June 1989

Histochemical and Cytochemical Localization of Calmodulin in Rat Osteosarcoma Cells (ROS 17/2.8) in Culture and in the Rat Alveolar Tissues.

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THE HISTOCHEMICAL AND CYSTOCHEMICAL LOCALIZATION OF CALMODULIN IN RAT OSTEOSARCOMA CELLS (ROS 17/2.8) IN CULTURE AND IN THE RAT ALVEOLAR TISSUES

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Dental Sciences at the University of Connecticut
1989
Master of Science Thesis

HISTOCHEMICAL AND CYTOCHEMICAL LOCALIZATION OF CALMODULIN IN RAT OSTEOSARCOMA CELLS (ROS 17/2.8) IN CULTURE AND IN THE RAT ALVEOLAR TISSUES

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1989
DEDICATED

I dedicate this thesis:
To my family, specially to my parents, grandparents, and my aunt who have supported me in all ways during my entire education.
To all of you, thanks for giving me the honor of being part of your lives.
ACKNOWLEDGEMENTS

I would like to recognize, appreciate and gratitude the aid from the following professionals:

Dr. Leslie S. Cutler, my major advisor, for his constructive criticism and guidance during the past two years.

Dr. Barbara Kream, Dr. Paul B. Epstein and Dr. Louis A. Norton, all members of my committee, for their advise and supply of the materials which made this thesis a reality.

Mrs. Connie Gillies for the provisions of some of the electron microscopy facilities.

Dr. Huw Thomas, for the provisions of the immunofluorescensce microscopy facilities.
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INTRODUCTION

Calmodulin

Calmodulin (CaM) is a thermostable, monomeric, globular protein with a molecular weight of 16,700 Daltons. In the majority of species, CaM contains 148 amino acids with a high proportion of acidic residues (fig.1), resulting in an isoelectric point of 3.9 to 4.3 (1-4). It was first described by Cheung (4), as an activator of the cyclic nucleotide phosphodiesterase. Consequently, other researchers refered to CaM as the: activator protein, modulator protein, calcium dependent regulator protein and troponin C-like protein.

CaM is now well accepted as the major calcium-binding protein in non-muscle cells (4). Calmodulin appears to be the most widely distributed (ubiquitous), extraordinarily versatile calcium binding protein found in most, if not all eukaryotic cells (4). CaM is a highly coiled molecule and has four putative calcium binding domains flanked by two stretches of two helixes with a large sequence of amino acids (1). CaM binds calcium with high affinity and specificity, serving as the major intracellular calcium receptor and having multifunctional regulation properties for many cellular functions (1,2). CaM invariably lacks tissue and species specificity (2), another suggestion
that it may be a universal regulator. Therefore, CaM is considered to be the primary physiological receptor for calcium in the eukaryotic cells.

**Literature Review**

**Bone Remodeling During Orthodontic Tooth Movement**

The mechanisms by which mechanical forces activate cells is not well understood. However, several decades of extensive research established the fact that cell activation occurs in, or through the plasma membrane (5). Some factors that mediate the effects of extracellular stimuli on their target cells are: calcium ions, adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP), (5). These factors, serve as intracellular second messengers in response to plasma membrane interactions with extracellular agents (first messengers).

The nature of the extracellular first messengers which bring about tissue remodeling during orthodontic tooth movement remains largely obscure. Indeed, bone remodeling is considered to be a multifactorial process involving different types of tissues, cells, many cell-derived factors, and many cell-mediated metabolic events. Each metabolic event occurs at its proper place and time.
Therefore, control of bone remodeling involves the complex interplay between stimulatory and inhibitory factors, various cellular organelles and compartments, intra and extracellular processes, organic matrix and inorganic ions (6).

In vivo and in vitro studies have demonstrated that bone remodelling is modulated by the classical mineral regulatory hormones (parathyroid hormone, 1,25-dihydroxy vitamin D), local chemical mediators (prostaglandin E₂, osteoclast activating factor) and growth factors (epidermal and platelet derived growth factors) among others (5). It is known that several of these agents alter the metabolism of second extracellular messengers, like cAMP and calcium.

Specific CaM Distribution

Calmodulin is structurally conserved and functionally preserved throughout the animal and plant kingdoms (1,2,4,7,8). Calmodulin is a component of virtually every intracellular compartment, as well as, the plasma membrane of eukaryotic cells (8). CaM has been found associated with the plasma membranes of hepatocytes and the Purkinje and granular cells of the rat brain (9,10). CaM has also been associated with free ribosomes, smooth and rough endoplasmic reticulum, nuclear envelope, vesicles,
mitochondria (11), inner surfaces of post synaptic membranes, microtubules, microtubule-organizing centers, and the mitotic spindle apparatus (12).

In the rat CaM has been found in: cytoplasm, plasma membrane of liver parenchymal cells (12) intestinal epithelium (12), ciliated tracheal epithelium (13), skeletal muscle (13), cytoplasm and nucleus of adrenal cortex cells (12), heart (13), uterus (12), molar tooth germ (14), within matrix vesicles derived by budding from mineralizing cells of tibia epiphyseal shafts (15), mandibular bone (15), mature and mineralizing chondrocytes (15), new born calvarial osteoblasts and osteoclasts (16), ROS (17/2.8) osteosarcoma cells (17), ribosomes (17), rough endoplasmic reticulum (17), cytosol and plasma membrane of the supranuclear region of secretory ameloblasts (18), among other structures.

Calmodulin Functions and Suggested Mechanism of Action

The role of CaM in general is to detect the calcium concentration transients that occur during normal cell function and to then reflect the ambient calcium concentration via regulation of various and multiple processes (2).

In a resting cell, the available calcium concentration
is in the order of $10^{-7}$ M to $10^{-8}$ M. Activation of the cell, results in an increase in intracellular calcium to about $10^{-6}$ M, or slightly higher. It is known that CaM can detect calcium at this micromolar concentration and the calcium binding constants for CaM do in fact have the appropriate affinities. The large number of identified CaM-dependent processes within one cell, requires that CaM should be accessible to many cellular loci (1). The presence of CaM in the cytoplasm and the mechanism of regulation for most CaM dependent processes, that is regulation by calcium dependent association and dissociation, satisfy this requirement.

The majority of known CaM-dependent systems are enzymatic. The general mechanism for the activation (regulation) of a CaM dependent enzyme (E) is as follows:

$$n\text{Ca}^{2+} + \text{CaM} = \text{Ca}^{2+}(n).\text{CaM} + E = \text{Ca}^{2+}(n).\text{CaM*}.E$$

(inactive) (active tertiary complex)

The first step is an extracellular stimulus or messenger which induces the binding of calcium to CaM. This induces a large number of conformational changes in the protein (2), that favor interaction and activation of the CaM-calcium enzymatic complex, (CaM-Ca$^{2+}$.E*). Therefore, the inherent molecular flexibility of CaM may have a role in
it's functional versatility (17). This flexibility gives CaM the unusual capability of combining with and modifying the properties of a wide spectrum of enzymes of very different molecular characteristics, controlling multiple basic cellular processes (17). Indeed, 16 possible CaM-calcium complexes have been reported. The different conformers may be active toward different enzymes affording a variety of receptor specificities (1). Fractional occupancy of the calcium binding sites may dictate the systems regulated by CaM and help to explain how this protein can be involved in diverse biological processes (17).

Reduction of the intracellular level of calcium causes dissociation of the tertiary complex, and a loss of enzymatic activity. Of course, this type of reaction mechanism and the regulation or activation of a given enzyme depends on several factors. At limiting concentrations of CaM or calcium, the concentration of the Calcium-CaM complex will obviously be restricted. An increase in either calcium or available CaM will shift the equilibrium to favor the complex formation, and thus the apparent calcium sensitivity of a given CaM-dependent system can depend on the CaM concentration. The latter, in turn is governed by the presence and concentration of other CaM binding proteins and their binding affinities for CaM (1).
Specific Calmodulin-Calcium Regulatory Processes

As stated before, the number and variety of systems that are now thought to be regulated by CaM is remarkable. The protein controls the metabolism and functions of cAMP and calcium, coupling and integrating the two major cellular messenger systems on a molecular basis. As a mediator of the calcium signal, CaM ensures the coordinated regulation of metabolic pathways associated with calcium dependent functions (1).

Calmodulin as the key Calcium Receptor Protein

Means et al. (13) reported that CaM controls the effect of calcium as a second messenger in stimulus-response coupling. Increased or decreased intracellular calcium concentrations have been implicated as a messenger between the extracellular space and the cytosol. Kretsinger (20) suggested that the sole function of intracellular calcium is to transmit information, and that the target(s) of calcium functioning as a second messenger is one or more intracellular proteins. Examples of processes regulated by the information of signals of intracellular calcium are: platelet aggregation, neutrophil chemotaxis and phagocytosis, and mast-cell histamine secretion (1).
Specific Calmodulin-Calcium Enzyme Regulation

CaM has no known intrinsic enzymatic activity but it regulates a wide spectrum of enzymes that control many fundamental cellular processes (18). It has multiple binding or acceptor proteins, interacting at different average degrees of calcium occupancy (3). Therefore, the CaM-calcium complex represents the biologically active state of intracellular calcium and mediates the calcium regulation of a large number of fundamental intracellular, and perhaps extracellular, metabolic pathways through enzymatic systems in various tissues (1,18).

In addition to the RBC calcium-magnesium ATPase model of regulation, other researchers presented evidence in which CaM can also act indirectly on a variety of regulatory systems such as several protein kinases (7,20), guanylate cyclase (4), adenylate cyclase (1), brain cyclic nucleotide phosphodiesterase (1), phospholipase A2 (4), tryptophan hydroxylase (4), calcium-magnesium ATPase (1), myosin light-chain kinase (1), phosphorylase b kinase (1,4) NAD kinase (1,18), and brain membrane kinase (1).

Regulation of the Calcium-Magnesium ATPase by Calmodulin

Vicenzi (21) reported that CaM not only mediates the effect of the cytoplasmic calcium signal but also terminates the effects of the CaM-Calcium complex by
regulating the intracellular free calcium concentration via stimulation of calcium transport ATPases. Therefore, by regulating calcium transport mechanisms, CaM modulates its own function.

The extensively studied Calcium-Magnesium ATPase (calcium pump) in the human red blood cell represents a classical example of the CaM regulation on the calcium transport (22,23). Where as calcium readily enters the cells, it's movement between organelles and removal from cells is an active process (23). CaM was shown not only to stimulate the ATPase activity of the calcium pump, but the binding of the protein to the membrane also results in calcium extrusion (23,24).

The data accumulated for the red blood cell suggests that energy-dependent calcium removal is important in maintaining calcium homeostasis and that CaM is intimately involved in regulating this process. CaM should be able to regulate the intracellular calcium level in a rather specific manner.

At resting levels, free calcium concentrations are normally about $10^{-7}$ M (1). At such concentrations, the calcium binding sites on CaM are unoccupied. When a stimulus increases this level to 5 micromolar or 10
micromolar, the CaM ion-binding sites become filled, thereby converting the protein into an active conformation (4) triggering the various CaM dependent cell reactions (1). The Calcium-CaM complex associates with and activates the plasma membrane Calcium-Magnesium pump, terminating the cell activation by removal of calcium from the cytosol (23).

The operational Calcium-Magnesium ATPase then actively transfers calcium from the cytosol to the outside of the cell. When intracellular calcium levels are reduced below the micromolar range, the sites on CaM empty, reversing its conformation and its association with the Calcium-Magnesium ATPase complex (23).

Thus, CaM has an additional involvement in the regulation of the termination of the calcium message. CaM is a transducer of the intracellular calcium message and this certainly is the most straightforward analysis of its role. In the case of the regulation of the plasma membrane Calcium-Magnesium pump, however, the effect of its action is to help terminate the calcium message. Thus, high intracellular calcium leads to accelerated removal of calcium from the cytosol not only because calcium is a substrate for the pump but also by its regulatory action in binding to CaM (1).
Finally in 1988, Takakuwa and Mohandas (25) reported that calcium and CaM can regulate membrane stability through modulation of skeletal protein interactions which are dynamic in nature on intact membranes.

Regulation of Cyclic Nucleotides and Calcium Metabolism

Cyclic nucleotides and calcium are two major cellular messengers whose functions and metabolism are closely interwoven. For example, cAMP may sometimes serve as a second messenger and calcium as a the third messenger, whereas at other times, the roles of cAMP and calcium may be reversed. In addition, the cyclic nucleotides may exert their effect in concert with or in apposition to that of calcium.

Therefore, in controlling the metabolism and function of cAMP and calcium, CaM integrates the two messenger systems on a molecular basis (18). Specifically, CaM can stimulate the adenylate cyclase activity (ex. brain and kidney), resulting in an increase in cAMP synthesis. On the other hand, CaM also stimulates the phosphodiesterase activity which would be expected to terminate the cAMP signal (18). Thus, the system seems ideal for a pulse of calcium to be translated into pulse of cAMP with a termination signal.
Regulation of Thyroid Hormone Action on Calcium-ATPase Activity in Vitro

Davis et al. (26) found that this hormone action is CaM-dependent and the complex of CaM and enzyme is stimulatable by physiological concentrations of iodothyronines (27). It has been proposed that thyroid hormone action on the enzyme-CaM complex involves hormone-induced changes in membrane fluidity in the domain of the enzyme.

Possible Calmodulin Hormonal Control During Bone Remodeling

Several researchers (Wong (28), Peck and Kohler (29), Dziak, et al. (30)) suggested that CaM-calcium dependent reactions are involved in the PTH stimulation of bone cell cAMP. It has been shown recently that during the release of luteinizing hormone, the CaM concentration in the target cell cytosol decreased with a concomitant increase in the amount of CaM bound to the plasma membrane (29).

Calcium and calcium binding proteins, in particular CaM, play major key roles in the regulation of the biology of the mineralization process. CaM may provoke changes in phosphates and calcium concentrations in plasma, which affect bone remodelling and modify the response to parathyroid hormone in mineralized tissue.
osteoclastic and osteoblastic cells, via changes in CaM activity. PGE₂ may alter the subcellular distribution of Calmodulin and produce some of its effects through this action. They also stated that the CaM pool that is involved in the mediation of the PGE₂-induced effects on calcium may be intracellular; but the pool involved in the cyclic AMP effect may be membrane-bound and accessible to antagonists.

**Calmodulin-Vitamin D Physiology**

Bikle, et. al. (36) observed that 1,25 dihydroxyvitamin D stimulated an increase of CaM content in chick duodenal brush border membranes (without increasing total cellular levels) in parallel with an increase in duodenal calcium transport in vivo, and calcium uptake by brush border membrane vesicles in vitro.

The results suggest that 1, 25 dihydroxyvitamin D leads to a redistribution of CaM to the brush border membrane, permitting CaM to mediate 1,25 dihydroxyvitamin D stimulated calcium movement across the brush border membrane. Perhaps CaM interacts with a calcium carrier or channel in the membrane to facilitate calcium movement through the membrane down it's electrochemical gradient (36).
In 1986, Gronowicz et. al. (37) suggested that the synthesis of intracellular calcium binding proteins is a possible mechanism for the alteration of the cytoskeleton and cell morphology characteristics of the ROS 17/2.8 cells. The author presented evidence for the effect of 1,25 dihydroxyvitamin D3 on the cytoskeleton of rat calvaria and rat osteosarcoma (ROS 17/2.8) osteoblastic cells. Finally, it was reported that changes in the cytoskeleton, as well as, cell proliferation have been associated with altered gene expression and differentiation and could participate in the response of osteoblasts to 1,25 dihydroxyvitamin D3 (37).

Possible Calmodulin Regulation of Calcitonin Activities

Calcitonin is known to inhibit osteoclast activity both in vivo and in vitro (38,39). There is evidence that this inhibition is mediated through an increase in intracellular cAMP (39). Inhibition of secretion of rat calcitonin by CaM antagonists has been reported. Cooper and Borosky (40), suggested that CaM plays an important role in the exocytotic process leading to extrusion of calcitonin from the C-cell of baby rat thyroparathyroids and probably of PTH from the chief cell, as well.
Induction of Alkaline Phosphatase Activity by Calmodulin

Komoda et al. (41-43) demonstrated evidence of a time-dependent inductive effect of Calmodulin on the alkaline phosphatase activity (ALP) which was inhibited specifically by CaM inhibitors. They proposed that elevated cAMP concentration due to CaM functions may regulate the ALP.

Calmodulin Role in Plasma Membrane Transport

The biological function of the CaM-coated vesicle association is unknown at this time. However, several postulates are conceptually appealing. It may regulate the calcium ATPase reported in coated vesicles (44,45), mediated interactions between the coat structure, and the membrane, assist in the assembly of the coat structure, modulate interactions between coated vesicles and cytoskeleton elements, and/or regulate the clustering of receptor-ligand complexes into coated vesicles (44,45). The authors presented biochemical and immunological data which offer the possibility that CaM may regulate the calcium requirement for receptor-mediated endocytosis. They also showed, that radioiodinated CaM binds specifically to coated vesicles, in vitro.

Larsen et al. (46) revealed that CaM is a component of clathrin-coated vesicles isolated from brain and that the association is calcium dependent and of high affinity
(kd=10^{-9} M). CaM stimulated the active transport of calcium into inside-out vesicles of red blood cell membranes, acting at the internal side of the membrane bound ATPase (Regulation of plasma membrane calcium transport) (46).

The mechanism has not been defined, however, it was suggested that CaM is a soluble protein (not an intrinsic membrane-bound protein) (46). Thus the possibility that certain soluble proteins such as CaM, may interact with the cytoplasmic face of the plasma membrane to modulate active transport processes has been proposed. Activation of the plasma membrane calcium pump would serve to terminate the other calcium dependent functions. Other related studies have been published indicatory of CaM involvement in the granule-plasma membrane fusion process (44), presence of CaM in secretory vesicles (44), binding of the protein to human erythrocyte ghosts and inside out vesicles in a calcium dependent manner (47).

**Calmodulin Antagonists Actions in Bone Metabolism**

The binding of calcium to CaM exposes a hydrophobic domain, which is essential for interactions with its receptor proteins. The anti-CaM drugs appear to bind to this exposed domain and interfere with CaM-receptor interactions (indeed, the ability of these drugs to
inhibit CaM appears related to their hydrophobicity rather than to their clinical efficacy) (43). Detailed investigations of numerous analogues within these classes of drugs have revealed common structural features (i.e., a large hydrophobic region and a side-chain amino group, which is separated by four or more atoms), thus enabling the prediction of a generalized structure for a CaM inhibitor (43).

Historically, phenothiazines antipsychotics were the first drugs demonstrated to be CaM inhibitors (4). The ever-expanding list of CaM inhibitors now includes other antipsychotics, as well as, alpha-adrenergic blockers, antidepressants, antihistamines, antimalarial agents, cancer chemotherapeutic agents, local anesthetics, neuropeptides, and smooth muscle relaxants (4). Specifically, known CaM inhibitors include: phenothiazines (trifluoperazine, chlorpromazine) (1,40,41,43,48,49), propranolol (1), haloperidol (1), butyrophenones (1), diphenylbutyl-amines (16), pimozide (1), calcineurin (1,4), compound W-7 [N-(6-aminohexyl)-5-Cl-naphthalene sulphonamide] (1), and recently, a potent CaM inhibitor called Calmidazolium (R-24571, Jansen Pharmaceutical) (47).
In regards to possible roles for CaM antagonists in the bone metabolism, Singh and Padmanabhan in 1979 (49) demonstrated the effects of chlorpromazine (CPZ) on skeletogenesis. The authors found that when a single dose of CPZ (100mg/kg) was administered to pregnant rats on the 14th day of gestation (sperm positive=day 0), an inductive intrauterine and extrauterine growth retardation was observed. Ossification was delayed by 1 to 3 days in the long bones of the extremities, by 1 day in the scapulae and by 2 to 3 days in the ileum (49).

In 1985, Komoda et. al. (41) presented evidence of the inhibitory effect of CPZ and trifluoperazine (TFP) on bone formation by in vivo and in vitro studies. Specifically, these drugs showed a dose-dependent decrease in the alkaline phosphatase activity (ALP), as well as, a suppression of collagen synthesis in calvariae of rats. These in vivo and in vitro findings, suggested that CPZ inhibits osteoblastic cell function, reflecting its mechanism of suppression in bone formation.

In addition to the effect of CPZ and TFP on osteoblastic cell functions, an increased serum calcium and phosphate levels in drug treated rats was observed, by a single injection of these antipsychotics. Therefore, these results may also indicate the possible involvement of
these tranquilizers in bone resorption where the increased serum calcium and phosphate levels could be caused by their release from bone tissues resulting from stimulation of bone resorption (41). The authors attributed the drug's actions to a rather specific inhibitory effect on the ALP and not to general cytotoxicity, (the enzyme activity was decreased further at higher concentration of the drug, but on-the-other hand, the DNA and protein contents stayed relatively constant throughout the experiment).

In 1986, Cooper and Borosky (40) reported an in vivo and in vitro inhibition of rat calcitonin by CaM antagonists. Studies were designed to investigate the potential importance of CaM in the release of calcitonin (CT), in response to an increase in the concentration of extracellular calcium. The results showed that CaM antagonists (TFP, compound W-7, CPZ, haloperidol) can inhibit the in vitro secretion of CT and release of PTH as well.

In 1988, Komoda et al. (43) studied the acute effect of CPZ on metabolic changes in rats. The drug was found to markedly suppress the radioactive calcium incorporation into the calvarium and ileum in vitro; increased the calcium and phosphate (P) levels in the serum; increased calcium, (P) and gamma-carboxylglutamate in the urine;
reduced the amount of alkaline phosphatase activity in serum and urine; reduced serum concentrations of ionized calcium, CT, 25-hydroxyvitamin D₃, 1,25-dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃. C-T scan analysis of the vertebrae showed 14% loss of bone density in the young bone of the vitamin D-deficient rats treated for 10 days with 10 mg of CPZ/kg of body weight (experimental group), resulting in an enhanced action of bone resorption in vivo. There was no change in serum and urinary creatine levels during CPZ administration in vivo, indicating that the above phenomenon does not reflect renal failure (43). From the results of several markers for the generalized bone turnover in the serum, it is believe that CPZ effects may not be mediated through changes in the PTH or corticosterone levels, because in this experiment little if any changes of mid-PTH and corticosterone concentrations were observed.

Calmodulin: Possible Role in the Regulation of Exocytotic Membrane Fusion

CaM has been found to bind to pre-formed exocytosis sites and also being associated with the membranes of the osmoregulatory system (50). In 1980, Perachia, et.al. (51) reported that CaM could be involved in the regulation of ionic transport processes (endocytosis) and lysosomal functions associated with ciliary basal bodies. Localiza-
tion of Calmodulin on the inside surface of the plasma membrane of the sea urchin egg strongly suggested that CaM is involved in exocytosis of cortical vesicles and confers a high sensitivity for calcium on the exocytotic process. Involvement of a CaM-like protein in the uncoupling mechanism has been suggested (50-57).

**Neurohormone Regulation by Calmodulin**

Calmodulin stimulates vesicle neurotransmitter release and protein kinase activity (58). In neuronal dentrite, calcium transport in the smooth endoplasmic reticulum and mitochondria is believed to be regulated by CaM (58).

**Binding of CaM to Synaptic Vesicles (59)**

Cathecolamine release involves movement of secretory vesicles to the plasma membrane (exocytosis), requiring fusion of the secretory granule membranes with the plasma membrane and the subsequent extrusion of the soluble granular contents to the exterior.

The process of secretion is one of the calcium mediated events in which CaM seems to be involved. How CaM might be involved in secretion remains to be determined. However, the presence of calcium dependent CaM-binding in a variety of secretory granules and plasma membranes (60) may suggest that CaM is involved either with the transport of
granules to release sites or with the interaction between granules and plasma membranes during exocytosis. Alternatively, CaM might be involved in the process of retrieval of granule membranes after exocytosis. In this regard, high affinity binding sites for CaM have been found in coated vesicles from the brain (45). If exocytosis is a true contractile event, cytoskeleton proteins and their regulatory ones (ex. CaM) might be involved (44).

Role of CaM in Cancer or Transformed Cells

Evidence over the past several years shows that CaM levels are higher in transformed and rapidly dividing normal cells and tissues than in their quiescent normal counterparts (61,62), suggesting that CaM levels may have a role in cell growth and malignant transformation.

In 1987, Rasmussen and Means (63) suggested than in cells (or tissues) transformed by either oncogenic viruses, chemical carcinogens or hormone treatment, the level of CaM is consistently increased and that change of concentration of CaM was a result of an enhanced rate of CaM synthesis.

In 1982, when Wayne et. al. (64) compared several fast-growing Morris hepatoma tissue lines with normal adult
liver tissue, the following observations were made: CaM activity was increased in the cytoplasm and decreased in the membranes of the tumor cells. Total calcium was increased three to five fold in the tumors. Cyclic AMP phosphodiesterase activity was increased, whereas cyclic GMP phosphodiesterase activity was decreased. Therefore, it is probable that the CaM-calcium complex is very active in these rapidly growing tumors and may contribute to an overall decrease in the tumor cell's ability to respond to cAMP and an overall increase in the ability to respond to cGMP. Also increased levels of CaM were observed in fibroblasts transformed by Rous Sarcoma Virus due to an increase in the rate of CaM synthesis.

Antagonists of Calmodulin inhibited cell replication of VX2-L cell line (carcinoma cell line) (64). Transformation of cells to malignancy appears to be one general mechanism causing a specific increase in the intracellular content of CaM. The initial report of such a change was made by Waterson et al. (62), who suggested that transformation of chick embryo fibroblasts by Rous Sarcoma Virus (RSV) resulted in an elevation of the activator protein of phosphodiesterase (CaM). In these experiments, the protein was quantitated by densitometric scanning of cytoplasmic proteins distributed on polyacrylamide gels. A similar study by La Porte et al. (65), utilized an identical
system but, measured CaM by its ability to stimulate phosphodiesterase. Both reports suggested a doubling in the concentration of CaM in the transformed compared with the non-transformed cells. Cell cycle progression and cell transformation is usually accompanied by characteristic alterations in cell morphology, metabolic rate, cyclic nucleotide metabolism, and intracellular calcium levels (1). All of these processes are considered to be regulated by CaM. In addition, transformed cells lose the requirement both for anchorage to a substrate and for the high levels of extracellular calcium required for proliferation by their non-transformed counterparts (61). Therefore, many of the properties typical of transformed cells could conceivably be a result of increased intracellular CaM levels.
Hypothesis

Calmodulin (CaM) is the major intracellular calcium receptor. The distribution of CaM changes during functional stresses on alveolar tissues, such as those experienced when mechanical forces are applied to the teeth.

Corollary:
1. In cells with CaM, the intracellular distribution of this protein will change under stress conditions.
2. Specific cells within tissues under stress will show increased concentration of CaM while others will show decreased concentration.

General Objectives
1. To begin to design a potential model for possible future characterization for the role and mechanism of action of CaM in the bone remodelling process induced by orthodontic tooth movement or bone pathologic conditions.
2. To study the relative distribution of CaM in the different cellular and subcellular compartments of bone active cells by two different approaches at the higher possible resolution level of immunocytochemistry.
3. To demonstrate the efficacy of the proposed methods in/on the tissues to be studied.
Specific objectives

1. To define the location of CaM in the maxillary bone structures of male Sprague Dawley rats using light microscopy immunofluorescence methodology.

2. To define the intracellular location of CaM in ROS (17/2.8) osteosarcoma-derived cells, using immunocytochemistry at the light and electron-microscopic levels.
MATERIALS AND METHODS

Rationale of Approaches Used

Since CaM appears to be a central regulator of calcium mediated processes in cells, its location inside the cell may help determine its sites of action. It has been demonstrated in the literature that the direct immunofluorescence in combination with the Protein-A gold approaches are well defined and established techniques developed for a rapid assessment and location of intracellular antigens for in vivo and in vitro systems. The procedures are relatively fast, versatile, reproducible and allows the screening of a large number of cells under a variety of experimental conditions. Specifically, the techniques have been used successfully to localize CaM showing a very high resolution, sensitivity and specificity.

The techniques present a high resolution method for the direct in situ localization of unique antigenic sites in specific organelles. Although many biochemical studies have examined CaM and CaM-binding proteins, relatively little is known about the localization of these molecules at the cellular level.
An *in vitro* tissue culture model (ROS 17/2.8 cells) was used as a test system to localize CaM. The reason for using ROS cells is based on their potential osteoblast lineage and their use as a model for the study of the regulation of the bone remodeling process (66). The ROS 17/2.8 cells were derived from a rat osteosarcoma and have several properties related to their origin from bone: elevated alkaline phosphatase levels (an indicator of bone formation and turnover), high sensitivity PTH stimulation of adenylnate cyclase, vitamin D receptors (showing vitamin $D_3$ regulated synthesis of osteocalcin), bone gamma-carboxyglutamic acid protein synthesis, among other characteristics (66). ROS 17/2.8 cells were also used because: they are a convenient and economical source of bone-like cells; the published studies by Dr. Paul Epstein in which CaM has been purified from the ROS 17/2.8 cells by fluophenazine sepharose affinity chromatography (17); the ROS 17/2.8 cells were available from Dr. Barbara Kream.

**Immunofluorescence Study in Rat Alveolar Bone Cells**

One hundred male Sprague Dawley rats (Hilltop Animal Lab, Indianapolis) weighing approximately 200g each, were maintained under the same environmental and nutritional conditions. All rats were under general anesthesia with Ketamine 40 mg/Kg + 10mg/Kg Xylazine, for 1 hour (67).
Fifty rats served as a control group, while the other 50 animals were subjected to orthodontic tooth movement, by the insertion of a piece of elastic band (.25mm in thickness) between the maxillary right first and second molars (modified Waldo's technique) (fig. 2-3). The maxillary right quadrant of the rat's dentition was arbitrarily chosen as the experimental side and the maxillary left quadrant as an internal control. After three days of orthodontic tooth movement, all rats were sacrificed. Maxillae from both experimental and control groups were dissected (fig. 4), fixed overnight with Zenker's fixative, and decalcified for five days with 0.5M EDTA. Specimens were dehydrated with a graded series of ethanol (70%, 95%, and (2x) in 100%) for thirty minutes, each. Finally, specimens were transferred to xylene for fifteen minutes prior the one hour and three changes embedding in paraffin. Sections of 4 microns were prepared from the mesial root area of each first maxillary molars.

For the immunological procedures, the primary antibody (sheep anti-native bovine testes Calmodulin) was obtained from Biomedical Technologies, Inc., Stoughton, Ma.(BT-570) and precipitated and purified by affinity chromatography. The secondary antibody (rabbit anti-sheep IgG-FITC) was supplied by Organon Teknika Co., PA.,( Cat.no.1214-0082).
The paraffin sections were preincubated with 1.5% highly purified bovine serum albumin (cat. no. A-7030 Sigma, ST Louis), dissolved in phosphate buffer solution (PBS) (pH=7.4) for thirty minutes, incubated for one hour at 37°C with a (1:10) sheep anti-rat CaM IgG (primary antibody), washed for fifteen minutes with PBS (3X-5 min, pH=7.4) and incubated with the secondary antibody (1:100 rabbit anti-sheep IgG with fluorescein isothiocyanate-conjugated), for thirty minutes. Finally, the sections were washed in PBS, for thirty minutes, (3x-10 min).

The antigen-antibody reaction, as well as, the immune complex between the primary and the secondary antibodies, was assesseed and their specificity demonstrated, by various immunocytochemical experiments:

1. Incubation of sections directly with the secondary antibody omitting the primary antibody to identify non-specific binding of the secondary antibody to the tissue sections.

2. Incubation of sections with a non-reactive antibody (IgG) previously absorbed (neutralized) with an excess of it's pure antigen (5-10 fold excess), instead of the active antiserum, followed by the secondary antibody to verify the specificity of the antigen-antibody interaction by revealing any non-specific binding of the secondary antibody to the tissue sections. The pre-absortion of this
neutralized antibody was achieved through an incubation of antigen with antibody for 30 minutes at 37°C and then overnight at 4°C by using an empirically defined 5-10 fold excess of the optimal ratio of the antigen to antibody interaction.

3. Incubation of sections with non-immune normal sheep serum followed by the secondary antibody to verify the specificity of the antigen-antibody interaction.

4. Incubation with a non-sense antibody (anti-human alpha amylase, cat. no. A-8273) obtained from Sigma, ST Louis and directed against antigens known to be absent in the ROS 17/2.8 cells, followed by the secondary antibody to verify the specificity of the antigen-antibody interaction.

* To avoid or decrease possible non-specific binding of the antibodies to the cells structures, antigen-affinity purified antibodies were used, but also a preabsorption of the secondary antibodies on the cells prior to its use was employed (68). Specifically, 300 microliter of the FITC-secondary antibody were carefully spread over the cells and incubated at 37°C for one hour (in the dark for the FITC). The supernatant containing the IgG molecules was collected and the procedure repeated on a second sample of cells. Finally, the IgG was collected and diluted to appropriate working concentrations.
For the examination with the fluorescence microscope the evaluator limited the field of observation to the mesial root of the first upper molar (mesial and distal surfaces), and to the inter-radicular septum of the same tooth. The preparations were examined and photographed using Kodak Ektachrome ASA 200 film on a Nikon Optiphot microscope equipped for fluorescence photography. The evaluator was not informed of which group was being examined.

**Cell Culture of the ROS 17/2.8 Cells**

ROS 17/2.8 cells, a permanent line in continuous culture since April, 1980 and obtained from a rat osteosarcoma (69), were cultured in F-12 medium supplemented with 5% fetal bovine serum (FBS) and 0.1 mg/ml Kanamycin sulfate in a humidified atmosphere of 95% air and 5% carbon dioxide (70). Cells were fed every 3-4 days with the mentioned culture medium.

**Immunofluorescence Study in ROS 17/2.8 Cells**

Cells grown on round coverslips (18 mm diameter) obtained from Will Scientific, Inc., Rochester, NY (cat. no. 2-180) and 12-wells tissue culture dishes obtained from Costar, Cambridge, MA (cat. no. 3512) for 4 days at 37°C. Most procedures were performed at room temperature, or otherwise specified. Cells were counted on a hemo-
cytometer and washed extensively with Dulbecco's PBS (pH=7.0) containing: 8.0g sodium chloride, 0.2g potassium chloride, 1.15g sodium phosphate (dibasic), 0.2g potassium phosphate (monobasic) per liter.

Cells were either fixed with freshly prepared 2% paraformaldehyde for 30 min or with cold absolute methanol for 5 minutes at -20°C (68,71) and subsequently rinsed (3X) thoroughly with PBS. Cells fixed with 2% paraformaldehyde were made permeable with 0.1% NP-40 (Sigma, St Louis) in stabilizing buffer (1mM EGTA, 100mM PIPES, 1% Polyethylene glycol) for 30 min and followed by further rinsing in stabilizing buffer. All fixed cells were rinsed (3X) with PBS containing 1% BSA obtained from Sigma, ST Louis, (cat. no. A-7030). All samples were then rinsed with PBS and incubated with 1.5% BSA for 30 min.

For the indirect immunofluorescence staining a polyclonal sheep anti-bovine testes CaM IgG diluted (1:10) with PBS (72) and obtained from Biomedical Technologies, Inc., Stoughton MA, (cat. no. Bt-570), was used for one hour as the primary antibody incubation. The IgG was affinity purified on a bovine testes on CaM sepharose column. Following this incubation, the cells were rinsed (3X) with PBS.
A rabbit anti-sheep IgG (H&L chains) conjugated to fluorescence isothiocyanate (FITC) (Organon Teknika Corporation, West Chester, PA cat. no. KS 116-02-F1) was used as the secondary antibody. This IgG was isolated by ammoniumsulphate precipitation followed by ion exchanged chromatography in PBS (pH=7.2) and conjugated to FITC according to the method of The Feltcamp Immunology (73). After conjugation, free FITC was removed by means of dyalisis and gel filtration. Before being used for the secondary incubation, the IgG-FITC was again purified in our laboratory by passage through a Sephadex G-25 column.

The cells were finally incubated with a 1:100 dilution of the secondary antibody in PBS for half an hour and then rinsed (3X) with PBS. The experimental and control samples were mounted on glass slides using 1:10 PBS:glycerol (pH=7.0) and containing 0.05% phenylene diamine as an antibleach. The slides were allowed to dry for at least 12 hours at a temperature of 4°C.

The antigen-antibody reaction, as well as, the immune complex between the primary and the secondary antibodies, was assesseed and their specificity demonstrated, by various immunocytochemical experiments:

1. Incubation of sections directly with the secondary antibody omitting the primary antibody to identify non-
specific binding of the secondary antibody to the tissue sections.

2. Incubation of sections with a non-reactive antibody (IgG) previously absorbed (neutralized) with an excess of its pure antigen (5-10 fold excess), instead of the active antiserum, followed by the secondary antibody to verify the specificity of the antigen-antibody interaction by revealing any non-specific binding of the secondary antibody to the tissue sections. The pre-absorption of this neutralized antibody was achieved through an incubation of antigen with antibody for 30 minutes at 37°C and then overnight at 4°C by using an empirically defined 5-10 fold excess of the optimal ratio of the antigen to antibody interaction.

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* To avoid or decrease possible non-specific binding of the antibodies to the cells structures, antigen-affinity
purified antibodies were used, but also a preabsorption of the secondary antibodies on the cells prior to its use was employed (68). Specifically, 300 microliter of the FITC-secondary antibody were carefully spread over the cells and incubated at 37°C for one hour (in the dark for the FITC). The supernatant containing the IgG molecules was collected and the procedure repeated on a second sample of cells. Finally, the IgG was collected and diluted to appropriate working concentrations.

The preparations were examined and photographed using Kodak Ektachrome ASA 200 film on a Nikon Optiphot microscope equipped for fluorescence photography. All other chemicals otherwise not specified were purchased from Sigma (St Louis, MO).

Immunoelectronmicroscopy Study in ROS 17/2.8 Cells

Cells were fixed with freshly prepared 1% paraformaldehyde diluted in distilled water or with .1M phosphate-buffered, 1% glutaraldehyde solution. After fixation, all cells were rinsed with .1M phosphate-buffer (pH=7.4), dehydrated in a graded series of ethanol (70%, 90%, and 2X in 100%), rinsed twice with propylene oxide and embedded in Poly Bed 812. Thin sections were mounted on nickel grids and processed for immunocytochemistry.
Cell Staining Procedure

The tissue sections were etched with .3 ml of sodium metaperiodate on a parafilm dish for 30 min and rinsed (3X) with distilled water. To block non-specific binding, incubation for 30 min with 1% BSA (.01g/ml) prepared in TBS (pH=7.4) was performed. Sections were incubated in a 1:10 dilution of the primary antibody (sheep anti-bovine testes CaM) rinsed (3X) with TBS, incubated in 1% BSA-TBS solution for 10 minutes and incubated for 30 minutes with Protein-A Gold (15nm) obtained from Jansen Pharmaceuticals (cat. no. 23.703.35). The protein-A gold was diluted 1:20 with TBS. Finally, the sections were rinsed three times with TBS and five times with distilled water. The sections were dried for 10 min and stained with methanolic uranyl acetate.

The antigen-antibody reaction, as well as, the immune complex between the primary and the secondary antibodies, was assessed and their specificity demonstrated, by various immunocytochemical experiments:

1. Incubation of sections directly with the secondary antibody omitting the primary antibody to identify non-specific binding of the secondary antibody to the tissue sections.

2. Incubation of sections with a non-reactive antibody (IgG) previously absorbed (neutralized) with an excess of
it's pure antigen (5-10 fold excess), instead of the active antiserum, followed by the secondary antibody to verify the specificity of the antigen-antibody interaction by revealing any non-specific binding of the secondary antibody to the tissue sections. The pre-absorption of this neutralized antibody was achieved through an incubation of antigen with antibody for 30 minutes at 37\(^{\circ}\)C and then overnight at 4\(^{\circ}\)C by using an empirically defined 5-10 fold excess of the optimal ratio of the antigen to antibody interaction.

3. Incubation of sections with non-immune normal sheep serum followed by the secondary antibody to verify the specificity of the antigen-antibody interaction.

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* To avoid or decrease possible non-specific binding of the antibodies to the cells structures, antigen-affinity purified antibodies were used, but also a preabsorption of the secondary antibodies on the cells prior to its use was employed (68). Specifically, 50 microliter of the Protein-A gold were carefully spread over the cells and incubated
at 37°C for one hour. The supernatant containing the IgG molecules was collected and the procedure repeated on a second sample of cells. Finally, the IgG was collected and diluted to appropriate working concentrations.

Management of Possible Technical Problems, Pitfalls Artifacts During The Research (74)

1. The use of nickel grids avoided oxidation of the metal and consequent contamination of the tissue sections.

2. To obtain maximum specificity of the immunoreaction and to reduce potential source of background, highly purified and very specific immunoreactive agents were used. Using high-titer antibodies allowed the use of lower concentrations of the secondary antibodies while still retaining good specific label patterns. This results in lower levels of non-specific binding of the secondary antibodies to the background.

3. To test the non-specific staining several control experiments were run in parallel with the experimental tissue sections.

4. Drying of the sections or evaporation of the reagents during or between the incubations was avoided to decrease any artifactual adsorption and/or clustering of the colloidal gold.

5. To decrease the potential for background staining, the incubations were performed by floating the grids on the
different reagents to avoid exposure of both sides of the grids to the antibodies or to the protein-A gold complex. In addition, preincubation with highly purified 1.5% BSA (cat. no. A-7030, Sigma, St Louis) also reduced the background staining.

6. Experimental dilution controls were performed, both for the primary antibody and the protein-A gold complex since the concentration of the antisera and staining reagent can also influence the specificity of the reaction. In particular, high concentrations increase the background and may induce the formation of electron-dense deposits on the tissue sections and their intense labeling by gold particles, as well as the clustering of the gold particles.

7. Harsh rinsing of sections may remove or displace the specific labeling. Rinses were performed by gently floating the grids on the reagents. The use of a glass washing spot plate was used when jet washing was employed avoiding direct flow of the jet on the tissue sections.

8. The quality of the tissue and the sections was carefully controlled. Indeed, suboptimal ultrastructural preservation, necrotic cells, cellular debris, or broken structures have the tendency to adsorb the gold particles. Similarly, imperfections such as "chatter", scratches, or dirt particles will interfere with the washing and will accumulate the gold particles.
9. The resolution of the precise location of the antigen depends on the size of the gold particles. The smaller the particle the higher the resolution obtained. Theoretically, the optimal resolution which can be achieved is in the range of 5 to 15nm. However, the choice of the gold particle size should be made in accordance with the type of labeling required (75). In this research, 15 nano-meter gold particles were used.
RESULTS

In any histochemical study, the specificity of the staining technique is always open to question. The accessibility of immunoreactive sites is influenced by both location of the antigen and the penetrability of the tissue. In addition, it is well established that fixation and embedding procedures alter protein antigenicity (75); therefore, comparison of data from different structures may be hampered by the phenomenon of different penetration depths of the immunoreaction in loose and compact structures. For these reasons, it was necessary to determine optimal conditions for each antigen evaluated. A variety of fixation protocols and the effect on antigenicity assayed by ELISA analysis would be used for future studies. The fixation procedure which preserves maximal CaM antigenecity and provides good tissue architecture was used.

Alveolar Cells-Histological Results

Observation of the condition of the bone in normal rats in which no tooth movement was undertaken, revealed a randomness of bone trabeculae, a moderate cellularity and relative uniformity in the thickness of the periodontal membrane. In experimental animals subjected to the placement of the elastic, definite separation was obtained
in twenty-four hours, and maximal separation in three days.

The elastic changed the first molar from a normal distal drift to a mesial direction of movement and, of course, accentuated the normal distal movement of the second molar. The areas of pressure and tension at the cervical level, compared to the apical level, indicated that the elastic strip produced a tipping kind of movement.

At twenty-four hours after placement of the elastic, a definite reaction to stress, namely, changes in the width of the periodontal membrane, cellular infiltration into the bony plate adjacent to the ligament and changes in the vascularity of the periodontal membrane was observed. Pressure areas showed a narrowing of the periodontal space and a localized vasoconstriction, whereas, in areas of tension the opposite was true, that is, a widened space with slight vasodilatation.

After three days, the tissue response was near maximum with undermining resorption and bone apposition. It resulted in an interesting aspect, an occlusal adjustment observed in the lower non-experimental quadrant where a slight tipping movement occurred for a possible restoration of normal masticatory function.
Alveolar Cells: Immunofluorescence Results

Upon examination of the sections obtained from all the specimens, the presence of anti-CaM fluorescence was observed in different areas: tooth pulp (fig.5), root canal (fig.6,7), cemento-enamel junction (fig.6), alveolar mucosa (fig.6), periodontal ligament (fig.7,9-11), alveolar bone (fig.8), interradicular septum (fig.9), alveolar bone lacunae (fig.12) and maxillary first molar sulcular area (fig.13).

All the control sections (fig.14) studied showed some generalized non-specific areas of low level fluorescence.

ROS 17/2.8 Cells-Histological Characteristics

On basis of Dr. Kream's continuous evaluation of the characteristics and behavior of the ROS 17/2.8 cells it is reported that these cells consistently demonstrate osteoblastic properties. In culture, the cells form contiguous layers covering the surfaces of the plastic dishes as described by Rodan and Rodan (fig.15) (66).

The intracellular organelles of the ROS 17/2.8 cells are typical for cells actively engaged extensively in protein synthesis and secretion (66). The oval nucleus is located at some distance from the matrix-secreting surface and the space between the two is occupied by organelles involved
in protein synthesis and transport, abundant rough endoplasmic reticulum and Golgi apparatus (66).

**ROS 17/2.8 Cells-Immunofluorescence Results**

Previously Immunofluorescence data in cultured cells has suggested that CaM is widely distributed including cytoplasmic structures (1,4,76), membranes (1), nuclear structures (12), as well as extracellular space (76). In this study the ROS 17/2.8 cells presented a surprising intense immunofluorescence staining in the nuclear compartments (fig.16,17) but very faint cytoplasmic labeling. The control sections (fig.18-21) demonstrated no nuclear staining and very faint cytoplasmic labeling maybe attributed to fixation procedures.

In the system used in this study, the experimental group revealed an amplified sensitivity of the immunofluorescence technique to detect anti-CaM location in the nucleus where as the controls demonstrated a decreased level of detection in terms of the amount of CaM present in the ROS 17/2.8 cells.
Electron-microscopy Study: Protein-A gold Technique in the ROS 17/2.8 Cells

Description of the Protein-A Gold Immunocytochemistry

Protein-A is a cell wall constituent produced by most strains of *Staphylococcus Aureus* (74,75) and consists of a single polypeptide chain having a molecular weight of 42,000 Daltons, (77). The protein-A-immunoglobulin (Ig G) interaction is a rapid pseudo-immune reaction (reaches saturation level in about 30 minutes) and does not interfere with the binding of the antibody to its antigen. The high specificity of this technique is based on the particular and unique ability of protein-A to interact and bind with high affinity to the Fc fragment of Ig G from almost all mammals (75).

Multiple antigens can be localized in a tissue section by a simple extension of the immunolabelling procedure (75). Protein-A gold complexes are very specific reactive immunoreagents (75). Theoretically, the resolution of the Protein-A technique is determined by: the distance between the antigen binding site and the Fc-terminal end of the Ig G molecule (10 nm), and by the size of the Protein-A molecule diameter of the gold particle used (75). The combination of ultra-thin sections and the Protein-A marker offers optimal conditions for a sensitive
immunoreaction (77). This technique represents a powerful and versatile method which can be successfully applied to protein secretion and intracellular distribution.

Advantages of the Protein-A Gold (PAG) Technique (74)
1. PAG is a negatively charged hydrophobic sol, formed by electron dense properties (strong emission of secondary electrons easily visualized). It is suitable for high resolution electron-microscopic studies on intracellular antigens for both TEM and SEM.
2. Low non-specific binding.
3. Useful for multiple marking studies ex: (double labeling in the same tissue section, is possible).
4. Since the marker is particulated, it does not mask the labeled organelle, thus allowing for good identification, high resolution and the possibilities of quantitative evaluation.
5. Valuable tool for ultra-structural analysis of cell and tissue morphology.

Although it is reported in the immunofluorescence literature that in the cultured cells most of the protein is uniformly distributed throughout the cytoplasm (72), some of it has also been associated with nuclear compartments (12).
The fixation procedures used in this study resulted in an overall good preservation of the cellular structures and the protein-A gold technique demonstrated the specific localization of Calmodulin in the ROS 17/2.8 cells. As illustrated in the experimental electron-micrographs (fig.22-26) specific anti-CaM-Protein-A gold particles were observed on the nuclear and cytoplasmic structures of the rat osteosarcoma cells when compared to the control electron-micrographs (fig.27-30). The statistical analysis of the experimental vs the control sections is demonstrated in (Table I).

The nuclear compartment demonstrated the highest localization of anti-CaM-Protein-A gold particles with a mean=28.01 while the cytoplasm showed a an average of 20.0 gold particles per section studied. The surrounding plastic revealed a insignificant number of protien-A gold particles (Table 1).

For the examination of the control sections, randomly selected areas were arbitrarily chosen for the photograph. The control sections, revealed no immunolabeling on the nucleus and virtually no immuno-staining was found in the cytoplasm or the embedding material (Table 1).
DISCUSSION OF THE RESULTS

Immunofluorescence Study in the Periodontium of the Rat Alveolar Structures

The observation that anti-CaM fluorescence staining was not found in the controls but present in the experimental samples may reflect a possible specific reaction of the immuno-cytochemical procedures. The presence of CaM in the tooth and periodontium may suggest that this multifunctional calcium binding protein is involved in enzymatic processes associated with bone growth and remodeling. However, though possible valuable indicators of function-specific CaM location, these experiments do not allow assessment of CaM in a specific type of cell. Therefore, the design using light microscopy or even with a biochemical analysis can't be used to test the hypothesis of CaM re-distribution in cells within tissues maintain under stressfull conditions.

If and how calmodulin is involved in the bone remodeling mechanism after applying orthodontic tooth forces has yet to be clarified. Therefore, further studies as the study of dose related-inhibitory effect of calmodulin as a mediator of the cell division and transformation mechanisms would be realized.
Immunofluorescence Study in the ROS 17/2.8 Cells

The finding of CaM mainly in the nuclear components is consistent with some recent reports in the literature (12, 72,78). Several immunofluorescence studies have reported nuclear staining of CaM in culture cells (72,78), as well as in the nucleus of liver adrenal cortex cells (12). In 1980, Harper et. al. (12) observed that the administration of hormones such as ACTH produced a large increase in nuclear CaM staining relative to dexamethasone-treated rats. It seems that CaM may change its intracellular location to specific organelles in order to respond to certain functional demands.

In the same study, the authors (12) reported that increased nuclear CaM localization occurred in liver after hormone stimulation. They suggested that hormones may act not only through the regulation of the intracellular calcium but through regulation of CaM as well. Especially intriguing is the possibility that hormone regulation of nuclear function could be accomplished through alterations of CaM location.

The functional significance of nuclear CaM in the ROS 17/2.8 cells is unknown at this time. Although several studies have reported a consistent pattern of nuclear localization of CaM, in most cases, we can only postulate
as to the significance of CaM localization in any particular cellular compartment.

The fraction of all cellular CaM actually localized by the immunofluorescence technique is unknown. Soluble CaM is surely lost from the tissue during washing steps, so that only a small percentage of total CaM is visualized. It is appealing to postulate that active CaM is preferentially retained in tissue section, because the formation of CaM-receptor complexes are known to occur only in the presence of calcium \(^{1,4}\). Thus, calcium-CaM complexes would be more likely to be bound to a component of the cellular structure than would calcium-free CaM. Therefore, the calcium-CaM complex might be less likely to be removed during washing procedures.
SUMMARY AND CONCLUSIONS

Immunofluorescence-Immunoelectron-microscopy Studies

In conclusion, from the experience obtained in this research project, there are many potential pitfalls in immunogold labelling with small gold probes or with immunofluorescence labelling, which can give non-specific binding problems. Although it is difficult to eliminate non-specific binding completely, application of the strategies suggested in these experiments yields acceptably low backgrounds and clear labelling patterns at both the light microscopy and ultrastructural levels.

The indirect immunofluorescence and the immunoelectron-microscopy (protein-A gold) techniques demonstrated a similar specificity to localize CaM in the ROS 17/2.8 cells. The decreased immunolabeling found in the cytoplasm of the ROS 17/2.8 cells by the immunofluorescence technique might be related to the fixation procedures used in the procedure. Another possible explanation to this finding is that the intense nuclear staining obtained in the nucleus may obscured the cytoplasmic labeling and provoked a contrast of immunofluorescence between the nucleus and the cytoplasm.
Despite little or no immunofluorescence staining of the cytoplasm, significant immunogold labelling of the cytoplasm was found. This apparent contradiction may reflect the increased sensitivity of the protein-A gold procedure vs the immunofluorescence technique or maybe due to differences in fixation procedures. Because a careful morphometric analysis was not realized in these preliminary studies (ex. number of gold particles counted in a corrected unit area), it is possible that the relatively high level of immunostaining seen in the cytoplasm, is a statistical artifact.

Calmodulin Possible Re-distribution in Normal or Transformed Cells

Compartmentalization of CaM has been suggested by studies demonstrating that the ratio of CaM content in soluble versus particulate cell fractions differs between tissue types, as well as, by studies with fluorescent labeled CaM reagents in intact or transformed cells (61). The concept of regulation through CaM interaction-dependent redistribution is also supported by evidence suggesting that the spectrum of CaM-binding proteins may change during cell growth, transformation, differentiation and ectopic response to stimulation by hormones or other factors (61).
Several lines of evidence suggests that CaM may dynamically vary its distribution between soluble and particulate fractions as cell divide. These observations support the concept that shifts in CaM location to different subcellular fractions may represent a change in intracellular CaM distribution. The cytosol-to-membrane ratio of CaM may also varies with cell density in some cultures, and different distribution of CaM have been observed in quiescent versus dividing cells (61).

The random diffuse distribution found in the immunoelectron-microscopy study does not of course, directly imply a freely soluble condition, since much of this CaM is maybe bound to proteins that are themselves diffusely distributed. The association of CaM with membranes or other immobilized cytoplasmic elements in a non-concentrative fraction would also be consistent with this data. However, the presence of CaM in vesicles or structures rapidly formed and collapsed in seconds, suggests that at least some of the protein or the proteins to which it is bound move through the cytosol freely (21).

Significance of the Project to the General Health Field
1. Similar studies with CaM have not been conducted in bone cells.
2. This research may be used in the future as a
preliminary approach to study the bone remodeling mechanism using CaM regulatory substances to ultimately optimize the rate of tooth movement during orthodontic treatment. The intelligent use of this knowledge, will permit us to modify the orthodontic methodology in order to achieve an optimum tissue response through the developing of innovative approaches in the future. Therefore, accurate and precise control of tooth movement can be optimized not only with the proper use of mechanics but with knowledge of the subsequent biological response.

3. The practical contribution of this research to pharmacology may well be a better understanding of the actions of drugs and a more enlightened search for better ones. Information in how certain drugs affect biochemical pathways controlled by CaM should stimulate further progress in therapeutic research. It is suggested that calcium blockers and anticalmodulin antagonists might be powerful anticancer and/or antihypercalcemic agents for malignant bone diseases, such as, osteosarcomas. It may be possible in the future, the development of effective treatments designed to modify the CaM levels in the human body.

4. In terms of the orthodontic-pharmacology fields, patients under antipsychotic drug treatment would be advise to take the neccessary medical precautions to avoid secondary unexpected reactions to the orthodontic tooth
movement treatment.

**Unanswered Questions and Suggested Future Investigations:**

The biological role of CaM in bone cells remain largely obscure. But, extrapolating from its known role in other cellular systems, this protein is likely to regulate various calcium dependent cell processes in bone. In further studies, therefore, CaM binding proteins or CaM binding enzymes should be investigated in mineralizing tissues.

At present, most of the data concerning CaM regulation must be considered as phenomenological. What is now required is a concerted effort to understand the chemical basis for each of CaM's actions. This will require isolation and purification of the enzyme systems regulated by this protein. Indeed, some success has already been achieved along this lines.

The histochemical and cytochemical demonstration of CaM in ROS 17/2.8 cells, as well as, alveolar cells give a tool to compare structural differences if any between normal (i.e. calvarial cells) and transformed cells during various stages of their cell cycle. It is hope that this project will aid in answering some questions of cellular transformations in relation to any possible CaM
activities.

In summary, it is difficult to predict whether CaM redistribution is a cause or sequence of a secretory or metabolic process since, it is so widely distributed: part cytosolic, in part reversibly bound to subcellular structures (1,13,) and intra-extracellularly distributed in sperm and testis, (79).

The question of fundamental importance, whether CaM is the major calcium binding protein regulating bone apposition and resorption, remains to be answered. The answer awaits future investigations. Further clarification of the calcium-CaM interactions in cancer cells will be of great assistance in understanding what role this metal-protein complex might play in cancer cell growth.
**TABLE-I**

RESULTS OF THE PROTEIN-A GOLD TECHNIQUE IN THE ROS 17/2.8 CELLS *

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>NUCLEUS</th>
<th>CYTOPLASM</th>
<th>EMBEDDING MATERIAL</th>
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**EXPERIMENTAL GROUP-STATISTICS:**

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<tr>
<td>SEM</td>
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<td>(+/- 3.6)</td>
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**CONTROL GROUP-STATISTICS:**

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<th>MEAN</th>
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<tbody>
<tr>
<td>SEM</td>
<td>0</td>
<td>(+/- .11)</td>
<td>(+/- .09)</td>
</tr>
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</table>

**LEGEND * **

1. Results were obtained by counting the number of gold particles found in cells from 18 random pictures from the experimental group and 18 random pictures from the control group. Most pictures were taken at the same level of magnification for evaluations.
2. Results are reported as the mean +/- the standard error of the mean (SEM).
Fig. 2—Photograph demonstrating the insertion of an elastic band (.25mm thickness) between the first and second rat maxillary molars to induce orthodontic tooth movement taken from Waldo (1953).
Fig. 3-Drawing showing the effects of the insertion of the elastic band (.25 mm thickness) between the first and second maxillary molars of the Sprague Dawley rat taken from Roberts (1974).
Fig. 4—Photograph showing a dissected maxilla from a Sprague Dawley rat demonstrating the retention of the elastic band between the first and second maxillary molars three days after insertion of the elastic.
Fig. 5—Photograph of the rat maxillary molar after the insertion of the elastic between the first and second maxillary molars. Immunofluorescence staining (arrow) is seen on the cells bordering the pulp chamber (PC).
Fig. 6—Photograph showing immunofluorescence staining even without the insertion of the elastic between the first and second maxillary molars. Immunofluorescence can be observed on: the root canal of the molar (A), cemento enamel junction (B) and alveolar mucosa (C). A faint anti-CaM labelling was found on the alveolar bone (D).
Fig. 7—Picture of a section of the periodontium of a Sprague Dawley rat after the insertion of the elastic between the maxillary molars. Immunofluorescence staining can be observed on the alveolar bone (A), periodontal ligament (B) and root canal of the rat maxillary first molar (C).
Fig. 8—Photograph revealing generalized immuno-labelling of the alveolus (A) of a Sprague Dawley rat even without any induction of tooth movement.
Fig. 9—Picture showing immuno-labelling after the insertion of the elastic between the maxillary molars. Immuno-fluorescence can be seen on the periodontal ligament (A,C) and interradicular bone (B) surrounding the mesial and distal roots of the rat maxillary first molar.
Fig. 10—Photograph of the anti-CaM immuno-fluorescence after the induction of tooth movement. Staining was found on the periodontal ligament (B) between the first (A) and second (C) maxillary molars.
Fig. 11 - Picture revealing immuno-labelling of a section of the periodontal ligament (B) without the insertion of the elastic. The root of the maxillary molar (A) is on the left and the periodontal ligament can be observed on the right.
Fig. 12—Photograph demonstrating specific immuno-staining on the alveolar bone area of resorption (B). Observe the bone (A), the periodontal ligament (C) and the root of the first maxillary molar (D). Orthodontic tooth movement was induced against the immunolabelled area of resorption.
Fig. 13—Photograph showing specific immunofluorescence staining of the sulcular area (arrows) of the rat maxillary first molar. No elastic was inserted.
Fig. 14—Photograph of a control specimen showing insignificant amount of non-specific immunofluorescence staining of the rat maxillary first molar and the periodontium. Picture was taken under the same conditions considered for the experimentals.
Fig. 15—Photograph demonstrating the contiguous layers formed by the ROS 17/2.8 cells in culture. Courtesy of Dr. Barbara Kream.
Fig.16—Photograph demonstrating intense nuclear (N) immuno-staining and faint generalized cytoplasmic labelling of the ROS 17/2.8 cells (Magnification=20X).
Fig. 17—Photograph of a frozen section preparation showing an intense nuclear (N) immunofluorescence staining and some cytoplasmic (C) labelling of the ROS 17/2.8 cells (Magnification=40X).
Fig. 18—Photograph of a control incubation of the ROS 17/2.8 cells with a pre-absorbed primary antibody instead of the experimental primary antibody where no nuclear (N) staining and insignificant amount of cytoplasmic (C) labelling can be observed (Magnification=10X).
Fig. 19—Photograph of a control incubation of the ROS 17/2.8 cells with normal sheep serum instead of the experimental antibody where no nuclear (N) staining and insignificant amount of cytoplasmic (C) labelling can be observed (Magnification=10X).
Fig 20—Photograph of control incubation with a non-sense primary antibody instead of the experimental antibody revealing a very faint cytoplasmic (C) staining but no nuclear (N) labelling of the ROS 17/2.8 cells. Cells were prepared as a frozen section (Magnification=40X).
Fig. 21—Photograph of a control incubation with only the secondary antibody revealing a very faint cytoplasmic (C) staining but no nuclear (N) labelling of the ROS 17/2.8 cells (Magnification=40X).
Fig. 22—Electron-micrograph of a section of the ROS 17/2.8 cells showing anti-CaM nuclear (N) labelling on the cell on the left. An area of densely distributed gold particles can be observed on the cytoplasm (C) of the other cell in the field. Note the absence of particles between the cells (Magnification=6600X).
Fig.23—Electron-micrograph of a section of a ROS 17/2.8 cell demonstrating anti-Calmodulin immuno-labelling on the nuclear components including the nucleolus (No) (Magnification=10,500X).
Fig. 24—Electron-micrograph of a portion of a ROS 17/2.8 cell showing gold particles in the nucleus (N), on nuclear membrane (NM) and some in the cytoplasm (C) (Magnification=10,500X).
Fig. 25—Electron-micrograph demonstrating cytoplasmic labelling of the rat osteosarcoma cell on the left. Immuno-staining can be seen in the nucleus (N), and cytoplasm (C) of the cell on the right of the field. Only few gold particles can be observed on the embedding material (EM) (Magnification=13,500X).
Fig. 26—Electron-micrograph of a section of the nucleus (N) and cytoplasm (C) of a ROS 17/2.8 cell. Nuclear and cytoplasmic immuno-staining was observed (Magnification=25,000X).
Fig. 27—Electron-micrograph of a control incubation with a pre-absorbed antibody instead of the experimental antibody demonstrating absence of anti-CaM immuno-labelling on the ROS 17/2.8 cell (Magnification=8900X).
Fig. 28—Electron-micrograph of a control incubation with normal sheep serum instead of the experimental antibody showing no immuno-staining on the nucleus and cytoplasm of a ROS 17/2.8 cell (Magnification=8900X).
Fig. 29—Electron-micrograph of a control incubation with only the secondary antibody. Absence of anti-CaM labelling was found on the section (Magnification=8900X).
Fig. 30—Electron-micrograph of a control incubation with a non-sense primary antibody instead of the experimental antibody. The section revealed a possible non-specific immuno-labelling of one gold particle (arrow) on the cytoplasm of a ROS 17/2.8 cell (Magnification=8900X).
REFERENCES


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