in 35-mm-diameter Petri dishes (Falcon, Becton Dickinson, Mississauga, Ontario, Canada) for calcium measurements. Rat2 cells (ATCC CRL 1764; American Type
Culture Collection, Rockville, MD) were incubated in Dulbecco's modified Eagle's medium (DMEM; high glucose) containing 10% fetal bovine serum (FBS) and a 1:10 dilution of the same antibiotic solution described above. The cells were propagated, maintained and prepared for calcium measurements as described above. According to the ATCC, Rat2 cells are phenotypically normal by all criteria tested and are diploid. They have been used in these experiments because of their ease of passaging and their phenotypic similarity to human periodontal cells based on collagen synthesis, collagen phagocytosis, osteopontin synthesis and morphology (Hui et al., 1997).

**Mitochondrial Depletion**

Rat2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; high glucose) containing 10% fetal bovine serum (FBS) and a 1:10 dilution of the same antibiotic solution described above. Cells were grown in the presence of 100 ng/mL of ethidium bromide for 20 passages as described (Biswas et al., 1999). The dose was increased gradually to 1000 ng/mL between passages 21-30. The cells were maintained at 1000 ng/mL of ethidium bromide for at least 4 weeks before using for experiments.

**[Ca\(^{2+}\)]\(_i\) measurement**

For measurement of whole cell [Ca\(^{2+}\)]\(_i\), cells on glass cover slips were incubated at 37°C with 3 μM fura 2/AM in bicarbonate-free medium for 25 minutes. The attached cells were washed twice with bicarbonate-free calcium buffer which contained 145 mM NaCl, 5 mM KCl, 5 mM MgCl\(_2\), 10 mM d-glucose, 10 mM HEPES, and 1 mM CaCl\(_2\), with pH adjusted to 7.4 and an osmolarity of 291 mosM. CaCl\(_2\) was omitted from the buffer
solution where indicated. Inspection of cells by fluorescence microscopy demonstrated no compartmentalization of dye nor were estimates of $[Ca^{2+}]_i$ changed by loading of cells with fura 2AM in Pluronic detergent. Consequently, estimates of $[Ca^{2+}]_i$ using these loading methods very likely represent cytoplasmic $[Ca^{2+}]_i$ and not simply calcium ion concentration in membrane-bound compartments such as endosomes. Measurements of $[Ca^{2+}]_i$ were made on single cells using a Nikon Diaphot II inverted microscope optically interfaced to an epifluorescence spectrofluorimeter and analysis system (Photon Technology International, London, ON) operating on a 386SX personal computer. Fura 2-loaded cells were excited at alternating (approximately 100 Hz) wavelengths of 346 and 380 nm from dual monochromators with slit widths set at 2 nm. Emitted fluorescence was collected by a 40X quartz 1.30-NA oil-immersion Nikon Fluor objective and passed through a 520/30-nm barrier filter (Omega Optical, Brattleboro, VT). Signals from the photomultiplier tube (model D104; Photon Technology) were recorded at ≥ 5 points/s. A variable-aperture intrabeam mask was used to restrict measurements to single cells.

Estimates of $[Ca^{2+}]_i$ independent of the precise intracellular concentration of fura2 were calculated from dual-excitation emitted fluorescence according to the equation of Grynkiewicz et al. (1985) (i.e., $[Ca^{2+}]_i = K_d \times Sf2/Sb2 \times (R-R_{\text{min}})/(R_{\text{max}} - R)$ (nM), where R is fluorescence ratio, $R_{\text{max}}$ is maximum R, $R_{\text{min}}$ is minimal R, and Sf2/Sb2 is the ratio of the fluorescence intensity of the free and bound dye at 380-nm excitation). The dissociation constant ($K_d$; 305 nM) and Sf2/Sb2 ratio (4.09) were calculated from 11 excitation wavelength scans of 1 μM fura 2 free acid (Molecular Probes) in buffers with $[Ca^{2+}]$ ranging from 0.0 to 39.8 mM ($Ca^{2+}$ calibration kit; Molecular Probes). $R_{\text{max}}$ of
346/380 was measured after saturation of intracellular fura 2 with Ca\(^{2+}\) by adding 3 \(\mu\)M ionomycin to allow equilibration with extracellular Ca\(^{2+}\). \(R_{\text{min}}\) was measured in ionomycin-treated cells after complete dissociation of fura 2 from [Ca\(^{2+}\)]\(_{i}\) by adding 2 mM EGTA to the calcium buffer bathing the measured cell and was estimated at 0.568.

**Dye loading with mag-fura 2/AM**

Dye loading and data collection is similar to that described for fura 2/AM except that intensity values are not converted to [Ca\(^{2+}\)]\(_{\text{SERCA}}\) values. Instead, the ratio of fluorescence intensity collected at 346/380 nm was used to calculate changes in [Ca\(^{2+}\)]\(_{\text{SERCA}}\) as described previously (Hofer et al., 1998).

**Dye loading with rhod-2/AM**

Rhod-2 is a single wavelength excitation and single wavelength emission dye. As ratio imaging cannot be used to overcome problems of dye leakage and photobleaching over time with this dye, the fluorescence intensity of rhod-2 was measured in small (\(\sim 4\mu\)m\(^2\)) sampling grids in the lamellipodia of well-spread cells. Nucleoli (which stain brightly with rhod 2) were not included in the measurement. Background fluorescence and adjustments for time-dependent photo-bleaching were made for each measurement calculating the bleach-induced rate constant (i.e. time-dependent loss of fluorescence) in untreated cell samples that were not treated with collagen-coated beads.
Dye loading and colocalization with rhod-2, MitoTracker Green and JC-1

Rat2 fibroblasts were co-loaded with 100 nM MitoTracker Green and 4.5 μM Rhod-2/AM in 0.005% pluronic gel for 30 minutes in a 37°C incubator containing 5% CO₂. The cells were washed twice and left in calcium buffer before imaging. The magnitude of fluorescence of stained samples was observed on an inverted microscope (Nikon) equipped with a CCD camera (Pentamax) and analyzed using Winview (Princeton) software program or photographs were taken.

Collagen coating of beads

Experiments with collagen-coated beads were conducted with cells grown in T-25 tissue culture flasks. All bead incubations with cells were conducted at 37°C with 5% CO₂, 95% humidity. Aliquots (80 μL) of either yellow-green fluorescent (excitation max = 490 nm, emission max = 515 nm) or non-fluorescent 2 μm diameter polystyrene beads were incubated with 1 mL of a 2.9 mg/mL acidic bovine collagen solution to produce collagen-coated beads. To neutralize the collagen solution to pH=7.4 and thereby induce fibril assembly on beads, an aliquot of 1 N NaOH was added to 1 mL of collagen with beads and mixed by vortexing. The beads were incubated at 37°C for 10 minutes, followed by centrifugation at 8000 x g for 2 minutes. The supernatant was removed and the pellet of coated beads was resuspended in 1 mL phosphate-buffered saline (PBS). After sonication of the coated beads to produce single bead suspensions, the number of beads per mL was estimated by counting beads in an aliquot of the sample with a hemocytometer. Cells were incubated with collagen-coated fluorescence beads at a ratio of 2 beads per cell for phagocytosis experiments and assessed by flow cytometry.
For calcium experiments, non-fluorescent collagen-coated beads were added to cells at a ratio of ~4 beads per cell.

**Flow cytometric analysis of phagocytosis**

Binding of collagen-coated beads to cells was assessed by flow cytometry. Cell samples were analyzed with a FACStar Plus flow cytometer (Becton Dickinson FACS Systems, Mountain View, CA) at a sheath pressure of 11 psi with an Innova 70 argon laser (Coherent Laser Products, Palo Alto, CA) at a light regulation mode setting of 250 mW and a wavelength of 488 nm. Emitted fluorescence was directed through a short pass 560 beam splitter (all filters and beam splitters from Omega Optical Inc., Brattleboro, VT) and a 530DF30 filter for green fluorescence in channel 1. Photomultiplier tube voltage settings were determined for each experiment on the basis of thresholds established from negative and positive control samples for each sample that was analyzed. To reduce the inclusion of cells with loosely bound beads during the process of phagocytosis measurements, cells were washed with calcium and magnesium-free PBS and trypsinized.

**Flow cytometric analysis of mitochondrial membrane potential ($\Psi_m$).**

Changes in the $\Psi_m$ were analyzed using 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolecarbocyanine iodide (JC-1). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Psi_m$ and forms J aggregates which have characteristic absorption and emission spectra (Smiley *et al.*, 1991). Untreated cells and cells treated with CsA were incubated in 3 mL of PBS supplemented with 10%
serum containing 0.5 μM JC-1 for 1 hour. As a positive control for reduction of \( \Psi_m \), a group of cells were treated with the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) after labeling with JC-1 (Zamzami et al., 1995). Cell suspensions were prepared for flow cytometry (Kulkarni and McCulloch, 1995) and the 488-nm line of an argon ion laser was used for excitation. Orange and green emitted fluorescence were collected through 585/42 (FL2)- and 530/30-nm (FL1) bandpass filters. The FL1-FL2 compensation was 4% and the FL2-FL1 compensation was 44%. Flow cytometry analysis was performed on a FACSTAR Plus flow cytometer and analyzed using LYSYS II software (Becton-Dickinson Immunocytochemistry Systems, Mountainview, CA). After gating out small-sized (i.e. noncellular debris), 10,000 events were collected for each analysis. Bivariate plots of FL2 versus FL1 were used to analyze \( \Psi_m \). Forward scatter was used to estimate relative cell volume. Based on forward and side scatter, and JC-1 fluorescence levels of untreated Rat2 fibroblasts, cells were gated into high and low FL1 and FL2 populations. The percentages and mean fluorescence intensities of these populations were compared to establish the effect of increasing concentrations of CsA.

**Immunoblotting**

For assessments of the mitochondrial-specific protein cytochrome oxidase I, the immunoblotting methods of Marusich et al. (1997) were used. Whole cell extracts were prepared by rinsing trypsinized Rat2 cells with calcium and magnesium free (CMF) PBS containing protease inhibitors (0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 1 mM PMSF). Cell pellets were solubilized for 30 min at 5 \( \times \) 10^6 cells/mL in CMF-PBS
containing the above protease inhibitors plus 1.5% lauryl maltoside at 4°C, centrifuged for 20 minutes at 16,600 x g, and the supernatants were saved. Equivalent amounts of protein, determined by Bradford assay, were separated on 10% polyacrylamide gels and transferred electrophoretically onto 0.2 μm nitrocellulose membranes in CAPS transfer buffer (10% methanol in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11) on ice. The blots were blocked with 5% non-fat dried milk powder in CMF-PBS, incubated overnight at 4°C with anti-human cytochrome oxidase-1 monoclonal antibody diluted in 5% milk CMF-PBS to 1:1000, washed with 0.05% Tween in CMF-PBS, incubated for 1 hour in horse radish peroxidase conjugated goat anti-mouse IgG at 0.2 μg/mL in 5% milk CMF-PBS and washed with 0.05% Tween in CMF-PBS. Blots were processed for chemiluminescence, exposed to X-ray film, developed and analyzed.

Data Analysis

Means and standard errors of the means were computed for phagocytic, calcium, and mitochondrial membrane potential experiments. Comparisons between two groups were performed with the unpaired Student's t-test. Comparisons between more than two groups were done using ANOVA.
Model System - Fibroblast

nucleus

CCB

CsA
Figure 5. Model system. Upon interaction with mitochondria, CsA will alter the calcium permeability of this organelle which in turn is likely to impact on calcium fluxes in the SERCA. The central question is: what is the effect of this perturbation on collagen phagocytosis? CCB - collagen-coated beads; CsA - cyclosporin A; SERCA - smooth endoplasmic reticulum calcium ATPase pump.
IV. Results

Effect of CsA on collagen bead phagocytosis

In HGF incubated with collagen-coated beads, there was abundant binding of beads that was inhibited ~2-fold with as low as 10 nM CsA (1 hour pre-incubation with CsA; 3 hour bead incubation; Fig. 6A). The phagocytic capacity of HGFs treated with CsA was compared to that of Rat2 fibroblasts treated with CsA to rationalize the use of Rat2 fibroblasts for all subsequent experiments. Rat2 cells were incubated with increasing doses of CsA prior to three-hour incubations with collagen-coated beads, a protocol that enables quantitative evaluation of the bead-binding step of phagocytosis. After the addition of 10 nM CsA, there was a significant and comparable reduction in mean % phagocytosis in both HGFs (p<0.05) and Rat2 fibroblasts (p<0.05; Fig. 6B). CsA doses which are substantially lower than those used therapeutically in humans (~200µM) produced a significant decrease in the phagocytic capacity of Rat2 fibroblasts (p<0.05 for 10-10,000 nM CsA; Fig. 6C). Dissipation of the mitochondrial membrane potential using CCCP (p<0.02) or inhibition of ATP formation using sodium azide also led to significant decreases in phagocytosis similar to those seen with CsA (p< 0.01), suggesting an involvement of mitochondrial function in the bead-binding step of phagocytosis.

[Ca^{2+}]_i and [Ca^{2+}]_{SERCA} responses to collagen beads

As integrin ligation with extracellular matrix ligands stimulates calcium rises (Schwartz, 1993) that may be important in the bead binding step of phagocytosis, the response of [Ca^{2+}]_i to collagen bead-induced phagocytosis in HGFs was
A

HGF + CCB

HGF + 10 nM CsA + CCB

B

Mean % Phagocytosis

untreated

10 nM CsA

C

Mean % Phagocytosis

untreated 10 nM CsA 100 nM CsA 1 μM CsA 10 μM CsA CCCP Sodium Azide
Figure 6. A. Flow cytometry - analysis of the collagen bead binding step of phagocytosis after incubation with cyclosporin A in human gingival fibroblasts (HGFs). B. CsA (10 nM) causes a marked decrease in the phagocytic capacity of HGFs. Data are means ± SEM (n=3). C. CsA causes a dose-dependent inhibition of phagocytosis. To study the possible involvement of mitochondria, CCCP (10 μM) and sodium azide (1%) caused a marked decrease in the phagocytic capacity of Rat2 fibroblasts. Data are means ± SEM (n=3). For figs. 6 B and C, *, p<0.05; †, p<0.02; ‡, p<0.01 when compared to vehicle-treated cells. ANOVA testing for untreated cells and increasing doses of CsA revealed that the groups are significantly different at p=0.001.
examined. The ratiometric calcium-sensitive dye fura 2 was used to measure 
$[Ca^{2+}]_{i}$ as it is relatively insensitive to photobleaching and dye leakage is minimal over time. In
Fig. 7A the insert depicts a representative tracing obtained when collagen-coated beads 
were added to HGF cells and $[Ca^{2+}]_{i}$ was measured over time. As cells were incubated 
with beads at a ratio of $\sim 4:1$ (beads:cell), multiple asynchronous bead-cell interactions 
occur over 1500 seconds and consequently the decrease in calcium to basal levels 
occur later (not shown). The mean $[Ca^{2+}]_{i}$ rise above baseline was similar in HGFs 
(124 ± 31 nM) and Rat2 fibroblasts (130 ± 9 nM; $p>0.02$). The rise to maximum levels 
of $[Ca^{2+}]_{i}$ also proceeded over a similar time course. The similarity of Rat2 fibroblasts 
and HGF responses to collagen beads rationalized the use of Rat2 fibroblasts for all 
subsequent experiments which measured levels of calcium within the cell.

When CsA was added to HGFs or Rat2 cells, $[Ca^{2+}]_{i}$ rose steadily over time and 
exhibited a dose-dependent increase of $[Ca^{2+}]_{i}$. The addition of CsA, even in small 
doses, induced significant increases of $[Ca^{2+}]_{i}$ compared to the DMSO vehicle ($p<0.05$ 
for 10 nM CsA; Fig. 7B). When Rat2 fibroblasts were pre-treated with 10 nM CsA for 1 
hour, there was a significantly reduced rise of $[Ca^{2+}]_{i}$ in response to collagen bead-
induced phagocytosis (3.4-fold; *p*<0.01; Fig. 7C).

The levels of $[Ca^{2+}]_{i}$ were investigated when intracellular stores of calcium were 
depleted using 1 µM thapsigargin or when intracellular calcium was chelated using 
BAPTA/AM. After addition of thapsigargin to either Rat2 fibroblasts or Rat2 fibroblasts 
pre-treated with CsA, there was a robust rise of $[Ca^{2+}]_{i}$ in both groups. The post-

Figure 7. A. Collagen-coated beads (CCBs) cause an equivalent rise of [Ca\(^{2+}\)]\(_i\) in human gingival fibroblasts (HGFs) and Rat2 cells. Note that the time required to reach maximum values are also similar. In contrast, cells stimulated with poly-L-lysine coated beads did not show any significant rise above baseline. Data are means ± SEM (n=5). Insert shows an increase of [Ca\(^{2+}\)]\(_i\) induced by collagen-coated beads in HGFs. B. Treatment with increasing concentrations of CsA induces dose-dependent increases of [Ca\(^{2+}\)]\(_i\) in HGF. Data are means ± SEM (n=5). *; p<0.05; †, p<0.01 when compared to DMSO. Inset demonstrates representative tracing when CsA is added to human gingival fibroblasts loaded with fura 2/AM (2 μM) and [Ca\(^{2+}\)]\(_i\) is recorded. C. The addition of CCBs to fibroblasts in a calcium-containing medium induces a substantial calcium flux (~130 nM Ca\(^{2+}\)) which is reduced 3.4-fold by CsA (10 nM). As expected, thapsigargin (Tg; 1 μM) induces a large calcium increase which is not affected by pre-treatment with CsA (10 nM). After depletion of intracellular calcium stores by thapsigargin, the collagen-induced calcium response is reduced ~3.7-fold (post-Tg + CCB). In contrast, there is no significant reduction of collagen-induced calcium increase in CsA-treated cells that are then treated with thapsigargin. Evidently CsA is not acting primarily at a thapsigargin-sensitive locus in the response to collagen beads. Data are means ± SEM (n=5). *, p<0.05; †, p<0.01 when compared to Rat2 + CCB; **, p<0.05 when compared to Rat2 fibroblasts treated with 10\(^{-8}\) M CsA. Inset shows pronounced increase in [Ca\(^{2+}\)]\(_i\), produced by thapsigargin and a very small rise due to the addition of collagen-coated beads after thapsigargin. D. Pre-incubation of Rat2 fibroblasts with CsA induces decreased [Ca\(^{2+}\)]\(_{SERCA}\) response to collagen-coated beads. Rat2 cells were loaded with [Ca\(^{2+}\)]\(_{SERCA}\) indicator mag-fura 2 and measured by ratio fluorimetry. Data are means ± SEM (n=5). †, p<0.02 when compared to Rat2 + CCB.
thapsigargin response to collagen bead-induced phagocytosis was small in both groups but showed a larger reduction of \([\text{Ca}^{2+}]_i\) in Rat2 fibroblasts (3.7-fold; \(p<0.05\)) than in Rat2 fibroblasts pre-treated with CsA (2-fold). Calcium chelation with BAPTA/AM (5 \(\mu\)M) almost obliterated the \([\text{Ca}^{2+}]_i\) rise induced by beads (Fig. 7C).

When 2 \(\mu\)M mag-fura 2/AM was used to load cells the response of \([\text{Ca}^{2+}]_{\text{SERCA}}\) to collagen bead-induced phagocytosis was measured with ratio fluorimetry, pre-treatment with CsA produced a \(>5\)-fold reduction in the rise of \([\text{Ca}^{2+}]_{\text{SERCA}}\) (\(p<0.02\)) over a similar time span as vehicle-treated controls (~1100 seconds; Fig. 7D).

**Dependence of phagocytic response on calcium signalling**

The addition of thapsigargin to release calcium from internal stores and to inhibit store uptake dramatically reduced the phagocytic capacity of Rat2 fibroblasts (10.2-fold; \(p<0.01\)) a reduction that was comparable to the 8-fold decrease seen when Rat2 fibroblasts were pre-treated with CsA (\(p<0.02\); Fig. 8). Pre-treatment with CsA (10 nM) produced no additional inhibition of phagocytosis. A similar sized reduction of phagocytosis was obtained after pre-treatment with BAPTA/AM. These data indicated that intracellular calcium is important in regulating the bead-binding step of phagocytosis.

**Mitochondrial calcium ([Ca\(^{2+}\)]_m)**

Rat2 fibroblasts were co-loaded with MitoTracker Green [which specifically stains mitochondria, Babcock et al. (1997)] and rhod 2/AM [which reportedly stains
untreated

1μM Tg

10 nM CsA + 1μM Tg

5 μM BAPTA/AM

Mean % Phagocytosis
Figure 8. Effect of agents that inhibit calcium signalling on phagocytosis in Rat2 fibroblasts. Cells treated with thapsigargin (Tg; 1μM) or BAPTA/AM (5 μM) show 10-fold reduction of phagocytosis. Pre-incubation with CsA produces no additional inhibition of phagocytosis compared to thapsigargin alone. Data are means ± SEM (n=3). †p<0.02; ‡p<0.01 when compared to untreated cells.
mitochondrial calcium, Babcock et al. (1997)] to determine if these dyes co-localize and thereby assess if rhod 2 can be used to monitor changes in $[\text{Ca}^{2+}]_{\text{mito}}$. There was marked co-localization of rhod 2 and MitoTracker Green (Fig. 9Ai). After staining with rhod-2/AM for 30 minutes, Rat2 fibroblasts or Rat2 fibroblasts pre-treated with CsA for 1 hour exhibited an equivalent time-dependent decrease of $[\text{Ca}^{2+}]_{\text{mito}}$ in response to collagen beads at $>10$ minutes (Fig. 9Aii, iii). Linear regression showed that mitochondrial calcium was lost at a similar rate in both controls and CsA-treated cells. However, in control cells (i.e. not pre-treated with CsA), there was marked and non-linear reduction of mitochondrial calcium loss in the 5-10 minute range (Fig. 9B).

**CsA prevents loss of mitochondrial membrane potential ($\Psi_m$)**

As CsA is thought to affect the permeability of the MPTP (Zamzami et al., 1996), I evaluated the efficacy of CsA to block the loss of $\Psi_m$ that occurs following an apoptotic stimulus (Kulkarni et al., 1998). Suspension-induced apoptosis (anoikis) was used as the stimulus (Kulkarni and McCulloch, 1994) and JC-1 aggregate formation was used to estimate the mitochondrial membrane potential. The addition of increasing concentrations of CsA to Rat2 fibroblasts in suspension was accompanied by an elevated mitochondrial membrane potential ($p<0.01$ for 1 and 10 $\mu$M CsA; $p<0.05$ for 100 nM CsA; Fig. 10). Thus CsA appears to preserve $\Psi_m$ as described (Zamzami et al., 1996) which indicates that an important locus of action for CsA is the MPTP of fibroblasts.
Mean Fluorescence Intensity (x 105)

Time (minutes)

0 10 15 20 25 30

Baseline, non-stimulated + CCB, t=10 minutes

(II)

50 nm Mitotracker Green

4.5 μM Rhod-2/AM

(A)

+ CCB, t=25 minutes + CCB, t=10 minutes

(B)

(Rex 10 nm Ca)

(Rex Inhibitors)
Figure 9. A. (i) Co-localization of MitoTracker Green and rhod 2 dyes in Rat2 fibroblasts. Cells were co-loaded with both dyes and then imaged by fluorescence microscopy using filters appropriate for either dye. (ii) Stimulation using collagen-coated beads (CCB) in Rat2 fibroblasts stained with rhod 2 induces a decrease in $[\text{Ca}^{2+}]_{\text{mito}}$ over time. Arrows indicate decreased rhod 2 staining in discrete regions of the cell. Note that in the left panel, discrete mitochondria stained with rhod 2. In the right panel, after addition of collagen beads (2 fluorescent beads can be seen on left margin of cell) there is a generalized reduction of rhod 2 fluorescence. (iii) Stimulation using CCBs induces a decrease of $[\text{Ca}^{2+}]_{\text{mito}}$ in Rat2 fibroblasts treated with CsA. B. The addition of collagen-coated beads to Rat2 and Rat2 fibroblasts treated with 10 nM CsA induces a time-dependent decrease in $[\text{Ca}^{2+}]_{\text{mito}}$. Data are means ± SEM (n=5). Linear regression equations and Pearson correlation values for Rat2 and Rat2 fibroblasts treated with CsA, respectively, are $y=-0.060x + 2.22, r=-0.97$ and $y=-0.18x + 4.84, r=-0.98$. 
Figure 10. CsA prevents the loss of mitochondrial membrane potential of Rat2 fibroblasts in suspension. Controls are untreated cells. Analysis was conducted by flow cytometry of JC-1-loaded Rat2 cells in suspension to promote dissipation of $\Psi_m$ and thereby induce anoikis (suspension-induced apoptosis). Data are means ± SEM (n=3). ‡, p<0.01 when compared to untreated cells.
Mitochondrial-depleted cells

The data in Figs. 6, 9 and 10 indicated that mitochondria may be an important organelle for regulation of SERCA Ca\(^{2+}\) and also of collagen bead phagocytosis. Consequently, mitochondria-depleted Rat2 cells were produced using well-established methods (Biswas et al., 1999) to determine if reduction of the numbers of mitochondria would affect collagen bead-induced calcium signalling and phagocytosis. I first verified if the standard protocol for mitochondrial depletion (Biswas et al., 1999) would in fact reduce the numbers of mitochondria.

Rat2 fibroblasts or Rat2 fibroblasts treated with 1000 ng/mL ethidium bromide were stained with JC-1 (0.5 \(\mu\)M) for visualizing mitochondria with a fluorescence microscope. The EtBr-treated cells showed much smaller numbers of mitochondria (Fig. 11Ai). Staining with rhod 2 AM (4.5 \(\mu\)M) to assess mitochondrial calcium stores also showed a large decrease of fluorescence in the EtBr-treated cells (Fig. 11Aii). Notably, rhod 2 staining of nucleoli was unchanged, indicating that the EtBr treatment specifically depleted mitochondrial calcium stores, presumably due to the greatly reduced numbers of mitochondria. Western blotting demonstrated that treated cells also contained less of the mitochondria-specific protein, cytochrome oxidase I (Fig. 11Aiii).

As shown in figure 11B, mitochondria-depleted cells exhibited 50% less collagen bead binding than normal cells in the absence of CsA (p<0.05). The addition of CsA caused a dose-dependent decrease in the mean % phagocytosis of collagen-coated beads in Rat2 fibroblasts treated with ethidium bromide (p< 0.05 for 100 and 1000 nM CsA) similar to that seen in untreated cells. The mitochondria-depleted cells
Figure 11. A. Treatment of Rat2 fibroblasts with ethidium bromide for over 30 passages of continuous culture induces inhibition of mitochondrial DNA synthesis and reduced synthesis of the mitochondria-associated protein COX I. (i) Staining of Rat2 fibroblasts and Rat2 fibroblasts treated with 1000 ng/mL ethidium bromide (Rat2EtBr) with the mitochondrial specific dye, JC-1. (ii) Staining of Rat2 fibroblasts and Rat2EtBr fibroblasts with rhod 2/AM shows no change in rhod 2 staining of nucleoli but greatly reduced fluorescence in the cytoplasm. (iii) Western blot for COX I protein in Rat2 and Rat2EtBr fibroblasts shows ~3-fold reduction of this mitochondrial-specific protein. B. Phagocytic capacity of Rat2 and Rat2EtBr fibroblasts. All cells were incubated with collagen-coated beads at a ratio of 2:1 (beads:cell) for 1 hour after indicated treatment. Treatments: 1- untreated; 2- 10 nM CsA; 3- 100 nM CsA; 4- 1 μM CsA; 5- 10 μM CsA; 6- 10 nM CsA + 1 μM Tg (15 mins); 7- 1 μM Tg (15 mins.); 8- 5 μM BAPTA/AM (30 mins); 9- 500 μM CCCP (30 mins); 10- 1% sodium azide (30 mins); all CsA treatments were conducted for 1 hour. These data shows that compared to untreated controls, CsA induces a more marked inhibition of phagocytosis in normal Rat2 fibroblasts than in ethidium bromide-treated fibroblasts. The thapsigargin, BAPTA and CCCP effects are also proportionately less in the ethidium bromide-treated cells. Data are means ± SEM (n=3). * p<0.05 when compared to Rat2EtBr fibroblasts, **, p<0.05 when compared to Rat2 fibroblasts.
displayed reductions of phagocytosis after treatment with thapsigargin, BAPTA/AM, CCCP or sodium azide (Fig. 11B) but the magnitude of the reduction was ~ one-half that of normal Rat2 cells.

**Calcium signalling in mitochondria-depleted cells**

Rat2 or mitochondria-depleted Rat2 fibroblasts were loaded with fura 2/AM (2 μM) and treated with CCCP (10 μM) for 10 minutes to depolarize mitochondria and block oxidative phosphorylation. As estimated by ratio fluorimetry, CCCP reduced \([\text{Ca}^{2+}]_i\) responses to collagen beads in Rat2 fibroblasts by ~90% (p<0.01). In contrast, collagen bead-induced \([\text{Ca}^{2+}]_i\) responses in mitochondria-depleted cells were < 10 nM and were reduced only an additional 5 nM by CCCP (Fig. 12A).

As shown in Fig. 7D and Fig. 12B, treatment of Rat2 fibroblasts with CsA markedly reduces the maximal \([\text{Ca}^{2+}]_{\text{SERCA}}\) response to collagen beads. In contrast, when mitochondria-depleted Rat2 fibroblasts were treated with CsA, there was no significant reduction of maximal \([\text{Ca}^{2+}]_{\text{SERCA}}\) in response to beads (Fig. 12B; p<0.2). Further, there was no significant difference between Rat2 fibroblasts and mitochondria-depleted fibroblasts with or without CsA treatment (p>0.2).

The data shown above indicate that mitochondria regulate calcium signalling in response to collagen beads but when the numbers of mitochondria are reduced, SERCA stores continue to exhibit a response to beads that is unaffected by CsA. I assessed if calcium stores in the remaining mitochondria of
Fibroblasts + CCB
Fibroblasts + CCCP + CCB

Rat2 fibroblasts
Rat2EtBr fibroblasts

Mean change in $[\text{Ca}^{2+}]_i$ (nM)

B

Mean change in fluorescence ratio (346/380 nm)

Rat2 + CCB
Rat2 + CsA + CCB
Rat2EtBr + CCB
Rat2EtBr + CsA + CCB

Time to maximum $[\text{Ca}^{2+}]_{\text{SERCA}}$ (seconds)
Figure 12. A. Incubation with CCCP inhibits \([\text{Ca}^{2+}]_i\) response to collagen-coated beads (CCBs) in both Rat2 and Rat2EtBr fibroblasts. CCBs induce only a minimal \([\text{Ca}^{2+}]_i\) response in Rat2 fibroblasts cultured with 1000 ng/mL ethidium bromide (Rat2EtBr). Data are means ± SEM (n=5). †, p<0.01 when compared to Rat2 fibroblasts + CCB. B. Pre-incubation with 10 nM cyclosporin A (CsA) induces a large decrease in \([\text{Ca}^{2+}]_{\text{SERCA}}\) in Rat2 cells but not in Rat2EtBr cells responding to CCBs. \([\text{Ca}^{2+}]_{\text{SERCA}}\) was measured using ratio fluorimetry of mag-fura 2-loaded cells. Data are means ± SEM (n=5). †, p<0.02 when compared to Rat2 fibroblasts + CCB.
mitochondria-depleted cells would still discharge $\text{Ca}^{2+}$ if stimulated with collagen beads. Mitochondria-depleted Rat2 fibroblasts were loaded with rhod 2/AM (4.5 $\mu$M) and fluorescence intensity was recorded over 30 minutes after initiation of collagen-bead induced phagocytosis as described (Fig. 9B). In cells treated with vehicle or CsA, the fluorescence intensity of rhod 2 decreased following stimulation with collagen-coated beads (Fig. 13A). As expected, the baseline fluorescence intensity due to rhod 2 reporting of $[\text{Ca}^{2+}]_{\text{mito}}$ was significantly lower in mitochondria-depleted cells compared to control Rat2 cells. Linear regression of fluorescence intensity data showed that $[\text{Ca}^{2+}]_{\text{mito}}$ in Rat2 fibroblasts dissipated at the same rate as $[\text{Ca}^{2+}]_{\text{mito}}$ in Rat2EtBr fibroblasts between 10-30 minutes. In contrast to mitochondria-depleted cells which showed a linear reduction of fluorescence between 0-30 minutes, control cells exhibited a pronounced 2-fold reduction 5-10 minutes after bead incubation which thereafter showed a similar linear reduction of fluorescence as the ethidium bromide-treated cells (Fig. 13B). After treatment with 10 nM CsA for 1 hour, the control Rat2 fibroblasts lost mitochondrial calcium at a linear but faster rate than Rat2EtBr fibroblasts (slope = -0.18 vs. slope = -0.055). The marked loss of mitochondrial calcium in the 5-10 minute range after stimulation with collagen beads was notably absent after CsA pre-treatment.
Figure 13. A. (i) Stimulation with collagen-coated beads (CCB) in Rat2EtBr fibroblasts stained with rhod 2/AM induces a decrease in $[\text{Ca}^{2+}]_{\text{mito}}$ of the few remaining mitochondria. In this micrograph I have included fluorescence beads (bright circles at cell periphery) to illustrate bead size and binding. Arrows point to rhod 2 stained mitochondria and indicate decreased rhod 2 staining. (ii) Stimulation with CCBs induces a decrease of $[\text{Ca}^{2+}]_{\text{mito}}$ in Rat2EtBr treated with CsA. B. CCBs induce decreased $[\text{Ca}^{2+}]_{\text{mito}}$ in Rat2 and Rat2EtBr. In normal Rat2 cells, CCBs induce a > 2-fold reduction of rhod 2 fluorescence between 5-10 minutes which decreases linearly thereafter (10-30 minutes). Mitochondria-depleted cells exhibit a linear reduction of rhod 2 fluorescence which has the same slope as the normal cells from 10-30 minutes. Linear regression equations and Pearson correlation values for Rat2 and Rat2EtBr fibroblasts, respectively, are $y=-0.060x + 2.22$, $r=-0.97$ and $y=-0.090x + 2.88$, $r=-0.90$. C. Addition of CCBs to Rat2 or Rat2EtBr fibroblasts pre-treated with 10 nM CsA induces decreased $[\text{Ca}^{2+}]_{\text{mito}}$. Note the continuous linear reduction of rhod 2 fluorescence in the normal Rat2 cells and the absence of the sharp drop from 5-10 minutes. Linear regression equations and Pearson correlation values for Rat2 and Rat2 fibroblasts both treated with CsA, respectively, are $y=-0.18x + 4.84$, $r=-0.98$ and $y=-0.055x + 2.16$, $r=-0.78$. 
Control fibroblast

Collagen-coated bead

↑↑↑

phagocytosis

nucleus

Fibroblast treated with CsA

Collagen-coated bead

↓↓↓

phagocytosis

CsA

nucleus
Figure 14. Schematic diagram of fibroblasts with and without CsA treatment. Summary changes of $[Ca^{2+}]_r$, $[Ca^{2+}]_{SERCA}$, $[Ca^{2+}]_{mito}$ and rate of phagocytosis are represented by arrows. Subsequent to collagen bead-induced phagocytosis, different calcium responses are observed after treatment with CsA. In control cells, $[Ca^{2+}]_{mito}$ decreases initially at a faster rate than CsA-treated cells. CsA sharply reduces whole cell calcium and SERCA calcium responses to collagen bead stimulation. I propose that binding of CsA to mitochondria inhibits mitochondrial release of calcium and therefore uptake by the SERCA store and a consequent reduced calcium response in the cytosol to collagen beads. The downstream effect is decreased phagocytic efficiency in CsA-treated fibroblasts which contributes to loss of collagen homeostasis and results in gingival overgrowth.
V. Discussion

Current data indicate that administration of CsA results in gingival overgrowth in as low as 17% and as high as 81% of cases available for study (Hefti et al., 1994; Friskopp and Klintmalm, 1986). The mechanism by which CsA induces gingival overgrowth is unknown but some of the modes of action of CsA as an immunosuppressant are understood (Kay, 1989). CsA inhibits the MPTP (Crompton et al., 1988) in the mitochondrial membrane, and more recently, has been shown to act upon the internal calcium stores and inhibit calcium release (Park et al., 1999). Further, several functional interactions between mitochondria and internal calcium stores have been characterized (Landolfi et al., 1998). With this background in mind, I propose that upon entry into the cell, CsA interacts with mitochondria to affect the normal functional relationship of mitochondrial calcium stores and other internal stores of calcium (Fig. 4). As calcium signalling is important for integrin functions related to binding of extracellular matrix molecules (Schwartz, 1993), this deregulation is likely to affect collagen phagocytosis. Below I will discuss in more detail the functional relationships between calcium deregulation and phagocytic processes.

Effect of CsA on phagocytosis

HGFs have been used previously to study collagen phagocytosis in vitro (McCulloch and Knowles, 1993). These cells are the principal synthetic and degradative cell in normal gingiva (Narayan and Page, 1993; Everts, 1996) and consequently are closely linked to the deregulation of collagen metabolism seen in drug-induced gingival overgrowth. I used Rat2 fibroblasts primarily for my studies since I showed that these
cells exhibit very similar calcium and phagocytic responses as HGFs. As Rat2 cells is a stable and easily propagated cell line, problems of cell death and the phenotypic variability associated with primary isolates were minimized. Data from previous collagen phagocytosis studies also demonstrate the utility of Rat2 cells for phagocytic studies and the similarity of the two cell types (Hui et al., 1997). For example I showed that addition of 10 nM CsA caused a significant and similar decrease in phagocytosis (>50%) for both cell types. Further, in Rat2 fibroblasts and HGFs, I showed that addition of CsA, in concentrations less than those administered in vivo (Kay, 1989), caused a significant decrease of phagocytosis compared to untreated cells. These data describe the final end-point effect of CsA upon fibroblasts studied here, (i.e. due to some mechanism, CsA inhibits the collagen phagocytic capacity of gingival fibroblasts). CCCP, a proton ionophore which dissipates the mitochondrial membrane potential (Zamzami et al., 1995) and eventually promotes cell death (Kulkarni et al., 1998), also reduced phagocytosis as was seen with CsA treatment. The mitochondrial dysfunction caused by CCCP suggests the importance of mitochondria in phagocytic function. Indeed the involvement of ATP production by mitochondria was shown by treating cells with sodium azide which also caused a similar decrease of collagen bead phagocytosis.

**Phagocytosis and \([\text{Ca}^{2+}]_i\) signals**

When \([\text{Ca}^{2+}]_i\) was monitored during collagen bead-induced phagocytosis, HGFs and Rat2 fibroblasts exhibited a gradual similar rise in \([\text{Ca}^{2+}]_i\) over 30 minutes but with no return to baseline over this monitoring period, presumably because of the prolonged period of bead-cell interactions and the progressive cellular contacts with more than one
bead. After intracellular calcium stores were depleted by thapsigargin, collagen bead-induced phagocytosis was still able to invoke a small but measurable calcium rise, an effect that was seen in both Rat2 fibroblasts and Rat2 fibroblasts pre-treated with CsA. If calcium was released into the cytosol through a thapsigargin-insensitive route, collagen phagocytic stimuli may effect calcium release from other calcium stores or this increase may be due to calcium influx from the extracellular medium (Schwartz, 1993). Notably, the post-thapsigargin response in CsA-treated cells was not as large as untreated control cells, suggesting that CsA interacts with thapsigargin-insensitive sites which may cause an increase of $[\text{Ca}^{2+}]_i$ due to phagocytosis. I propose that since treated cells have more of these binding sites engaged by CsA, they cannot respond fully to phagocytic stimuli. The chelation of $[\text{Ca}^{2+}]_i$ with BAPTA also caused a significant decrease in the $[\text{Ca}^{2+}]_i$ response compared to untreated cells and the magnitude of this response was similar to that following thapsigargin. These data are consistent with previous findings showing that $[\text{Ca}^{2+}]_i$ responses to phagocytic stimuli are not due solely to release from SERCA stores (Bengtsson et al., 1993). Indeed as SERCA calcium stores were actually increased after collagen bead stimulation, it is possible that a large part of the rise of $[\text{Ca}^{2+}]_i$ due to phagocytosis originates from non-SERCA stores. Small contributions may also come from both the extracellular medium and from other, as yet unidentified, stores within the cell.

CsA induced a relatively rapid, dose-dependent rise of $[\text{Ca}^{2+}]_i$, likely reflecting the transport of CsA into cells and its interaction with binding sites or receptors that regulate intracellular calcium stores (LeGrue et al., 1983). Addition of thapsigargin to cells pre-treated with CsA induced the same robust increase in $[\text{Ca}^{2+}]_i$ as seen in untreated cells.
However, collagen bead-induced phagocytosis after internal store depletion by thapsigargin produced a reduced response of \([\text{Ca}^{2+}]\text{SERCA}\) in Rat2 fibroblasts (p<0.05) and a similar rise in Rat2 fibroblasts pre-treated with CsA. This finding indicates that pre-treatment with CsA caused calcium release from thapsigargin-insensitive stores. As collagen beads stimulated uptake of calcium into the SERCA, and as this uptake was inhibited by CsA, it seems likely that mitochondrial calcium is released by collagen bead stimuli and that the released calcium is taken up by the SERCA.

\[\text{[Ca}^{2+}]_{\text{SERCA}} \text{ response to collagen phagocytic stimuli}\]

As discussed above, pre-incubation of Rat2 fibroblasts with CsA caused a significant decrease in the \([\text{Ca}^{2+}]_{\text{SERCA}}\) response to collagen beads. Previous data by Schwartz (1993) indicate that a large part of the increase in \([\text{Ca}^{2+}]_i\) may be due to calcium release from the SERCA stores. However, when mag-fura was used to observe the behaviour of calcium within SERCA stores, I found an increase after collagen bead-induced phagocytosis over 30 minutes. Therefore, both \([\text{Ca}^{2+}]_i\) and \([\text{Ca}^{2+}]_{\text{SERCA}}\) were increased after collagen bead-induced phagocytosis. My expectation was that release of SERCA stores of calcium would provide the increased \([\text{Ca}^{2+}]_i\), seen experimentally. However, when cells were pre-treated with CsA, \([\text{Ca}^{2+}]_{\text{SERCA}}\) and \([\text{Ca}^{2+}]_i\) were reduced compared to untreated cells. Thus CsA may act initially to release calcium from intracellular stores thereby reducing the calcium ion pool for uptake by the SERCA. In this context, mitochondria in untreated cells may sense high local concentrations of calcium and act to buffer \([\text{Ca}^{2+}]_i\), as has been shown earlier (Rizzuto et al., 1993). Accordingly, in mitochondria-depleted cells, CsA treatment did not alter \([\text{Ca}^{2+}]_{\text{SERCA}}\) presumably
because productive interactions between mitochondrial and SERCA-related stores were restricted (Biswas et al., 1999).

$[\text{Ca}^{2+}]_{\text{mito}}$ responses to collagen phagocytic stimuli

Collagen bead-induced phagocytosis provoked a time-dependent decrease of $[\text{Ca}^{2+}]_{\text{mito}}$ in untreated Rat2 cells or Rat2 cells pre-treated with CsA but there was a much more rapid loss at early times (5-10 minutes) in untreated cells. Pre-incubation with CsA or mitochondrial depletion attenuated the dissipation of mitochondrial calcium in the 5-10 minute range. This result may be a reflection of the initial obstruction to calcium efflux due to inhibition of the MPTP by CsA, an effect that is probably overcome as calcium ions exit via other channels or pumps (Bernardi, 1999). My data on CsA inhibition of suspension-induced reduction of $\Psi_m$ indicate that CsA was indeed acting upon the MPTP and consequently the supposition that CsA is altering the conductance of the MPTP seems reasonable.

Phagocytosis in mitochondria-depleted fibroblasts

Compared to untreated Rat2 cells, Rat2 fibroblasts treated with ethidium bromide exhibited similar reductions of phagocytic efficiency as seen in cells treated with CsA. This suggests that mitochondria play a role in regulating collagen phagocytosis. When the rate of dissipation of $[\text{Ca}^{2+}]_{\text{mito}}$ due to collagen bead-induced phagocytosis was compared between these two cell types, there was no significant difference. This result appears reasonable since the ethidium bromide-treated cells have a lower number of mitochondria but the function of the remaining mitochondria is not altered, otherwise the
cells would not proliferate in culture. A similar rate of mitochondrial calcium dissipation was seen in Rat2 fibroblasts treated with CsA, emphasizing that although reduced in number, these mitochondria function normally.

The major conclusion of this thesis is that mitochondrial calcium signalling is an important determinant of collagen bead phagocytosis and that consequently, CsA-mediated gingival overgrowth may be mediated by the same or a similar pathway. Fig. 14 illustrates a model integrating data obtained for changes of $[\text{Ca}^{2+}]_{i}$, $[\text{Ca}^{2+}]_{\text{SERCA}}$, $[\text{Ca}^{2+}]_{\text{mito}}$ and phagocytic efficiency. Consistent with my overall conclusion, subsequent to collagen bead-induced phagocytosis, very different calcium responses were observed in Rat2 fibroblasts or Rat2 fibroblasts treated with CsA. $[\text{Ca}^{2+}]_{\text{mito}}$ decreased at a faster rate initially in the untreated cells. Both $[\text{Ca}^{2+}]_{\text{SERCA}}$ and $[\text{Ca}^{2+}]_{i}$ increased to a greater extent in controls than CsA-treated Rat2 fibroblasts. I propose that the effect of CsA on mitochondria is transmitted to the SERCA stores, which then "relay" the information and dampens the phagocytosis-induced calcium signal in the cytosol. The effect of this perturbation on the whole cell is a decrease of phagocytic efficiency which, in the CsA-treated gingival fibroblast, results in gingival overgrowth. While the actual sequence of events is not shown by these data, the importance of signaling interactions between discrete calcium stores is evident. When the sequence and regulation of these phagocytic signaling events is deciphered, and the exact effect of CsA on the mitochondria as a fibrosis-inducing agent is outlined, modifications to CsA therapy could be approached more rationally. For example, if CsA could be modified so as to reduce fibrosis while retaining its immunosuppressant properties, this would be a significant clinical advance.
VI. **Conclusions**

1. CsA inhibits collagen bead phagocytosis dose-dependently in human gingival and Rat2 fibroblasts. Phagocytosis is decreased in mitochondria-depleted Rat2EtBr fibroblasts, suggesting that mitochondria play a role in the regulation of collagen phagocytosis.

2. Collagen-coated beads induce equivalent calcium rises in both human gingival and Rat2 fibroblasts. Part of the calcium increase is due to thapsigargin-sensitive and thapsigargin-insensitive stores. Mitochondria depleted Rat2EtBr fibroblasts show reduced rise of $[Ca_i]$ in response to collagen-coated beads. CsA prevents the loss of mitochondrial membrane potential of Rat2 and Rat2EtBr fibroblasts in suspension indicating that CsA blocks the mitochondrial membrane permeability transition pore.

3. Depletion of internal calcium stores or dissipation of mitochondrial membrane potential greatly reduce phagocytosis in both Rat2 and mitochondria-depleted Rat2-EtBr fibroblasts.

4. Pre-incubation of Rat2 fibroblasts with CsA causes a decrease in $[Ca^{2+}]_{SERCA}$ which is probably related to CsA-induced release of calcium from internal stores. In Rat2-EtBr cells, CsA treatment does not alter $[Ca^{2+}]_{SERCA}$ because productive interactions between mitochondrial and SERCA-related stores are restricted. Collagen bead-induced phagocytosis induces a time-dependent decrease in $[Ca]_{mito}$ in both Rat2 and Rat2 fibroblasts treated with CsA. Calcium release from mitochondria is provoked by collagen phagocytosis in both cell types.
VII. References


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