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Effects of Calcitonin Gene-Related Peptide on Human Pulpal Cells IN VITR

Catherine P. Terzoglou

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THE EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON
HUMAN PULPAL CELLS IN VITRO

Catherine P. Terzoglou
D. D. S., University of Athens, 1989

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THE EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON HUMAN PULPAL CELLS IN VITRO

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1992
To my parents
To Spyros
ACKNOWLEDGMENTS

A number of people have played an essential role for the completion of this project.

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LITERATURE REVIEW

A. TOOTH DEVELOPMENT

Odontogenesis is an integrated process of events that lead to the formation of a mature tooth (Ruch, 1984). It involves cell segregation, differential cell division, cell migration and specific spatial distribution of terminally differentiated cells. Although the molecular mechanisms that are involved in tooth development have not been elucidated yet, there is evidence that epigenetic factors control the different stages of tooth development (Thesleff and Humerinta, 1981). Tissue interactions occur when "two or more tissues of different history and properties become intimately associated and as a result alterations of the developmental course of the interactants occur" (Grobstein, 1956).

Tooth development is the result of both instructive and permissive tissue interactions between oral epithelium and ectomesenchyme of the first branchial arch. The ectodermally derived oral epithelium will give origin to the enamel organ that is going to form the enamel of the tooth, while the neural crest derived mesenchyme will give origin to the dental papilla that is going to form dentin, pulp, periodontal ligament and alveolar bone (Ruch, et al., 1983).

From a histologic perspective odontogenesis starts early during the craniofacial growth when the maxillary and mandibular processes are migrating mesially to contribute to the developing face. It can be divided in three distinct phases: i) initiation ii) morphogenesis and iii) cytodifferentiation (Kollar, 1985).
1. Initiation

Early during development a thickening of cells of the oral epithelium, the dental lamina, can be noticed. At specific areas which determine the position of the future teeth the dental lamina invaginates into the mesenchyme of the developing alveolar process thus, creating the dental primordia (Bhussy and Sharawy, 1990).

The oral epithelium seems to control the initiation phase. Tooth bud formation was elicited in vitro after recombination of mouse premigratory neural crest cells with mandibular ectoderm but not with limb bud epithelium of non dental origin (Lumsden, 1985). It has been demonstrated that mouse epithelium from the first branchial arch before day 12 is able to elicit tooth bud formation from non odontogenic mesenchyme of the second branchial arch in both homotypic and heterotypic recombinations (Mina and Kollar, 1987). The oral epithelium instructively induces the ectomesenchymal cells to become dental papilla cells.

2. Morphogenesis

During this phase the developing tooth structure assumes its morphologic pattern of organization. The epithelial part of the tooth bud develops into the concave enamel organ which further differentiates into the outer and inner dental epithelia which are separated by the stellate reticulum and stratum intermedium. The ectomesenchymal tissue below the enamel organ condenses and gives rise to the dental papilla. The
developing tooth structure progressively assumes a cusp-like morphology. The junction of the inner and outer enamel epithelia clearly demarcates the enamel organ from the dental papilla (Bhussy and Sharawy, 1990).

Both enamel organ and dental papilla are necessary for tooth development. If the tissues are separated, tooth-like structures can not be formed in vitro (Koch, 1967; Kollar and Baird, 1970). The dental papilla cells control this stage of development. Dental papilla cells from 13-17 day old mice have been shown to induce epithelia of non-dental origin to form enamel organ (Kollar and Baird, 1970). If enamel organ and dental papilla are separated and cultured alone, development is arrested and the enamel organ can express a variety of epithelial configurations ranging from random invasiveness to keratinization. When the enamel organ and the dental papilla are placed again in close approximation they regain their original configuration and continue their developmental course (Kollar, 1976).

During morphogenesis the extracellular matrix mediates tissue interactions between the cells of the enamel epithelium and the dental papilla.

Several components of the extracellular matrix are necessary for morphogenesis. The extracellular matrix was first implicated in the developmental process when it was demonstrated that collagenase administration in cultures of tooth germs disrupted morphogenesis (Koch, 1968). Several drugs affecting the normal production of collagen, proteoglycans and glycoproteins, or cell mitosis have been shown to play similar inhibitory roles. The inhibitory effects of αα dipyridyl, β-aminopropionitril and L-azetidine on collagen production and morphogenesis were eliminated and morphogenesis resumed after
removal of the inhibitory agent (Galbraith and Kollar, 1974; Kollar, 1973) or addition of procollagen to the culture medium (Kollar, 1978). These data suggest that the interactions that occur during this phase of development are permissive. The extracellular matrix seems to be a mediator rather than a controlling influence of histogenesis. Cell-cell and cell-matrix interactions result in a functional spatial tissue pattern that will permit cytodifferentiation and the successive ordered deposition of the hard tissue matrices (Kollar and Lumsden, 1979).

3. Cytodifferentiation

During this phase terminal differentiation of the odontoblasts and the ameloblasts occurs according to a temporo-spatial pattern (Ruch, et al., 1972) Experimental data suggest that the genetic programs of both the odontoblasts and the ameloblasts require a minimum number of cell cycles before overt differentiation can occur (Ruch, et al., 1976). The odontoblasts are post-mitotic cells of neural crest origin. Their embryologic origin has been established in the amphibia (Chibon, 1966). Migrating neural crest cells were distinguished in the developing alveolar process and mitotic figures were also identified in the dental papilla (Schoenwolf and Nichols, 1984).

The association of dental papilla with inner dental epithelium is important for odontoblast differentiation. Heterotypic homospecific associations between dental papilla and non dental epithelium resulted in functional odontoblast differentiation after the epithelium assumed the characteristics of inner dental epithelium (Kollar, 1972). Moreover, when
dental papilla cells were associated in vitro with isochronal dental epithelium odontoblast differentiation occurred while, when they were associated with younger inner dental epithelium the odontoblastic cells depolarized, underwent another mitotic cycle and then secreted predentin (Ruch, et al., 1971; Ruch, et al., 1976). Taken together these data emphasize the temporal nature of odontogenesis and the existing interdependence between the sequential developmental stages.

Specific cell-matrix interactions seem necessary for the cytological and functional differentiation of odontoblasts (Koch, 1967). Specific elements of the basement membrane probably deposited by the cells of the inner dental epithelium mediate these interactions. The basement membrane is a dynamic interface demonstrating modifications in its composition that appear to be causally related with the different stages of odontogenesis (Ruch, 1985). Reassociation of dental components after trypsinization and subsequent removal of the basement membrane temporarily inhibited cytodifferentiation and did not allow it to proceed before a new basement membrane was secreted by cells of the inner dental epithelium (Thesleff, et al., 1981; Thesleff, et al., 1978). It is likely that time and space specific information encoded in the basement membrane is recognized transduced and interpreted by the cell membranes of adjacent cells (Ruch, 1987).

Once the odontoblasts become fully differentiated and secrete the first layer of predentin, the preameloblasts polarize and subsequently differentiate into functional ameloblasts that start secreting enamel proteins, simultaneously with the initiation of the mineralization of the dentin matrix (Bhussy and Sharawy, 1990). The presence of functional odontoblasts is a prerequisite for ameloblast differentiation (Ruch et al.,
Dental papilla is the controlling influence that determines the developmental fate of the epithelially derived enamel organ. Dental papilla cells have been shown to induce ameloblast differentiation from non-odontogenic epithelia and even from chick epithelium (Ruch et al., 1973; Kollar and Baird, 1970).

During ameloblast differentiation the basement membrane is broken down, allowing direct interaction between the ameloblasts and the odontoblastic process (Katchburian and Burgess, 1983; Slavkin and Bringas, 1976). The space previously occupied by the basement membrane will delimitate the future dentino enamel junction (DEJ).

**B. DENTINOGENESIS**

The term dentinogenesis refers to the period of active dentin deposition during tooth development, despite the fact that dentin continues to be deposited throughout the lifetime of a tooth.

During the last mitosis of preodontoblasts the mitotic spindle is positioned perpendicular to the basement membrane thus, giving rise to two superimposed daughter cells (Osman and Ruch, 1976). Only the cell that is in contact with the basement membrane differentiates into a functional odontoblast, while the other daughter cell contributes to the formation of the subodontoblastic layer (Ruch, 1987).

During its differentiation the odontoblast progressively elongates, polarizes and starts synthesizing and secreting predentin (Takuma and Nagai, 1971). At this terminal phase, the odontoblast can be clearly divided into two morphologically distinct parts with different functional
roles, the cell body and the odontoblastic process. The cell body, which is responsible for the synthesis of the elements of the organic matrix, is rich in mitochondria, Golgi apparatus and rough endoplasmic reticulum. Distended saccules can be seen at the mature surface of the Golgi apparatus (Frank, 1979; LeBlond and Weinstock, 1976). Intracellular contacts, such as gap and tight junctions and desmosomes have been demonstrated to exist between the odontoblast cell bodies (Holland, 1975; Koling, et al., 1981). The odontoblastic process, which is responsible for the secretion of the odontoblast products in the extra cellular space, has fewer cytological organelles. It is rich in fine filaments, microtubules and secretory vessicles. Two types of vessicles can be distinguished, the electron dense vessicles, containing filamentous granular material, and the coated vessicles possibly involved in pinocytosis (Frank, 1979).

Cytoskeletal elements of the odontoblastic process are important for cytoskeletal function as well as for the transport of granules in and out of the process (Thomas, 1979). The odontoblastic process arborizes extensively below the dentino-enamel and cemento-enamel junctions. Its size and shape varies at different stages of dentinogenesis. It has been suggested that the odontoblastic process with its structural elements and its side branches might direct the formation and orientation of the organic matrix (Kaye and Herold, 1966).

1. Dentinal Collagen

Odontoblasts secrete collagen, proteoglycans, glycoproteins and phospholipids. Collagen is their main product accounting for almost 90%
of the inorganic matrix (Butler, 1985; Mac Dougall et al., 1985; Becker et al., 1986).

Dentinal collagen consists mainly from collagen type I \([\alpha_1(I) \alpha_2(I)]_2\) and small amounts of collagen I trimer \([\alpha_1(I)]_3\) and type V collagen (Lesot, et al., 1981; Lesot and Ruch, 1979). However, during biosynthesis of the organic matrix of dentin collagen I trimer is secreted in large amounts but it is not retained during mineralization and maturation of the tissue. No type III collagen has been detected in dentin although it is abundantly made in the pulp (Lesot, 1981).

The genes that encode collagens type I and type V belong to the family of fibrillar collagen genes (Barsh, et al., 1984; DeWet, et al., 1987). Each protein is approximately 1000 amino acids long and has a characteristic triple helical structure. The intron-exon structure is maintained and the differences in the size of the genes are due to differences in intron size (Byers, 1989). Transition exons exist on both the amino and carboxy terminal sides of the chain containing sites of proteolytic cleavage. The organization of the genes belonging to the family of fibrillar collagens is similar. The \(\alpha_1(I)\) chain is encoded by a 18 kb \(\text{COL}_1\text{A}_1\) gene, located on chromosome 17 (Sunderraj, et al., 1977) while, the gene for the \(\alpha_2(I)\) chain \(\text{COL}_1\text{A}_2\) is 40 kb long and is located on chromosome 7 (Huerre, et al., 1982).

The primary sequence of the \(\alpha\) chains is Gly-X-Y where X and Y can be any amino acids. Due to its small size glycine is the only amino acid that can fit into the interior crowded positions of the helix. The stability of the chain is provided by hydrogen bonds between the amide groups of glycine and the carboxyl group of the X amino acid (Berg and Prockop, 1973). Three chains assemble together and form a triple helical molecule. The triple helical structure seems to be a requirement for the transport of type I
collagen beyond the rough endoplasmic reticulum (Jiminez, et al., 1973). It also provides resistance to degradation by most proteases and allows collagen to be an extremely stable structure in the extracellular environment (Harwood, et al., 1976).

Collagen production is controlled at many levels but the molecular mechanisms involved are not clearly understood. Since the genes for chains $\alpha_1$ and $\alpha_2$ of type I collagen are located in different chromosomes their physical relationship during cell cycle is not known. They are present in equal number but $COL_1A_1$ produces twice as much steady level mRNA than $COL_1A_2$, probably due to a difference in their transcriptional efficiency (DeWet, et al., 1983). As a result two pro$\alpha_1$ (I) chains are synthesized for each pro$\alpha_2$(I) chain (Lian, et al., 1986). Regions located upstream in the collagen gene and in the first intron seem to be able to regulate collagen synthesis (Bornstein and McKay, 1988; Schmidt, et al., 1986). It has been suggested that different promoters are responsible for the regulation of the genes coding pro$\alpha_1$(I) and pro$\alpha_2$(I) chains (Lichtler, et al., 1989). Collagen mRNA are synthesized in the nucleus as long precursor molecules that are spliced, capped and polyadenylated before being transported to the cytoplasm (Avvedimento, et al., 1980). There is no adequate information regarding control of mRNA translation. However, there is evidence that the amino and carboxy terminal propeptides can influence mRNA translation (Bennett and Adams, 1987). A variety of peptides and growth factors can influence collagen gene production (Rashcow, et al., 1987; Roberts, et al., 1986). Ascorbic acid has been found to enhance type I collagen production by stimulating many stages of its biosynthesis (Pinnel, et al., 1987; Sandell and Daniel, 1988).
Following secretion the procollagen triple helices are converted into tropocollagen after excision of the amino and carboxy terminals through the action of specific procollagen peptidases (Prockop and Tuderman, 1982). The nascent collagen molecules spontaneously self assemble into fibers in the extracellular fluid, close to the cell surface. Their structure resembles staggered arrays where each tropocollagen molecule is separated by the adjacent ones by 400 Å gaps and the adjacent ones are displaced by 680 Å (Hulmes, et al., 1983). The gaps allow the formation of cross-links and the deposition of apatite during mineralization.

Cross-linking within the collagen molecule itself and between different molecules occurs during the maturation stage (Eyre, et al., 1984; Tanzer, 1976). Dentinal collagen like collagen from other mineralized tissues exhibits a higher amount of cross-links than soft tissue collagen (Bonar and Glimcher, 1981). The dentinal collagen molecule has a more complete hydroxylation than other types of collagen, thus contributing to a higher amount of cross-links (Kuboki and Mechanic, 1982; Veis and Schlueter, 1964). The major reducible inter molecular cross-link of dentinal collagen is hydroxy-lysine-5-ketonorleucine which links the carboxy end of the molecule to the triple helical region of other molecules (Mechanic, et al., 1971). Another major transductional bond pyridinoline cross-links two carboxy terminal ends of $\alpha_1$ (I) chains (Fuzimoto, 1980; Kuboki, et al., 1981). The high amount of cross-linking might account for the increased stability of the dentinal collagen molecule and its resistance to protease activity (Light and Bailey, 1981).
2. Collagen Biosynthesis in Odontoblasts

Odontoblasts are able to synthesize type I collagen from the early stages of their polarization (Cournill, et al., 1979; Thesleff, et al., 1979). In situ hybridization experiments show increased amounts of proα₁(I) mRNA with concomitant low amounts of proα₂(I) mRNA during the early stages of odontoblast differentiation thus, suggesting enhanced production of type I collagen trimer. As development proceeds proα₁(I) mRNA levels increase while proα₂(I) mRNA levels decrease, indicating a shift towards type I collagen synthesis (Andujar, et al., 1991). Light and electron immunocytochemical studies of collagen distribution during dentinogenesis in the developing mouse molar, have shown only type I collagen immunoreactivity in the odontoblast zone and the predentin, while type III collagen immunoreactivity was limited to the sheath of Hertwig and the mesenchymal pulpal tissue (Andujar, et al., 1988). It seems that augmentation of gene expression for type I collagen and type I collagen trimer is a characteristic feature of odontoblast differentiation (Andujar, et al., 1988; Ruch, 1985).

Collagen synthesis in odontoblasts has been studied by means of electron microscopy, histochemistry and autoradiography following [3H] proline injection in rats (Weinstock and LeBlond, 1974). Procollagen chains are synthesized in the ribosomes of the rough endoplasmic reticulum. Three procollagen chains assemble to form a procollagen molecule. At that point and prior to folding of the molecule prolyl and lysyl hydroxylases catalyze the hydroxylation of the proline and lysine residues, while collagen galactosyl transferase catalyzes the transfer of galactose to certain hydroxyl sites and glycosyl transferase mediates the
formation of galactosyl hydroxy lysine residues. Also, some oligosaccharides are added to the triple helical molecule. The procollagen molecules assemble in aggregates in the Golgi saccules. These are progressively transformed in presecretory and secretory granules which will be subsequently released into predentin. The whole process takes about ninety minutes. In the extracellular space the tropocolagen chains assemble into fibers which progressively acquire their mature form.

3. Non-collagenous Proteins

In addition to collagen, predentin contains non-collagenous proteins which account for approximately 10%. These comprise phosphoproteins, proteoglycans and glycoproteins (Linde et al., 1980). The amount of non-collagenous proteins is higher in dentin than in predentin, suggesting that they may play an important role in the mineralization process (Linde, 1984).

Phosphoproteins constitute the major component of the non-collagenous proteins (Veis and Schlueter, 1964). Dentin phosphoprotein (DPP) mainly consists from aspartic acid and serine. The majority of the serine residues, approximately 80%, are phosphorylated (Butler, et al., 1981; Fujisawa, et al., 1984). DPP is believed to play a major role during mineralization due to its ability to bind tightly the calcium ions (Lee, et al., 1977). The secretion of DPP is one of the characteristic features of the odontoblast phenotype (Dimuzio and Veis, 1978). Autoradiographic study of DPP synthesis after injection of $^{32}$P phosphate and $^{3}$H serine showed that the biosynthetic process takes place in the Golgi apparatus of the
odontoblast cell body. DPP is secreted into dentin 30' after injection and by 90' it can be traced in the dentin side of the predentin-dentin junction (Weinstock and LeBlond, 1973).

Some investigators have reported that both in vitro and in vivo translated mouse DPP have the same molecular weight as mature DPP (MacDougall, et al., 1985). However, a number of studies contradict the above data. The molecular mass of newly secreted DPP was demonstrated to be 150 kDa, while the one of DPP extracted from mature rat dentin was only 90 kDa (Maier, et al., 1985). It has been suggested that extracellular processing involving proteolytic cleavage and/or phosphate degradation by b-elimination mechanism might be implicated in the DPP maturation process (Dimuzio, et al., 1985; Marsh, 1986). This hypothesis was also supported by in vitro translation of bovine DPP mRNA by the xynopus oocyte system, which estimated the nascent DPP to be a single molecule which only has a size of 100 kDa (Ibaraki, et al., 1991). However, further studies are required in order to clarify this matter.

Proteoglycans comprise an important component of the non collagenous fraction of predentin. The predominant proteoglycans secreted in predentin are chondroitin-6-sulfate, chondroitin-4-sulfate, dermatan sulfate and keratan sulfate (Clark, et al., 1965). It has been suggested that proteoglycans influence fibrillogenesis and predentin matrix organization (Hjerpe and Engfeldt, 1973).

Finally, the organic matrix contains small amounts of lipids that account for 0.2-0.3% of the dry waste and 1.7% of the organic mass (Dirksen and Ikels, 1964; Manzoli and Gelli, 1968). Dental lipids consist of phospholipids, "free cholesterol", cholesterol esters and triglycerols that comprise approximately 90-95% of the total lipid content, while the rest is
made from free-fatty acids and diacylglycerols (Ellingson, et al., 1977; Prout and Odutuga, 1974). Lipids are necessary components of cell membranes and thus participate in membrane formation (Ennever, et al., 1977). Besides the matrix vesicles that appear in the initial stages of mineralization are rich in lipids (Anderson, 1969).

C. MINERALIZATION

Mineralization of dentin starts after an initial layer of predentin approximately 10 μm wide has been deposited. Hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) is the main inorganic component of dentin (Posner and Tannenbaum, 1984). The crystals of hydroxyapatite are needle shaped with a thickness of approximately 5 μm and a length of approximately 20 μm (Steve-Bocciarelli, 1973). The mechanism involved in the initiation of mineralization and initial crystal formation is still unclear. Scientific evidence suggests that "nucleation" and matrix vesicles formation results from cellular activity and/or specific interactions between different molecules in the extracellular matrix and the subsequent attraction of calcium and phosphate ions. The matrix vesicles contain high amounts of alkaline phosphatase, an enzyme that has been extensively associated with biomineralization (Bernard, 1972; Eisenman and Glick, 1972). After their initial formation the crystals progressively grow until they reach a mature stage (Nylen, et al., 1963). The foci of mineralization grow into spherical structures, the calciospherites, which fuse with adjacent spheres thus advancing the mineralization front (Boyde and Jones, 1972; Katchburian, 1977; Posner, et al., 1980).
Both collagen and non-collagenous proteins along with specific cellular activities play an important role in the regulation of mineralization.

Collagen is the most obvious element to be involved in the mineralization process. It seems to provide the proper matrix for the biochemically directed cell actions (Neuman, 1980). The unique structure of collagen is an important requirement for its calcifiability. There is an intimate relationship between the mineral phase and the collagen fibers. The mineral crystals are located within and around the fibrils exhibiting the same axial periodicity as the collagen molecules (Bevelander and Nakamara, 1966). Electron microscopic and low angle x-ray and neutron diffraction studies show the crystals filling in the spaces of the collagen fibrils (White, et al., 1977). The long axis of the crystals is located in a parallel direction to the one of the collagen fibrils (Glimsher, 1981). It has been shown that the intermolecular space of dentinal and bone is twice as large as tendon collagen, thus providing the necessary space for the calcification process (Katz and Li, 1973). It has been suggested that collagen allows crystal deposition and limits the amount deposited (Glimsher, 1981).

Non-collagenous proteins, and in particular phosphoproteins, play an important role in the mineralization process. They seem to influence the rate at which mineral is being formed (Veis and Schlueter, 1964). The high affinity of phosphoproteins for calcium ions along with their ability to form links with collagen give them a unique contribution in the mineralization process (Carmichael, et al., 1971; Lee and Veis, 1980). Phosphoproteins are secreted by the odontoblast directly at the mineralization front and have been associated with the collagen fibers that are located in the mineralizing dentin and in the predentin-dentin.
junction (Weinstock and LeBlond, 1973). Also, phosphoproteins bind calcium and contribute in the formation of hydroxyapatites with a higher degree of crystallinity (Linde, 1984).

Finally, lipids as structural elements of the secretory vesicles influence the normal progress of mineralization at the initial stages (Ennever, et al., 1977).

D. DENTIN

Dentin is a mineralized connective tissue consisting of approximately 70% inorganic content, 18% organic content and 12% water (Mjor, 1979). Dentin is permeated by dentinal tubules. The dentinal tubules are filled with fluid and contain the odontoblastic processes along with nerve endings (Anderson, et al., 1967). The cell bodies of the odontoblastic cells are located in the pulp border just below a layer of predentin thus, comprising an integral part of the pulp organ. Due to this intimate morphological and functional relationship the dentin and the pulp are considered as one functional entity (Ruch, et al., 1971).

Underneath the odontoblast zone is located the cell-free zone of Weil's which is followed by the cell rich zone. During dentinogenesis the cell-free zone is not discernable probably due to active cell migration. It has been suggested that the zone of Weil's might represent a space that the odontoblast has to go through before differentiation, or that it might just be a morphological feature of the pulp without any particular function (Avery, 1990). The cell-rich zone is mainly populated by fibroblasts endothelial cells and undifferentiated mesenchymal cells. Besides,
defense cells such as macrophages, mast cells, monocytes exist in the pulp (Avery, 1990).

During dentinogenesis after the odontoblasts secrete the predentin matrix they retreat towards the pulp leaving their process entrapped in the organic matrix of dentin. The depositon of dentin in a circular configuration around the odontoblastic process results in the formation of the dentinal tubules. The shape of the lumen resembles that of an inverted cone with a larger diameter of approximately 3 µm near the pulp, which progressively gets narrower and is only 1 µm long near the dentino-enamel junction (Bradford, 1967). The diameter of the tubules gets smaller with age, due to continuous mineral deposition and some tubules even get obturated. Besides, the number of tubules per area decreases as we move coronally. In human teeth there are approximately 65,000 tubules/mm² near the pulp, about 30-35,000/mm² in the middle and 10-25,000/mm² in the periphery (Avery, 1987; Forssell-Ahlberg, et al., 1975). The dentinal tubules run in a loose S shaped curvature that represents the spiraling path of the odontoblastic process (Bradford, 1967).

The first dentin that is deposited is termed mantle dentin and is located close to the enamel. Its organic matrix mainly consists from coarse collagen fibers, the Von Korf's fibers (Ten Cate, 1978; Ten Cate, et al., 1970).

The main part of dentin is called circumpulpal dentin and it can be divided into peritubular and intertubular dentin.

**The Intertubular dentin** constitutes the major part of dentin. The collagen fibers constitute the bulk of the organic matrix. Their organization shows varying complexity, ranging from entirely random to a circular orientation. The collagen fibers are deposited in a direction parallel to the incremental pattern of dentin but rather irregularly to the
other two dimensions (Johnson and Poole, 1967). The amount of intertubular dentin decreases in a pulpward direction mainly due to the increased number and diameter of the dentinal tubules close to the pulp. It is evenly mineralized except from one hypomineralized area located in a small distance from the predentin (Boyde and Jones, 1972; Schmidt and Keil, 1971).

The Peritubular dentin is a highly mineralized structure that lines the dentinal tubules. Peritubular dentin can not be found before a distance of 150-200 µm from the pulp in newly erupted teeth. The organic matrix of the peritubular dentin contains very few filamentous elements if any (Lester and Boyde, 1968; Takuma and Nagai, 1971). There is a difference in the mineral phase, the distribution of the density structure and the crystal size between peritubular and intertubular dentin. Peritubular dentin contains higher amounts of calcium, phosphate and magnesium. It is 40% mineral denser than intertubular dentin and its crystalline phase is homogenous and evenly dispersed (Jones and Boyde, 1984). The continuous dentin deposition throughout the lifetime of a tooth results in an increase of the thickness of peritubular dentin at the expense of the lumen of the tubule (Frank, 1959; Lester and Boyde, 1968).

1. Odontoblastic Process

The extent of the odontoblastic process into the dentinal tubules has been most controversial. Both light and scanning electron microscopy show tubule like structures to extend throughout the length of the dentinal tubules (Avery, 1987; Mjor, 1973). A few authors have
interpreted these structures to be odontoblast processes, while others could only see odontoblast processes extending into the inner third of dentin up to a distance up to 0.7μm from the pulp dentin border (Brannstrom and Carberoglio, 1972; Frank, 1968, b; Holland, 1978).

Transmission electron microscope studies of human dentin demonstrated that odontoblast process to extend up to the inner third of dentin, while an electron dense structure demarcating the border of the peritubular dentin, the lamina limitans, extended up to the dentino-enamel junction (Thomas, 1979). Comparison between scanning and transmission electron microscopy confirmed these tubule-like structures to be lamina limitans (Thomas and Carella, 1983; Thomas and Carella, 1984; Weber and Zaki, 1986).

On the other hand, the existence of odontoblastic processes close to the dentinino enamel junction has been reported in cases when the tissue was frozen with liquid nitrogen prior to fixation (Maniatopoulos and Smith, 1983). However, the above technique has been demonstrated to cause artifacts to the tissue (Rankin, 1988). Immunocytochemical staining for cytoskeletal elements like vimentin and tubulin demonstrated the staining to persist all along the dentinal tubules up to the dentino enamel junction (Sigal, et al., 1985; Sigal, et al., 1984). However, due to technique limitations it could not be distinguished whether the antibody was staining the lumen of the tubule or its wall, nor is it known whether staining persists after degeneration of the odontoblastic process.

One may conclude that despite the conflicting reports the majority of evidence favour the extension of the odontoblastic process into the inner third of dentin.
2. Secondary Dentin Formation

Secondary dentin comprises the circumpulpal regular dentin that is deposited during the later stages of the life of the tooth (Baume, 1974). Secondary dentin formation starts when the tooth is fully formed, after the end of the period of active dentinogenesis (Pindborg, 1970). The rate of formation of secondary dentin is 0.8-1 μm per day, being 75% less than the rate of primary dentin formation. Besides, the metabolic activity of secondary dentin quantitated by ATP production is similarly decreased by 70% (Luostarinen, 1971; Massler and Schour, 1946).

Secondary dentin is deposited in continuity with primary dentin and some investigators claim that there is a clear demarcation line separating the two of them. This is probably due to a change in the tubule direction (Stanley, 1981). Secondary dentin is deposited more on the ceiling and floor of the pulp chamber than on the sides, leading to a constriction of the pulp in an occlusal-apical direction with age (Philippas, 1961).

3. Tertiary Dentin Formation

a) Definition

Tertiary or reparative dentin is dentin that is deposited on the pulpal aspects of primary and secondary dentin corresponding to areas of external irritations (Baume, 1974). Tertiary dentin is the main defense mechanism of the tooth in response to noxious stimuli such as abrasion, attrition, caries or operative procedures. Tertiary dentin is more
amorphous than regular dentin (Sela, et al., 1981). When the irritant is weak tertiary dentin appears to be well organized, whereas in severe injuries it is irregular with less tubules present (Seltzer and Bender, 1975). The amount of tertiary dentin produced is analogous to the amount of dentin destroyed in the periphery and the presumable damage to the underlying odontoblasts and pulp.

Tertiary dentin is present in 36-73% of the mature carious teeth (Massler and Schour, 1946). It gets deposited at a rate of approximately 1.5μm per day (Weider, et al., 1955). Tertiary dentin is less mineralized than regular dentin and has a microhardness of 46 kn in contrast with 80 kn and 50 kn in primary and secondary dentin respectively (Luostarinen, 1971). Occasionally, uncalcified matrix can be noticed entrapped in the mineralized tissue, giving tertiary dentin a "Swiss cheese" appearance (Trowbridge, 1981).

Collagen fibers constitute the main component of the organic matrix of tertiary dentin (Sela, et al., 1981). Immunocytochemical studies showed that both type I (88%) and type III (12%) collagen and fibronectin are present in tertiary dentin and the underlying predentin (Magloire, et al., 1988). It has been suggested that type II collagen and fibronectin are secreted initially during wound healing and progressively the synthesis of these two peptides decrease, while type I collagen predominates (Grinnel, 1984; Kischer and Hendrix, 1983). This series of events might also take place during the deposition of 'scar tissue' by the pulp-dentin organ as a response to noxious agents (Magloire, et al., 1988).

Changes in enzyme activity have been noticed during tertiary dentin formation. Alkaline phosphatase activity, which is very high during active dentinogenesis is found to decrease by 30-80% in mature healthy
teeth and is more prominent in the odontoblast-predentin area (Laikko and Larmas, 1980). However, during mild injuries alkaline phosphatase activity is increased by 2-10 fold, while in deep injuries, involving pulp exposure, it disappears from areas corresponding to the deepest sites of the injury (Karjalainen and MacKinen, 1977). If the inflammation progress is inhibited, the alkaline phosphatase activity returns to normal levels. Thus, alkaline phosphatase is an appropriate marker for monitoring tertiary dentin formation. Besides, odontoblast ATP levels and ATP cleaving enzyme activities have been found to increase in mild injury (Karjalainen, 1979)

b) Histological characteristics

In an attempt to understand the cellular mechanisms involved in tertiary dentin formation and to investigate the healing capacity of the pulp, scientists have tried to simulate inflammation in in vivo experiments. The sequence of the pulp reaction to noxious agents seems to be the following: Initially there is an inflammatory and vascular migration at the injury site that leads to a clot formation. As the inflammatory reaction progressively resolves mesenchymal fibroblast-like and endothelial-like cells migrate in the area. An amorphous substance, the fibrodentin or osteodentin, that is extremely rich in collagen, is probably being deposited at the injury site by the fibroblast-like cells. When the injury site is sealed by fibrodentin and the pulp is protected from the irritating stimulus odontoblast-like cells differentiate and the newly deposited mineralized tissue acquires dentin morphology (Fitzerald, 1979; Schroeder, 1985).
Transmission electron microscipical studies performed one month after the pulp exposure, show that the coronal part consists mainly of mineralized, irregular bone-like tissue with cellular inclusions. Spherical foci of mineralization resembling matrix vesicles can be seen. On the pulpal side of tertiary dentin predentin-like tissue lined with odontoblast-like cells can be noticed (Fitzerald, 1979). Three months later tertiary dentin can be clearly distinguished into the irregularly mineralized coronal part and the dentin-like pulpal part, permeated by a few dentinal tubules, followed by predentin. Odontoblast-like cells can be seen at the predentin-pulp border. It seems that the degenerating cells and the demineralized dentin stimulate initial mineral deposition (Fitzerald, 1979).

The origin of these odontoblast-like cells is equivocal. They may be G2-blocked predetermined cells or mesenchymal origin pulpal cells that differentiate into odontoblasts after DNA replication. The hypothesis that G2-blocked cells might be the precursors of the odontoblastic-like cells was first proposed by Stanley (Stanley, 1962). These cells are thought to be located in the cell rich zone and may be the daughter cells of the last mitosis of the preodontoblasts that did not differentiate into mature odontoblasts (Ruch, 1984). They are already predetermined and enter the M phase without requiring DNA replication (Takuma and Nagai, 1971). This hypothesis was supported by the results of studies that did not find $^3$H thymidine labeled odontoblast-like cells after tooth grinding without pulp exposure (Cotton, 1968; Sveen and Hawes, 1968; Torneck and Wagner, 1980).

On the other hand, new odontoblasts might differentiate from undifferentiated cells of mesenchymal origin that are induced to become
odontoblasts, or from already differentiated cells that are induced to dedifferentiate and redifferentiate (Tziafas and Kolokuris, 1990; Yamamura, 1985). Studies in both, exposed (Yamamura, 1985; Yamamura, et al., 1980) and non exposed pulps (Fitzerald, et al., 1990) showed $[^3]$H thymidine labeled replacement odontoblast-like cells next to the tertiary dentin site. Both labeled and non labeled odontoblast-like cells have been shown to exist next to one another, suggesting that a) either both newly differentiated cells and $G_2$-blocked cells can give rise to odontoblast-like cells or b) that the precursors of the odontoblastic-like cells are only the mesenchymal origin cells which have incorporated $[^3]$H thymidine at different points in time. This difference in the time of the DNA replication might be due to an actual difference of the time of induction or to the heterogenicity of the cell population induced (Fitzerald, et al., 1990). The mesenchymal origin progenitor cells might be fibroblast-like or endothelial-like, since these two types of cells seem to be actively proliferating during the peak of the cell mitotic activity (Fitzerald, 1979). It has been suggested that these cells migrate from deeper sites of the pulp to the injury site (Fitzerald, et al., 1990). Taken together these data suggest that undifferentiated pulpal cells of mesenchymal origin are mainly responsible for tertiary dentin deposition after pulp exposure. It is possible that in mild injuries that do not involve pulp exposure, more differentiated odontoblast-like cells located adjacent to the injury site might also contribute to tertiary dentin formation.

Although calcium hydroxide is commonly used due to its ability to stimulate tertiary dentin formation (Heys, et al., 1980), the pulp has the potential to heal by itself. After mechanical exposure in germ-free rats the pulp tissue healed and tissue barrier occurred without application of
any active medicament (Kakehashi, et al., 1965). Besides, tertiary dentin formation and differentiation of odontoblast-like cells has been demonstrated in mechanically exposed pulps covered with bone morphogenetic protein (Nakashima, 1990) or demineralized dentin (Tziafas and Kolokuris, 1990). In control teeth that were covered with albumin some osteodentin could be noticed but no tubular dentinal structure could be seen. One can conclude that dentin and pulp are reacting as one biological and functional pluripotent entity. They seem to be intricately linked in biological functions such as morphogenesis, tissue maintainance and reaction to noxious stimuli, including pain perception and tertiary dentin formation.

E. DENTAL INNERVATION

Innervation of the teeth is carried out by the second (V₂) and third (V₃) branches of the trigeminal nerve. The second branch supplies sensation to the maxillary teeth as the superior alveolar nerve. The third branch which innervates the mandibular teeth divides into three smaller branches, the buccal nerve, the lingual nerve and the inferior alveolar nerve. The latter runs on the medial aspect of the mandible and enters the mandibular canal along with major vessels at the mandibular foramen (Avery, 1987). It has been shown that branches from the mylohyoid nerve also contribute to the innervation of the molar teeth and their terminal ends extend up to the incisors (Madeira, et al., 1978).
1. Dental Nerve Fibers

a) Dental sensory fibers

The dental sensory axons are bipolar neurons with their cell bodies located at the Gasserian ganglion and their central axons extending to the principal sensory pontine nucleus and the descending spinal nucleus of the brain stem (Byers, 1984). Although most sensory axons arise in the Gasserian ganglion, retrograde horseradish peroxidase staining implies that some axons originate in the masencephalic nucleus as well (Chiego, et al., 1980). Acetylcholine is the neurotransmitter found in both myelinated and unmyelinated sensory fibers. Neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP) have been shown to exist in many of the small axons either alone or in combination (Kukletova, et al., 1968; Olgart, et al., 1977).

The somatosensory fibers are characterized as myelinated Aδ fibers that have a diameter varying between 1-6 μm and small unmyelinated C fibers that have a diameter varying 0.25-1.75 μm (Pimenidis and Hinds, 1977). The dental somatosensory fibers are branches from larger parent axons, since their conduction velocity has been shown to increase after they leave the tooth (Dong, 1981). The individual sensory axons enter the pulp of the tooth along with the blood vessels through the apical foramen. It is close to the root apex that the sensory axons have their larger diameter and are seen to form bundles from both myelinated and unmyelinated fibers. After a course of 1-2 mm into the root pulp the sensory nerve trunks start to branch. The bulk of the dental innervation is seen in the coronal part of the pulp. The nerves seem to be relatively unbranched in the root and...
extensively arborized in the crown and particularly in the pulp horns. The fine nerve endings form Rashcow's plexus below the odontoblastic layer. Free nerve endings pass through the odontoblastic zone and some extend into dentin (Avery, 1987). The number of sensory axons varies depending on their particular location in the pulp and the maturation status of the tooth. There is a 1:3 ratio in the number of axons located in the root comparatively to the ones located in the crown (Holland, 1978). The number of axons also seems to increase steadily throughout the maturation process.

Both myelinated and unmyelinated nerves are ensheathed by Schwann cells. The ensheathment seems to be more complete and regular in the myelinated fibers, while it is incomplete or absent in the unmyelinated ones and often a group of axons is seen to be covered by one Schwann cell (Johnsen and Harchbarger, 1977). The Schwann ensheathement grows increasingly incomplete as the axons approach the Rasckows plexus and the cell free zone of Weil.

All sensory nerve endings in the pulp are free nerve endings (Byers, 1979). They lack myelin ensheathement and have a beaded appearance, with successive dilatations connected by thin axonal regions. The thin axonal regions are rich in microtubules and microfilaments, while dense and light vesicles predominate in the beaded regions (Arwill, 1967; Corpron and Avery, 1973). The nerve endings can be distinguished in small, medium and large. The existence of different sizes might be attributed to differences in the maturation status, or to the absence of Schwann cell ensheathement and/or basal lamina from the small ones, or to differences in the sensory functions (Byers, 1979). Nerve endings can be seen in close approximation with fibroblastic cells, odontoblastic cell

bodies and processes following them in their course into the dentinal
tubules (Frank, 1968, a)

b) Dental sympathetic fibers

Immunofluorescence studies have shown the existence of sympathetic
nerve fibers along the blood vessels of the dental pulp (Pohto, 1972). The
existence of adrenergic endings in the dental pulp of molar teeth in rats
has been demonstrated after administration of 5-hydroxy dopamine, a
false neuritransmitter. Each molar contained an average of 70 adrenergic
nerve endings that were distributed as follows: 35.5 (± 5.2) in the pulp
horns, 26.1 (± 2.4) in the central coronal pulp, 5.4 (± 0.7) in the bifurcation
and 5.6 (± 0.9) in the root pulp (Avery, et al., 1980). The majority of the
sympathetic endings appeared to be vascular related, some were located in
the odontogenic zone and very few were free endings. All sympathetic
axons appear to be unmyelinated and use noradrenalin and
vasoconstrictor intestinal peptide (VIP) as neurotransmitters (Byers,
1984).

2. Dentin Innervation

The extent of the dental innervation was not resolved for a number of
years, mainly due to limitations of the experimental methodology used
(Byers, 1984). a) The routinely used silver impregnation methods gave
conflicting results due to the presence of other argyrophilic structures in
the dentinal tubules b) Photomicrography was not successful due to the
increased thickness of the tooth sections that could be obtained. c) Tissue sampling techniques gave conflicting results due to the different distribution of the sensory axons in the pulp. d) Finally, histochemical studies were limited due to the susceptibility of the tissue to damage by decalcifying agents (Avery, et al., 1984).

Some investigators have claimed to see nerve endings close to the dentino-enamel junction, following freezing of the tissue with liquid nitrogen prior to fixation (LaFleche, et al., 1985). However, these results are probably artifactual, due to the effects of liquid nitrogen to the tissue (Rankin, 1988).

Early ultrastructural studies showed nerve-like cells to pass through the odontoblastic layer to the predentin and the inner part of dentin (Arwill, 1967; Frank, 1968, a). Nerves could be distinguished from the odontoblast processes because they contained cytoplasmic organelles such as mitochondria up to 20\(\mu\)m into dentin (Corpron and Avery, 1973). The proof that the nerve like cells were indeed sensory axons came from resection studies of the inferior alveolar nerve that caused degeneration of the nerve like cells into the dentinal tubules (Arwill, et al., 1973; Corpron, et al., 1972).

The above results were confirmed by means of anterograde axonal transport mapping technique. Anterograde axonal transport mapping in a reliable method for identifying sensory nerve endings (Cowan and Guenod, 1975; Byers, 1984). The technique is based on the fact that most proteins are synthesized in the nerve cell body or in the dendrites and they are transferred to the nerve endings via anterograde axonal transport. Thus, a radioactive protein could be detected in the nerve ending by autoradiography if adequate time was provided (Lasek, et al., 1968). The
injected radioactive probe, such as $^3$H proline, will only be taken in sufficient quantities by the cells adjacent to the injection site (Byers, 1984). Trigeminal nerve endings were shown into the inner dentin in rats (Byers and Kish, 1976; Fink, et al., 1975), cats (Byers and Matthews, 1981; Quinton-Cox, 1975; Weil, et al., 1975), dogs (Byers, et al., 1987 a) and monkeys (Byers and Dong, 1981; Byers and Dong, 1983). The free nerve endings are shown to extend up to 150 µm and possibly 200 µm into the dentinal tubules depending on their specific location. They are seen deepest into dentin at the cuspal areas and the extention progressively decreases as one moves towards the roots. The innervation density is similarly graded, the greatest being adjacent to the pulp horn, where up to 50% of the dentinal tubules can be innervated and progressively decreasing in the midcrown, intercuspal and cervical dentin. The least number of fibers is found in the root dentin (Byers, 1980). In the monkey, dentin innervation was noticed in healthy areas of the tooth where Rashkow's plexus, odontoblasts with their processes extending into the dentinal tubules and predentin zone could be clearly distinguished (Byers and Dong, 1983)

Some nerve endings can be seen below the odontoblastic layer, some appear to be located in close approximation with the odontoblast cell bodies and other continue their course entering the dentinal tubules (Byers & Kirsh, 1976). Nerves seem to follow an increasingly helical course forming a "groove" in the odontoblastic process either due to space constriction factors, or as a way of maintaining a specific cell-nerve position, necessary for sensation transduction (Frank, 1968, a).
3. Positional Relationships between Nerves Odontoblasts and Fibroblasts

Ultrastructural studies have shown a close proximity between the nerve endings and the odontoblasts (Frank, 1968, b). Some investigators have claimed to see gap junctions between the odontoblastic processes and the nerves in cat teeth (Holland, 1976, b; Matthews and Holland, 1975). The relative relationships between nerves and odontoblastic cell bodies and processes as well as between the structures themselves were studied by means of electron microscope autoradiography (Byers, 1977). The scanning electron microscope was equipped with a goniometer that allowed the electron microscope grid to be rotated up to 360° and tilted up to 45° until it could align with the electron beam. Gap junctions were only noticed between fibroblast like cells in the subodontoblastic plexus and between the odontoblast cell bodies. Wide appositions of approximately 200-350 Å with parallely apposed membranes existed between nerves and odontoblasts or fibroblasts. Close appositions of approximately 100-150 Å with parallely apposed membranes were observed between adjacent terminal axons or nerve endings. These close appositions might represent sites for electronic coupling that allow synchronization between nerves. It might also be the case that the cleft width is the minimum extracellular space that the free nerve endings require among them (Byers, 1977). It was noticed that when the electron grid was not parallel with the electron beam the wide appositions appeared as if they were gap junctions thus, providing a satisfactory explanation for the conflicting results found between different researchers.
4. Development of Sensory Innervation

The development of sensory innervation has been studied in mice by means of anterograde transport mapping technique after intraganglionic or intraperitoneal \([^3H]\) proline injection (Byers, 1980; Corpron and Avery, 1973) During amelogenesis labeled nerves could be seen among the odontoblasts while blood borne \(^3H\) proline was incorporated in the enamel matrix. When the tooth was ready to start its eruption process labeled nerves could be traced all over the crown area and they were seen entering the dentinal tubules at a distance of approximately 20-40 \(\mu m\) at the tip of the cusp. A few of them were extending up to 60 \(\mu m\). Thus, the crown was innervated before the tooth emerged in the oral cavity. When the tooth reached functional occlusion, numerous nerves could be seen in the crown dentin and especially at the cusp areas. At the time when dentinogenesis started to slow down innervation density began to increase considerably, and nerve-odontoblasts or nerve-fibroblasts associations could be distinguished (Fearnhead, 1963). The maximum intradentinal nerve length was approximately 100-160 \(\mu m\) and occurred around the 50th day from plug. Soon after the teeth reached occlusion, tertiary dentin formation initiated. The innervation of the tertiary dentin was sparse and often times absent. During the attrition periods the nerves maintained their original position even when they were only 35 \(\mu m\) away from the occlusal surface. As the pulp chamber became constricted the pulpal innervation started to shift apically.

Innervation depends on i) nerve growth ii) enclosure of nerves within newly formed dentin and iii) gradual loss at the cusp tip due to attrition and replacement by newly formed non innervated dentin. The nerve
fibers get entrapped into dentin as it develops (Avery, 1971) and once entrapped they continue their peripheral growth (Fearnhead, 1961). Development of dental innervation coincides with root formation and proceeds until the tooth reaches a mature stage. The maturation period lasts only four to five months in rats, while it takes six to nine years in humans. The coincidence of sensory maturation with root closure might be attributed to the fact that root closure leads to changes of the morphological and physiological conditions of the pulp chamber that are favourable for the development of innervation or it might be simple coincidence (Byers, et al., 1982).

5. Dentin Sensitivity

Dentin sensitivity has been attributed to dense pulp innervation (Trowbridge, 1986). The degree of dentin sensitivity seems to be related with nerve density. Clinical studies show that newly erupted teeth are less sensitive to electrical and thermal stimuli compared to mature ones (Fulling and Andreasen, 1976; Klein, 1978). The progressive acquisition of sensitivity coincides with the proportionate increase in the number of the nerve endings in the pulp and dentin. When the sensitivity of rat molars was determined by means of constant current monopolar stimuli and recorded via the evoked digastric muscle flexion reflex, it was shown that there was a direct correlation between dentin sensitivity and the number of the nerves extending into dentin (Byers, et al., 1982). There was no correlation between dentin sensitivity and the extent of the nerve endings into the dentinal tubules, or the width of the outer dentin between the
nerve endings and the occlusal surfaces. In addition, denervation experiments showed that the return of sensitivity corresponded to the number of nerves that reinnervated dentin (Berger and Byers, 1983 b; Berger, et al., 1983 a). Although primary teeth are oftentimes thought to be less sensitive than permanent teeth this is not really the case in primary teeth with intact roots. This is probably attributed to the fact that the innervation density is constant relative to the tooth size in both primary and permanent teeth, despite the fact that the primary ones have a smaller number of axons (Johnsen and Harchbarger, 1977).

6. Sensory Mechanisms

The dental pulp is a model for studying nociceptive mechanisms (Silverman and Kruger, 1987). Teeth are sensitive to electrical, chemical, thermal, mechanical and osmotic stimuli. The application of suprathreshold stimuli elicits pain. A number of theories have been proposed over the years in an attempt to explain the nociceptive properties of pulp and dentin. Although dentin is innervated, the nerve endings do not extend up to the dentino enamel junction. Therefore, a transducer mechanism has to exist to mediate the transfer of the external stimuli through dentin to the nerves (Byers, 1979).

The hydrodynamic transduction mechanism seems to be the most widely accepted theory which is in agreement with most scientific evidence. The main premise of the hydrodynamic theory is that different noxious stimuli can cause an outflow or inflow of the dentinal fluid,
which results in direct or indirect activation of the free nerve endings (Brannstrom, 1966). The flow rate of dentinal fluid has to be rapid in order to be able to elicit pain and it follows Poiseille's law (Branstorm, et al., 1979). Temperature changes lead to expansion or contraction of the dentinal fluid thus eliciting pain. Also, heat or reduced pressure cause fluid outflow, which under the action of capillary forces cause fluid to overfill the dentinal tubules and activate nociception (Brannstrom, 1968). Pain is also elicited after application of paper or glucose over cut dentinal tubules due to their capacity to absorb fluid (Brannstrom and Astrom, 1972). However, the action of desensitizing agents does not always correlate with their ability to inflict fluid movement (Anderson and Linden, 1973). However, more studies are required to further clarify this matter.

The activation of nerve endings by fluid movement might occur through one of the following mechanisms:

i) **Independent neural transduction** when the nerve endings get stimulated through their own deformation by fluid movement. In this case the nerves act as mechanoreceptors. Although the viability of the nerve terminals into the dentinal tubules has been questioned (Arwill, 1963), anterograde transport mapping techniques have provided proof that the nerve extensions are indeed viable and thus, are capable of reacting to noxious stimuli.

ii) **Odontoblastic transduction** when the odontoblasts stimulated directly by dentinal fluid either change the ionic balance, or by their independent stimulation lead to cell membrane movements that are detected by the nerve endings. The odontoblasts are the primary receptor cells that subsequently transduce the information to the adjacent nerves.
This hypothesis was first suggested in an attempt to explain the increased sensitivity close to the dentino enamel junction. However, scientific evidence does not support a primary receptor role for the odontoblast. Dentin sensitivity does not seem to be related to the number of the odontoblasts (Brannstorm, 1966). Pain can still be elicited by a dental probe even when the odontoblastic zone is necrotic (Brannstorm, 1966). Besides, no gap or tight junctions seem to exist between odontoblasts and nerves, suggesting that no electrical or chemical communication exists between these cells. Finally, if odontoblasts were the primary receptor cells one should assume that the odontoblastic processes extend up to the dentino enamel junction, which is a most controversial issue.

iii) A combined transduction mechanism, where both nerves and odontoblasts contribute to the reception and elaboration of the noxious stimuli. The odontoblasts might be responsible for the ionic composition of the dentinal fluid and the formation of the necessary framework that will hold the nerves in position for optimal transduction.

Other theories have also attempted to offer an explanation for dentinal sensitivity through piezoelectric effects (Liboff and Shamos, 1971), bioelectric potentials depending on ion composition of fluids of the oral cavity (Atkinson and Parker, 1969), thermal gradients (Matthews, 1977) or direct chemical stimulation (Horiuchi and Matthews, 1976; Matthews, 1976).

Although nociception implies that the tooth perceives all nociceptive stimuli as pain, there is a clear distinction between the type of pain elicited by hot and cold. Cold gives a sharp sensation of pain while hot provokes a dull ache. It has been shown that the receptors that respond to cold differ from the ones that respond to hot. The cold receptors look very much like
A-δ mechanoreceptors, while the later ones seem to be C dentinal fibers (Matthews, 1970; Matthews, 1977; Scott and Temple, 1965). Certain experimental data suggest that the pulp might primarily act as a mechanoreceptor. Direct histamine or substance P application over cut dentin did not cause signs of nociception, while application of acotinine substance, a mechanoreceptor stimulator, was able to activate the mechanoreceptors (Brannstorm, 1966). It has been shown that only three out of ten sensitive fibers responded to mechanical stimulus (Nahri, et al., 1982 a). However, there are still unanswered questions that require further investigation. It is still not known whether the difference in elicited sensation is due to the existence of two different kinds of receptor versus one pluripotent receptor, nor has the exact location where the transduction occurs in the nerves been demonstrated.

7. Non Sensory Nerve Functions

Except from their well-known function to provide sensation, nerves have also been attributed other functional roles.

a) Primary odontogenic role

Innervation has been suggested as one of the mechanisms that provide specificity for dental organization during the initiation phase of odontogenesis (Pearson, 1977). Circumstantial evidence implicates innervation in the earlier stages of tooth development. It has been reported that in recombination experiments with tissues from early stages
of development, when the trigeminal ganglion was included in the graft the incidence of development of the tooth germ was increased (Kollar, 1976). However, more recent data showed that the inclusion of the trigeminal ganglion in the grafted tissue did not affect tooth formation (Lumsden, 1984).

A selective distribution of nerve branches along the developing maxillary and mandibular processes of mice at day 10.5-11 of gestation, prior to the formation of the tooth bud has been demonstrated by means of a silver staining technique (Linder, 1979). However, there is not enough accuracy when one is trying to identify the future location of teeth during earlier stages of development. In certain sections fine nerve fibers were identified moving to the future incisor and molar regions, respectively. One main trunk was passing through the mesenchymal cells and a fine branch was extending to the oral epithelium (Kollar and Lumsden, 1979).

Once the tooth bud is formed the nerve appears to be displaced. It has been reported that no association of nerves with oral epithelium or dental papilla could be detected in the developing tooth bud (Al-Takriti and Alkinson, 1979). Quite surprisingly nerves were found close to the dental sac. However, this finding may be due to the limitations of the technique used. More studies are required to confirm these data.

Nerves may contribute in different ways in tooth development. They may be involved in the initiation phase, or in the interactions that take place during tooth development (Kollar and Lumsden, 1979). Besides, the presence of nerves might simply be attributed to neurotrophism (Singer and ., 1952).
b) Neurotrophism

Besides their nociceptive properties sensory nerves have been suggested to have neurotrophic functions as well, involving growth regulation, maintenance and repair of target tissues (Singer and ., 1952). The presence of nerves has been demonstrated to be an important factor in growth and regeneration of tissue. Neurotrophism has been considered to be the main regulatory mechanism during craniofacial growth and development (Moss, 1972). Axoplasmic flow has been shown to be essential for normal growth and development of the rat craniofacial complex (Behrents, 1975). When lesions were inflicted at the trigeminal ganglion in rats, development was impaired in the ipsilateral side involving incomplete growth of bone, soft tissues and teeth. Enamel and dentin appeared to have lost their normal pigmentation and had an unusual white colour. Interruption of the axoplasmic flow in the adult mouse results in a change in the homeostatic mechanisms of the pulp (Avery and Cox, 1977).

Thus, the trigeminal sensory fibers seem to have a dual role: on the one hand, they are responsible for nociception and on the other, they are required for normal growth and development.

During inferior alveolar nerve resection the nerve endings in both the dentin and the pulp showed intense signs of degeneration, while only a few odontoblasts seemed to be affected. Dentin deposition occurred in an irregular way with rapid calcification spikes and irregular dentin matrices located between odontoblasts and often enclosing them in the mineralized tissue (Avery, et al., 1974).
In cases where the pulp was wounded after inferior alveolar nerve resection the tertiary dentin formed appeared to be incompletely calcified. The deposition of tertiary dentin was uncontrolled and sometimes it almost filled the entire pulp cavity. However, no similar changes were noticed after resection of the cervical sympathetic ganglion (Avery, et al., 1972). Therefore, it seems that the sensory fibers of the dental pulp also play different roles than nociception and vascular tone control and are involved in cell proliferation, dentinogenesis and tertiary dentin formation (Byers, 1977; Chiego, et al., 1983; Pimenidis and Hinds, 1977).

It has been suggested that nerves are capable of stimulating cell mitotic activity (Singer and , 1952). The role of the peripheral nervous system in regulation of pulpal cell proliferation has been studied in rats in response to wounding or after unilateral resection of inferior alveolar nerve, cervical sympathetic ganglion and chorda tympani. The peak of cellular proliferation in normally innervated teeth was noticed 5 days post wounding and lasted up to 15 days. Nerve resections led to a change in the overall pattern of cell proliferation and also, between the experimental and the control sides. The 5 day peak was lost, a low cellular activity was noticed during the first ten days and the mitotic activity started being restored fifteen days later. The combined resection of the inferior alveolar nerve and the cervical sympathetic ganglion resulted in the lowest mitotic activity. Thus, it seems that both the sensory and the autonomic system are implicated in the regulation of the cell proliferation to wounding. Denervation seems to inhibit the normal pulp response to noxious agents, or it may affect epigenetic controls regulating odontoblast differentiation. After a few days an internal compensatory mechanism takes effect with
possible synergy of the autonomic and sensory nervous system (Chiego, et al., 1986).

8. Neuropeptides /Neurogenic Inflammation

   The afferent and efferent nerve impulses are transfered from one neuron to the other, or to the effector cell by chemical neurotransmitters. The classical neurotransmitters, including acetylcholine, γ-aminobutiric acid, noradrenalin and dopamine, are released at the neuronal synapses and are able to excite or inhibit the post synaptic cell. Recently a number of peptides are found to be localized in both the central and peripheral nervous system. Some neuropeptides have been shown to act as neurotransmitters while they may have some neurotrophic actions as well (Hokfelt, et al., 1980).

   The release of neuropeptides from the nerves seems to mediate the neurogenic response. Several pieces of evidence attest to this fact.

   Neuropeptides can act as neurotransmitters, or neuromodulators and can potentially stimulate cell growth (Dalsgaard et al., 1989). They include substance P, Somatostatin, Vasoactive Intestinal Peptide and calcitonin gene-related peptide (CGRP). Neuropeptides have been attributed a number of non sensory roles.

   Neuropeptides are capable of eliciting peripheral effects such as smooth muscle contraction and relaxation, vasodilation, increase of vascular permeability and stimulation of epithelial secretion in the gut and lungs (Hoekfelt et al., 1980).
Neuropeptides are important in controlling the vascular tone; SP and CGRP are potent vasodilators (Bayliss, 1901; Bernstein, et al., 1981). Also, neuropeptides have been shown to play an important role in survival of experimental flaps. Pretreatment with capsaicin reduces the flap survival rate from 44% to 14%, while administration of CGRP lead to a considerable increase of the survival rate to 73% (Kjartansson and Dalsgaard, 1987). Pretreatment with reserpine, that depletetes catecholamines from the adrenergic endings, including sympathetic post ganglionic fibers, increased the flap survival rate to 71% (Kjartansson, et al., 1987). The results of these experiments tend to suggest that sensory nerves play an important role under normal and ischemic conditions. The vasodilatory properties of the sensory nerves balance with the vasoconstrictory properties of the sympathetic nervous system and the removal of one of them may considerably change blood flow in the tissue.

Neuropeptides can act as mitogens. Substance P and neurokinin A have been shown to stimulate mitogenesis of smooth muscle cells by stimulating DNA synthesis through the phosphoinositol pathway, while CGRP can stimulate directly the mitogenesis of cultured umbilical vein endothelial cells (Dalsgaard, et al., 1989). Due to their mitogenic properties it has been suggested that neuropeptides should be considered to be growth factors that are capable of exerting their influence during embryogenesis, growth and development, wound healing, immune response or tumorigenesis (Dalsgaard, et al., 1989). Locally released neuropeptides may act as mitogens during wound healing. During neoplasia neuropeptides may act as endocrine or autocrine factors, regulating angiogenesis, hyperplasia or hypertrophy of the surrounding connective tissue or even inhibiting tumour growth.
Also, neuropeptides have been shown to enhance or suppress macrophage mediated tumoricidal and microbiocidal activities either alone or in combination with biological response modifiers, such as interferon-γ (Peck, 1987).

Inflammatory and tissue repair processes are modulated by neuropeptides through stimulation of leucocyte chemotaxis, regulation and release of inflammatory mediators and enhancement of cell proliferation (Payan, et al., 1987a). This reaction might be activated in the periphery through polymodal nociceptors to produce axon reflexes which result in an inflammatory response via release of neuropeptides and increase of vascular permeability (Jansco, et al., 1967). Repeated capsaicin treatment, that causes depletion of neuropeptides, simultaneously results in desensitization of the inflammatory effects. Sensory nerves might have a peripheral effector function through antidromic transmission in an axon reflex arangement. Neuropeptides such as SP, CGRP and NA have been localized in the nerves involved in axon reflex neurogenic inflammation (Forman, 1987). During inflammation in the rat paw an increase in the mRNA content of CGRP and SP could be noticed (Iadarola and Draisic, 1988).

F. CALCITONIN GENE-RELATED PEPTIDE

A novel neuropeptide CGRP seems to be involved in neurogenic inflammatory responses. CGRP is found in 30-50% of sensory ganglia with higher concentrations in the trigeminal ganglia thus, implicating it in the sensory nociceptive and defense mechanisms of the pulp.
1. The Prediction of a Novel Neuropeptide from the CT Gene

CGRP is a novel neuropeptide derived from alternative splicing of the calcitonin (CT) gene (Rosenfeld, et al., 1983). The existence of this neuropeptide was predicted after studying the properties of Medullary Thyroid Carcinoma (MTC) in rats. MTC is a tumour that produces high amounts of CT. Serial transplantations of several MTC tumour lines showed a shift in the production of CT almost 10 fold from 1.5% - 4.5% to less than 0.3%. These results were confirmed with immunocytochemistry that showed a dramatic decrease of CT positive cells from 95% in high calcitonin producing tumours (MTC_H) to less than 5% in low calcitonin producing tumours (MTC_L) (Roos, et al., 1986). The change in CT production coincided with a decrease in CT mRNA and the production of a new nuclear mRNA of similar molecular weight around 18,000 (Jacobs, et al., 1981). Hybridization experiments of labeled CT cDNA with mRNA of MTC_H or MTC_L showed a shift from the high producing 1050 nucleotides (n) long CT mRNA precursor to very low quantity, while a 1050-1300 n long mRNA was produced in 10-30 fold quantities (Rosenfeld, et al., 1981).

Both mRNAs were shown to be derived from the same gene (Rosenfeld, et al., 1982). In vitro translation of this novel mRNA in a cell free system from wheat germ embryos resulted in the production of a protein with molecular weight around 16,000. This new peptide was given the name of calcitonin gene related peptide (CGRP) (Amara, et al., 1982). Hybridization and arrest of translation experiments proved that this propeptide was indeed the one translated from the novel mRNA.
precursor (Rosenfeld, et al., 1982). The production of different protein products from the same gene could be the result of alternative splicing of a) the same primary mRNA transcript or b) it could be due to structural changes generated by differential transcription initiation (Young, et al., 1981) or polyadenylation sites (Early, et al., 1980) leading to two different mRNA encoding two different peptides, but derived from the same gene.

During fragmentation and hybridization mapping experiments it was indicated that the reactive regions for both CT and CGRP were present in common nuclear transcripts. They share a common amino terminal sequence of 227 nucleotides followed by the CT sequence and in the end the CGRP sequence at the carboxy terminus. It seems that the transcription proceeds through both CT and CGRP regardless of which protein is finally going to be secreted. A small 4,000 MW peptide was proven to be the immunoreactive peptide (Rosenfeld, et al., 1983).

Human CGRP (hCGRP) was first isolated and sequenced by means of fast bombardement mapping, a mass spectrometric approach (Morris, et al., 1984). hCGRP differs from the rat CGRP (rCGRP) in four positions in the amino acid sequence, three of which lead to charge changes. CGRP is a 37 amino acid peptide that has a disulfide bridge between positions 2,7 in the amino terminus and a phenylalanine amide amino acid in the carboxy terminus. Selective utilization of alternative polyadenylation sites in the mRNA precursor forms the basis for the switching in the production between CT and CGRP as has been shown by S\textsubscript{1}-nuclease mapping. It is possible that some cellular mechanism directly recognizes one of the two polyadenylation sites and proceeds into endonucleotic cleavage (Evans, et al., 1983).
2. The Calcitonin Gene Family

CGRP belongs to the family of peptides of the CT gene that include calcitonin (CT), katacalcin (KT) and CGRP. The CT gene is located in the short arms of chromosome 11 (Pzepiorka, et al., 1984). It has been mapped in p14-qter region of chromosome 11 by analysis of human rodent cell hybrids (Hoppener, et al., 1984).

Moreover, the CT gene expression provides a model for studying transcriptional and post transcriptional regulation mechanisms during development (Amara, et al., 1982). It also provides an example of a new regulatory mechanism for the diversity of brain neuropeptides. The CT gene has six exons and introns which are found between the functional domains. The first three are common in both CT and CGRP, exon four is only found in CT, while exons five and six are only found in CGRP (Evans et al., 1983). Recently a second mRNA encoding CGRP II has been identified. Screening of a DNA library of human MTC mRNA with a genomic hCGRP specific probe and nucleotide analysis revealed the existence of a second distinctly different hCT gene (hCT II gene) (Steenbergh, et al., 1985). Both genes seem to share many similarities but no exon four seems to be present in the hCT II gene. The hCT II gene has been found to be located on the short arms of chromosome 11 in the region q12-pter (Hoppener, et al., 1985).

hCGRP II differs from hCGRP I in three amino acids but these substitutions do not cause alterations in the biological activities of the peptides. A second CGRP has also been identified in the rat. rCGRP II is a 37 amino acid peptide amidated at the carboxy terminal end, that differs from rCGRP I by a single amino acid (Amara, et al., 1985).
3. Calcitonin

CT is an important hormone of ancient lineage. Calcitonin was first discovered when it was demonstrated that high calcium perfusion lead to a precipitous fall in plasma calcium, but removal of thyroid and parathyroid glands resulted in a continued rise in the plasma calcium (Copp, et al., 1962). It was concluded that a new hormone was discovered and it was named calcitonin because of its ability to control calcium in body fluids. Its origin was proven to be the thyroid gland due to a profound hypocalcemic effect of thyroid extracts (Hirsh, et al., 1963). CT is produced by the parafollicular C cells of the thyroid gland which have migrated from the neural crest thus linking CT with the neuroendocrine system (Copp, 1985). C cells have originated in the ultimobranchial body of the embryo which exists as separate gland in non mamals (Pearse and Colvaheira, 1967). CT is expressed to a different extent in the central nervous system of most species (MacIntyre, 1985). Immunocytochemistry showed CT to be localized in most vertebrates, in the brain of cyclostomes and protochordates and monocellular organisms such E. coli and C. albicans (MacIntyre and Stevenson, 1981).

CT is a 32 amino acid peptide that has a disulfide bridge between two cysteine residues in position 1 and 7 in the amino terminous and a prolinamide in the carboxy terminous (Gutterman, 1981). Currently, CT from fifteen species has been identified and it shows great variety in structure. There are three main chemical types of CT: rat primate, teleost and artiodactyl that share only nine common amino acids in the beginning and in the end of the molecule (MacIntyre and Stevenson,
1981). The common core in all are the cysteine, leucine, glycine and prolinamide residues (Gutterman, 1981).

The whole 32 amino acid CT molecule is required for biological actions. The active sites seem to reside in a cyclical loop of seven residues in the amino terminus as well as in the carboxy terminus where only restricted amino acid changes are permitted (Gutterman, 1981).

4. Tissue Distribution of CGRP

CGRP is the main product of the CT gene (Editorial, 1985). Recombinant DNA and molecular biology techniques showed that CGRP production was tissue specific. CGRP has been demonstrated in the brain and central and peripheral nervous system and consequently has been characterized as a neuropeptide. By using an antibody to its carboxy terminal sequence CGRP was found to be localized in the trigeminal ganglion, spiral sensory ganglia and terminals in the brain stem and the spinal cord, that relay pain or temperature sensations (Rosenfeld, et al., 1983). CGRP was absent from the autonomic ganglia. In the spinal cord CGRP is primarily localized in the marginal zone of substantia gelatinosa, with lesser amounts in the nucleus proprious, and ventral to the central canal, like most primary nociceptive afferents.

The tissue distribution of CGRP in the posterior horn of the spinal cord and trigeminal ganglion, strongly suggests the involvement of the peptide in sensory functions (Silverman and Kruger, 1987). Treatment with capsaicin, a neurotoxine specific for peripheral unmyelinated fibers, substantially eliminates CGRP- like immunoreactivity (CGRP-LI), while
treatment with 6-hydroxydopamine, a sympathetic neurotoxine, does not seem to have any effect on CGRP immunoreactivity. CGRP has also been seen in the pulp and dentin of teeth, in the taste buds of the tongue, in olfactory fibers, in the motor neurons of facial, hypoglossal nuclei and rostral parts of nucleus ambiguus. Thin beaded fibers were found in the heart, lung and gastro-intestinal tract. Thus, CGRP seems to be related only with sensory nerves and ganglia (Silverman and Kruger, 1987).

An S1 nuclease protection assay, using a 586 nucleotide long insert, confirmed that CGRP was indeed synthesized in the brain and the sites of biosynthesis were identified (Rosenfeld, et al., 1983). CGRP has also been found to be secreted in sufficient quantities in the thyroid gland in both animals and in man (MacIntyre, et al., 1984 b; Tchopp, et al., 1984).

In vitro cultures of trigeminal ganglion cells from rats show CGRP release. When the ganglion cells were depolarized by addition of potassium in the medium, CGRP production was increased 7-10 fold. However, when calcium was removed from the medium CGRP production returned to control values suggesting a calcium dependent high potassium-induced secretion of CGRP. (Mason, et al., 1984). Plasma CGRP is mainly released by the peripheral nerve terminals in young rats, while in older rats significant amounts seemed to be contributed by the thyroid gland (Bevis, et al., 1985)

CGRP II follows a similar pattern of distribution as CGRP I but is expressed in lower quantities in the brain, sensory ganglia and thyroid gland. It has been shown by means of immunocytochemistry that trigeminal ganglia contain 10 times more CGRP I than CGRP II and that CGRP II immunoreactivity accounts for less than 20% in the lateral medulla, hypothalamus and thyroid gland (Amara, et al., 1985). However,
we should be cautious in the interpretation of these results because of possible crossreactivity (Henke, et al., 1987).

5. Physiologic Actions of CGRP

The physiologic properties of calcitonin (CT) and CGRP should be examined in combination as well as under the context of the physiologic controls that regulate their relative expression (MacIntyre, 1985).

a) Vasodilation

CGRP is the most potent vasodilator known to date. In humans CGRP administration caused hyperemia that lasted longer than the one caused by histamin (Brain, et al., 1984). Intracerebroventricular administration of CGRP results in noradrenalin dependent sympathetic outflow, following a different pattern than other peptides studied and leading to tachycardia and elevated blood pressure. Intravenous administration of CGRP also results in tachycardia and causes a drop in the blood pressure due to vasodilation (Fisher, et al., 1983). Direct microscopic observation of cheek pouch after CGRP administration showed arterioal dilatation. The vasodilatory effects do not seem to be mediated by histamine release in the rabbit since no plasma protein leakage could be detected, nor can they be due to prostaglandin release since injection of indomethacin, a PG inhibitor, did not affect CGRP action. Also, it could not be attributed to secondary inhibiting vasodilator release in the skin. Immunoreactive CGRP has been detected in extracts of cat aorta suggesting a potent role of
CGRP in the regulation of the blood flow. CGRP has been shown to have a relaxant effect on artery preparations in vitro via an endothelial cell dependent mechanism (Brain, et al., 1984). CGRP action may not be uniform in large and small blood vessels. CGRP is able to stimulate cyclic adenylate production and cause vasodilation in a PG mediated way in endothelial cells of the human umbilical vein (Crossman, et al., 1987).

CGRP is the major product of the CT gene having physiologic control on blood pressure and vascular tone. CGRP circulates in the plasma of healthy individuals at 5 times higher levels than CT. There are no differences in CGRP plasma levels between males and females (Girgis et al 1985). Human CGRP was found in six excised medullary thyroid carcinomas (MTC) and in the plasma of ten patients with MTC. It is likely that CGRP might be responsible for some of the clinical symptoms observed in MTC (Morris, et al., 1984)

b) Neurosomatomotor role.

CGRP coexists with acetylcholine (Ach) in single neurons of the rat hypoglossal facial and ambiguous nuclei as well as in single motor neurons innervating striated muscle thus, suggesting a possible neurosomatomotor role for CGRP (Takami, et al., 1985 a). Immunocytochemical studies have shown CGRP to exist in neuromuscular junction of bicept branchi in the mouse and specifically, in the motor end plate terminal associated with mitochondria and small clear vesicles. When the nerve was stimulated in the presence of 10 \(^{-7}\) CGRP, contraction was significantly enhanced. Also, direct transmural stern stimulation had similar effects due to CGRP action. Addition of
curare a Ach antagonist, did not affect the magnitude of the contraction (Takami, et al., 1985 b). CGRP has been found to regulate Ach receptor synthesis in developing neuromuscular junctions due to direct stimulation rather than increased rate of protein synthesis. This increase is achieved by enhancement in the insertion rate of new receptors (New and Mudge, 1986). Also, CGRP possibly acts as a muscle trophic factor released by motor neurons in vivo thus mediating neuromuscular junction formation.

c) Calcium regulatory effects

CGRP has been shown to have calcium regulatory properties. CGRP has been found to have osteoclast inhibitor effects similar to CT but it is 1/500 - 1/1000 less effective (Yamamoto, et al., 1986). When the effect of calcium regulating hormones on bone resorption of isolated osteoclasts was studied in vitro, both human CT and CGRP cause a dose dependent inhibition of the mean area of resorption. CGRP was found to be $10^{-3}$ less potent than CT (Zaidi, et al., 1987 a). Taking into account the plasma levels of CGRP these data suggest that CGRP should not play any physiological role in the regulation of Ca levels in plasma. The hypocalcemic effect of CGRP might be achieved through its weaker binding to the CT receptor. CGRP might bind with a weaker affinity to the CT receptor. The whole molecule was required for action and no effects could be noticed after fragmentation (Zaidi, et al., 1987 b).

In rabbits low doses of CGRP have a CT-like effect while, high doses of CGRP have a PTH-like effect and lead to hypercalcemia (Tippins et al., 1984). CGRP was found to have direct effects on osteoblastic cells similar
to PTH but 10 it is times more potent. Besides, osteoblastic cell lines have been shown to produce CGRP (Zaidi, et al., 1987 b).

Also, CGRP has been found to have osteogenic stimulating effects by increasing the number and size of bone colonies in cultures of bone marrow cells at higher doses (Bernard and Shih, 1990). In common with PTH, CGRP causes a dose dependent stimulation of intracellular AMP levels in cultured bone cells but is 15-20 fold more potent (Crawford, et al., 1985).

CGRP was able to stimulate mitogenesis in endothelial cells of the umbilical vein (Dalsgaard, et al., 1989). It is a potent inhibitor of substance P (SP) degradation in a dose dependent way thus, potentiating SP effects (LeGreves, et al., 1985).

6. Physiologic Properties of CT

CT is secreted by the thyroid when the levels of calcium rise in plasma. Large differences exist in the CT plasma levels between men and women that are removed only during pregnancy and lactation (MacIntyre and Stevenson, 1981). However, it has been noticed that CT levels fall after menopause probably due to a deficit of ovarian hormones (Stevenson, et al., 1982; Taggart, et al., 1982), since chronic eostrogen administration restores CT and katacalcin (KT) levels (Marimoto, et al., 1980; Stevenson, et al., 1981).

The main action of CT is to exert a homeostatic function on the levels of calcium in plasma. CT restrains skeletal resorption during periods of calcium stress such as growth, pregnancy and lactation. CT also regulates
plasma calcium levels after meals (MacIntyre and Stevenson, 1981). Its hypocalcemic effect is achieved through direct inhibition of osteoclasts (Chambers, et al., 1985; Chambers and Moore, 1983; Murrills, et al., 1989). CT has been shown to inhibit osteoclasts motility (Chambers and Dunn, 1983). It also causes a decrease in the osteoclast number possibly due to a direct inhibitory effect on osteoclast precursors, or to an indirect effect of repeated acute inhibition. CT action is mediated by activation of the adenylate cyclase (Marx, et al., 1972).

Katacalcin is the C-terminal flanking peptide from the CT precursor that is secreted in equimolar quantities as CT. Although, initially it was suspected that KT might have calcium lowering properties alone, or in conjunction with CT (MacIntyre, et al., 1982) there is no experimental evidence to suggest such a function (MacIntyre, et al., 1984 b). Thus, the biological role of KT remains unknown (MacIntyre, et al., 1987).

7. Receptors for CT and CGRP

CGRP binding is time dependent, reversible and saturable and demonstrates high affinity for the brain (Inagaki, et al., 1986). $^{125}$I-CGRP has been identified in membrane fractions of brain homogenates and also in areas of the central nervous system with highest concentrations in the cerebellum and spinal cord (Inagaki, et al., 1986; Tchopp, et al., 1985).

Separate receptors for CGRP I and II have been identified in the cerebellum and spinal cord by means of receptor autoradiography due to different regional distribution and binding affinities (Henke, et al., 1987).
However, these results should be interpreted with caution due to possible crossreactivity between the two peptides.

In classic binding studies it has been shown that receptor binding is specific for CT, saturable, time and temperature dependent and varies with increasing concentrations of CT. A large number of CT receptors were shown to exist in both the central and peripheral nervous system, despite the fact that CT has not been shown to be synthesized in these locations by recombination DNA techniques. Although, the blood borne circulation of CT could justify to some extent the existence of CT receptors in the nervous system, the bulk of them possibly implicate the involvement of an endogenous ligand (Goltzman and Mitchell, 1985, a).

CGRP can be a very likely candidate for this role mainly due to the existing conformational similarities between the two small peptides and the fact that CGRP is synthesized in these regions of the central nervous system (Roos, et al., 1986). Binding studies using antibodies against hCGRP and salmon CT (sCT) in both human and rat has shown a distinct regional distribution of both. CGRP receptors were identified in large amounts in the human brain, spinal cord, pituitary and in lesser amounts in the thyroid, while CT receptors were found in abundance in the thyroid and the kidney (Fisher, et al., 1985).

Although there are distinct specific binding sites for CT and CGRP and no crossreactivity could be detected, each peptide inhibited binding at the high affinity receptor site of the other. Salmon CT was able to inhibit binding of $^{125}$I-rCGRP although full dose response curves showed that it had a substantially weaker effect than rat CGRP and vice versa (Goltzman and Mitchell, 1985, b). CGRP was unable to activate the adenylate cyclase system in tissues containing high concentrations of CGRP receptors but
was able to do so in tissues containing high concentrations of CT receptors, suggesting the existence of alternative mechanisms for enzyme activation for receptor binding and possibly some crossreactivity between CGRP and CT receptors (Goltzman and Mitchell, 1985, b). CGRP might serve as an endogenous ligand for the CT receptors existing in the central nervous system and might carry out effects previously attributed to CT. CGRP has a dual way of binding through cAMP dependent or independent mechanisms depending on which receptor is present in higher concentrations.

8. Immunocytochemical Localization of CGRP in the Pulp and Dentin

CGRP is widely distributed in the pulp. Immunocytochemical studies using rat antibodies have shown the existence of CGRP-positive nerve fibers in the pulp, dentin and periodontal ligament of teeth, as well as in the mandibular periosteum (Hill and Elde, 1988). CGRP has been observed in certain parts of the tongue, palate, nasal structure and skin (McCulloch, et al., 1986). CGRP-stained fibers can be traced from the inferior alveolar nerve as they enter the pulp as coarse bundles. The number of stained fibers progressively increases as one moves towards the coronal pulp where elaborate anastomosing can be demonstrated. Intense staining can be observed in Rashcow's plexus, from where thin processes can be seen entering the cell free zone of Weil. Several pass through the odontoblastic layer and predentin and continue their course into dentin. They can be traced up to 200 \( \mu m \) into dentin and this distance decreases as one moves from the cuspal to the radicular dentin. A similar staining pattern was
demonstrated in pulps from cats, rats, monkeys and human beings (Silverman and Kruger, 1987).

CGRP-containing fibers are of a sensory nature. Inferior alveolar nerve resection substantially eliminated CGRP-like immunoreactivity (CGRP-LI) in dentin, pulp, periodontal ligament and gingiva leaving only a few immunoreactive fibers. When the mandibular division of the trigeminal nerve was resected at its emergence point through the foramen ovale, total depletion of the immunoreactivity was achieved. Conversely, bilateral sympathectomy did not show any qualitative or quantitative change in the immunostaining (Silverman and Kruger, 1987). Capsaicin treatment leads to a distinct but variable decrease of CGRP-LI, possibly due to CGRP association with neural elements not susceptible to capsaicin (Silverman and Kruger, 1987). Anterograde axonal transport studies show that CGRP fibers are not only found in close association with pulpal vasculature but they also exist independently (Kimberly and Byers, 1988).

9. CGRP is a Mediator of Neurogenic Pulpal Inflammation

The pulp houses and nurtures cells that build, maintain and repair dentin in response to tissue damage. A variety of brain neuropeptides have been shown to exert diverse trophic and immunoregulatory influences in pulpal functions. Substance P, vasoconstrictory intestinal peptide and especially CGRP seem to mediate effector roles other than nociception. They play a role in the regulation of the blood flow as well as certain aspects of tissue repair (Kimberly and Byers, 1988).
The sensory properties of pulp and dentin change significantly during inflammatory conditions. Intradental cytochemical changes, modification of the sympathetic nerve activity (Edwall and Scott, 1971), physical distortion of nerve endings (Nahri, et al., 1982 a) and changes in the neuropeptide content of the sensory axons contribute to the creation of an altered environment during inflammation (Silverman and Kruger, 1989). Besides the nerve reaction to the noxious stimuli, tertiary dentin is being formed thus, providing a barrier that protects the pulp against the toxic agent. Nerve reaction to noxious stimuli and tertiary dentin formation seem to be causally related.

Free nerve endings have traditionally been thought to comprise a homogenous population responsible for nociception. However, there is growing scientific evidence that points out the fact that free nerve endings are highly diversified and contain neuropeptides that have been attributed a variety of non-sensory functions (Silverman and Kruger, 1989).

The widespread distribution of free nerve endings in ocular, nasal and oral epithelia known to mediate nociceptive protective reflexes can not account for the total number and distribution of sensory axons strictly for nociception. The density of the pulpal innervation can not be justified strictly by mediation of sensation and regulation of the blood flow. Besides, pulpal inflammation and other pathologic conditions that stimulate nociceptive afferents are only transiently related to pain. It may be that these axons also mediate an efferent response to noxious stimuli, a "nocefector " role that requires a prior afferent generation (Silverman and Kruger, 1989).

In that case small ganglion cells may represent the "prototype neuron" which detects noxious stimuli and mediates tissue protection and repair
via phenotypic expression of a variety of neurotransmitters and neuromodulators that help control circulation, immune responses, damage, infection and activation of metabolic effects related with inflammation (Kruger, 1987).

Sensory nerve fibers are the dominant population in the oral cavity, although some sympathetic nerves also exist. CGRP is a regulator of blood flow and tissue hydrodynamics and possibly has some pathophysiologic actions.

CGRP seems to be one of the most potent mediators of neurogenic inflammation. It is a potent vasodilator and modulator of leucocyte and macrophage function, it can inhibit substance P degradation or act as a mitogen for certain cell populations.

10. CGRP Reactions to Injury

There has been some conflict in the literature regarding nerve response to dental inflammation. It has been reported that the nerves remain intact in the inflammed area (England, et al., 1974). However, immunocytochemical studies have demonstrated that CGRP-stained nerve fibers were clearly destroyed in the area of acute inflammation and were proliferating underneath it (Byers, et al., 1987 a). Similar nerve sprouting was also shown in the alveolar bone in guinea pigs (Fish, 1939). Nerve growth appears to be inhibited in the inflammed area but stimulated in the adjacent areas.

The response of CGRP fibers to mild injury was studied by means of immunocytochemistry. An extensive increase of CGRP-LI fibers in the
Rashcow's plexus, the odontoblastic layer and dentin could be seen at 4 days. This increase was transient and disappeared at 21 days. It was more likely attributed to "sprouting" proliferation of new nerve fibers (Kimberly and Byers, 1988).

When severe injury leading to microabcess formation was inflicted in dental pulps the following histologic findings were noticed. At day 4 post-injury a dense CGRP-LI staining with increased numbers of terminal fibers 50μm long was noticed in a zone rich in fibroblastic and osteoblastic cells localized peripherally to the acute abcessed area. Intensive sprouting in this zone was noticed by day 7. Simultaneously, tertiary dentin formation was initiated at the edges of the lesions where approximation of CGRP sprouts was seen. While, tertiary dentin gradually covered the lesion, the CGRP-positive fibers decreased in number and innervation returned to normal pattern (Taylor and Byers, 1990).

Increased CGRP-positive nerve sprouting has been demonstrated under carious lesions (Yamamura, 1987), under pulp exposures (Kimberly and Byers, 1988) after orthodontic treatment (Kvinnsland and Kvinnsland, 1990), mild or severe pulp injury (Taylor, et al., 1988). CGRP-LI was considerably increased in the periapical area of an inflammed tooth before the appearance of the lesion itself (Khayat et al., 1988). The increased number of fibers might be one explanation for the hyperanalgesia during inflammation (Yamamura, et al., 1988).

Therefore it is clear that CGRP-containing fibers react to inflammation by increased sprouting. Sprouting may be one way of enhancing their effector influence in inflammation and tissue repair (Taylor, et al., 1988). It may be that the nociceptive stimulation by the CGRP sensory fibers
triggers a neurogenic inflammatory response through CGRP release and vasodilation. The fact that peripheral sensory fibers have the capacity to change their distribution patterns indicates a direct involvement in the inflammatory process. Tissue damage results in initial destruction or retreat of the nerves from the site of the injury while the approximate nerves proliferate thus mediating pulp healing (Kimberly and Byers, 1988; Taylor and Byers, 1990). A reduced healing potential was noticed after capsaicin treatment while, the healing response was substantially increased after CGRP administration (Silverman and Kruger, 1987). It is possible that the same growth factors that participate in tertiary dentin formation have a similar effect on sensory nerve fiber proliferation or, that the neuropeptides themselves can act as growth factors mediating tertiary dentin formation.

G. SUMMARY

The pulpal defense mechanism to noxious agents consists of stimulation of free nerve endings and tertiary dentin formation. Neuropeptides released in an axon-reflex way seems to mediate a number of non-sensory functions. There is evidence that CGRP-LI nerve fibers demonstrate transient increased sprouting adjacent to the inflammation area simultaneously with the migration of fibroblast-like and endothelial-like cells and tertiary dentin formation. However, the effect of CGRP on the pulpal cells, per se, in terms of mitogenesis and collagen synthesis, has not been investigated.
These data will help to elucidate the defense mechanism of the pulp and tertiary dentin formation. They will also help to establish CGRP mediated neurogenic inflammation as an essential defense mechanism of the pulp-dentin organ. Finally, they will attest to a neurotrophic-neuromodulator role of the sensory nervous system in tissue repair.

H. SPECIFIC OBJECTIVES

1. To establish cultures of human pulpal cells and characterize them in terms of proliferation kinetics, alkaline phosphatase activity and collagen synthesis.

2. To investigate the effect of CGRP on proliferation kinetics alkaline phosphatase activity and percentage of collagen synthesis on human pulpal cells in vitro.

3. To investigate the effect of CGRP on $\alpha_1(I)$ collagen mRNA levels.
MATERIALS AND METHODS

A. CELL ISOLATION AND CULTURE PROCEDURES

Human pulpal cells were grown from explants (Miller, et al., 1976; Pissiotis, 1990). Tissue was obtained from pulps of individuals of age 16-30 years old undergoing extractions. Teeth were obtained immediately after extraction and the periodontal remnants were removed after wiping the teeth with a gauze containing 2% potassium iodine. A longitudinal groove was made in each tooth with a diamond burr under running sterile saline and the teeth were split open with a sterile chisel. The pulp was aseptically removed and placed in a Petri dish containing a-MEM culture medium (Sigma) supplemented with 10% fetal calf serum (FCS, Hyclone) and 1% antibiotic antifungal (Sigma) (complete culture medium). The explants were dissected in small pieces and were transferred to a 25 cm\(^2\) culture dish covered with a thin layer of complete culture medium in order to facilitate attachment. Subsequently they were placed in an incubator, in a humidified atmosphere of 5% CO\(_2\)-95% air for 30 minutes. Explants were then fully covered with complete culture medium and returned to the incubator. After a period of 6-12 days cells started growing out of the explants. After the cells became confluent they were trypsinized with 0.08 trypsin/0.04 ethylenediamine-tetraacetic acid (EDTA, Sigma) in Hank's balanced salt solution (HBSS, Sigma), pH 7.2, and transferred to a new 25 cm\(^2\) culture dish. These cells were designated as "first passage cells, T\(_1\)". Only second passage cells, T\(_2\), were used for the experimental studies.
B. CELL PROLIFERATION STUDIES

Second passage human pulpal cells, T2, were plated in 24-well dishes at a concentration $1.5 \times 10^4$ cells/cm$^2$ in α-MEM complete culture medium, supplemented with 50 μg/ml ascorbic acid. Both the culture medium and the ascorbic acid were changed every other day.

Cells were trypsinized and counted in a hemocytometer using phase contrast microscopy. Growth curves plotting cell number versus time in culture were established and the growth rate at midlog phase was determined.

C. ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase (AP) activity was determined by means of a biochemical assay (Majeska and Rodan, 1982). The assay was based on the reaction of Na$_2$ p-nitro-phenylphosphate with AP in the presence of Mg$^{++}$. The relative amount of AP activity was determined by measuring the absorbance of p-nitrophenol at 405 nm in a Titertek multiscan absorbance spectrophotometer. Both total AP activity and AP activity per cell were estimated and plotted versus time in culture.

D. COLLAGEN SYNTHESIS

Collagen synthesis was estimated by means of a collagenase digestible protein assay (Diegelmann and Peterkofsky, 1972; Kream, et al., 1989;
Peterkofsy and Diegelmann, 1971). Cultures were pulsed with 10 μg/ml
[3H] proline (10-40 μCi/mmol, Amersham) for four hours. Both culture
medium and cell extracts were precipitated with 10% trichloroacetic acid.
An aliquot was digested with purified bacterial collagenase and the
amounts of collagenase digestible proteins (CDP) and non-collagenous
proteins (NCP) were determined. NCP was multiplied by a factor 5.4 to
correct for the fact that there are more proline residues in collagen
compared with other proteins. The percentage of collagen synthesis (PCS)
was calculated according to the formula PCS=CDP/(NCP x 5.4) + CDP, and
plotted versus time in culture.
CDP and NCP were normalized against the cell number and plotted versus
time in culture.

E. CHARACTERIZATION OF THE EFFECT OF CGRP

During midlog phase of growth, determined to be at day 8, human
calcitonin gene-related peptide I (CGRP, Calbiochem) was added to serum-
containing and serum-free cultures of human pulpal cells at
concentrations 10⁻⁶ M, 10⁻⁹ M, 10⁻¹² M and 10⁻¹⁵ M. The effect of CGRP on
cell proliferation, AP activity, collagen synthesis and procollagen mRNA
levels for the α₁ chain of type I collagen was examined 24, 48 and 72 hours
later.

Serum-free cultures: In serum-free cultures complete medium was
removed at least 20 hours before the addition of CGRP and the cultures
were supplemented with α-MEM containing 0.1% FCS, 1% antibiotic-antimycotic and 50 µg/ml ascorbic acid.

F. TOTAL RNA ISOLATION AND DNA PROBES

Total RNA from serum-containing cultures of human pulpal cells was isolated by phenol extraction (Chomczynski and Sacchi, 1987). Total RNA was extracted the day the cells were seeded, at day 8, and 24, 48 and 72 hours following addition of 10⁻⁶ M and 10⁻⁹ M CGRP.

The probe for the α₁ chain of human type I collagen (HF677) has been previously described (Chu, et al., 1982). HF677 is a 1.4 kb cDNA fragment (Eco R₁) from plasmid pBR 322. HF677 spans from residue 787 of the α₁(I) helix and extends into the 3' end untranslated region of pro α₁(I) mRNA. It codes part of the α₁(I) helical region, the C terminal telopeptide, the C terminal propeptide and 224 bp of the 3' non-coding region (Bernard, et al., 1983). HF677 has been shown to have no crossreactivity with mRNAs for α₂(I) and α₁(III) collagen chains (DeWet, et al., 1983; Rowe, et al., 1985). The cDNA probe was labeled with ³²P dCTP using the random oligonucleotide priming technique (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984).

G. NORTHERN AND DOT BLOT HYBRIDIZATION

Northern blot hybridization was used in order to confirm the specificity of the HF677 probe used. Five µg of total RNA were denatured
1% agarose gel and transferred to nitrocellulose paper by capillary blotting (Thomas, 1980). A BRL RNA ladder was used as size marker.

Quantitative changes in procollagen mRNA levels for the α1 chain of type I collagen following addition of 10⁻⁶ M and 10⁻⁹ M CGRP in serum-containing cultures of human pulpal cells were estimated by the dot blot hybridization technique. This technique is more quantitative than northern blot analysis in that it is not as sensitive to small variations in intactness of the RNA and in that the amounts are easily normalized to total poly A⁺ RNA. Total RNA aliquots mixed with 7.4% formaldehyde and 6x standard-citrate buffered solution (SSC) were heated for 15 minutes in a 65°C water bath (Mina, et al., 1991). Aliquots were serially diluted with 15x SSC and spotted into the nitrocellulose filter using the minifold apparatus. The blots were baked for 2 hours at 80°C under vacuum (Costanzi and Gillespie, 1987).

The same protocol for prehybridization and hybridization was used for both northern blots and dot blots. The nitrocellulose filters were prehybridized overnight in a 42°C. The prehybridization solution used was similar to the one described by Mina et al (1991) except for the fact that it contained 0.1% bovine serum albumin, 0.1% Ficoll₄₀₀, 0.1% polyvinylpyrrole, 1% glycine and 216 μg/ml of t-RNA. The filters were then hybridized for 18 hours at 50°C in a shaking water bath with a solution containing 30-60 x 10⁶ cpm ³²P. The hybridization solution was similar to the prehybridization solution except for the addition of 10% dextran sulfate and of the heat denatured probe. Following hybridization the blots were washed three times (5 minutes each) with 2x SSC, 0.1% SDS at room temperature followed by three additional washes (10 minutes each)
at 52° C. The relative levels of α1(I) procollagen mRNA on the filters were quantitated by scanning the dots on the autoradiograms using a Biorad scanning densitometer (Kosher, et al., 1986).

**Measurement of poly A+ RNA:** The same filters were subsequently used for hybridization with $\gamma^{32}$P labeled oligo (dT)$_{20}$. Filters were first stripped and then were prehybridized in 5x SSC for 1 hour. Approximately $5 \times 10^6$ cpm of end-labeled oligo (dT) probe was added to the hybridization solution which contained 0.1% FCS, 0.1% Ficoll$_{400}$ and 0.1% polyvinylpyrroline (Harley, 1987). Hybridization was done at room temperature for 6 hours. Filters were washed at room temperature for four times (15 minutes each) in 2x SSC, 0.1 % SDS. The relative levels of poly A+ mRNA on the filters were quantitated as described above and used to normalize the amount of α$_1$(I) procollagen mRNA to total poly A+ RNA.

**H. STATISTICS**

The results were analyzed with one-way analysis of variance (ANOVA). Pairwise comparisons were done using Sheffe’s test.
cultures was statistically different from the controls throughout the experimental period (Table 2). Micromolar amounts of CGRP were again able to stimulate a 2 fold increase in cell number. Addition of 10^{-15} M CGRP did not seem to have any effect on cell proliferation. Cell number progressively decreased over time and did not show any statistically significant difference compared with controls.

B. ALKALINE PHOSPHATASE ACTIVITY

**Baseline:** Total alkaline phosphatase (AP) activity of human pulpal cells over time in culture remained at low levels during the period of active cell proliferation and started increasing during and after the time the cells reached confluence (Figure 7). When AP activity was normalized against cell number a rather different plot was obtained (Figure 8). AP activity dropped significantly during the first six days of culture, remained at constant low levels until the cells were almost confluent and started to increase after the cells reached confluence.

**Characterization of the CGRP effect:** Addition of 10^{-6} M and 10^{-9} M CGRP stimulated total AP activity (Figure 9). AP activity peaked at 24 hours after the addition of CGRP and then progressively decreased. The total activity in the experimental cultures was statistically different from the controls throughout the experimental period (Table 3). AP activity per cell (Table 4) increased in experimental cultures 24 hours after the addition of the CGRP and then progressively decreased (Figure 10). After 48 hours
it reached control levels and after 72 hours it was statistically lower than the controls (Table 4).

**Serum-free cultures:** Total activity in control serum-free cultures remained at the same level during the experimental period. Addition of $10^{-6}$ M and $10^{-9}$ M CGRP was able to stimulate AP activity in serum-free cultures (Figure 11). The AP activity in the experimental cultures was significantly different from the controls throughout the experimental period (Table 5). AP activity per cell was increased in the experimental cultures 24 hours after the addition of CGRP and progressively decreased over time (Figure 12). The AP activity per cell in the serum-free control cultures showed a progressive increase over time and at 72 hours the control cultures had higher levels of AP activity per cell than the experimental cultures (Table 6).

**C. COLLAGEN SYNTHESIS**

**Baseline:** The amount of collagenous digestible proteins (CDP) and non-collagenous proteins (NCP) was determined and the percentage of collagen synthesis (PCS) was estimated. PCS showed a progressive increase over time in culture and reached levels as high as 9% between day 9 and when the cells became confluent (Figure 13). In post confluent cultures PCS decreased to levels around 6%.

**Characterization of the CGRP effect:** CGRP at concentrations of $10^{-6}$ M, $10^{-9}$ M and $10^{-12}$ M was able to stimulate PCS in a dose dependent manner
(Figure 14). PCS showed a peak 24 hours after addition of CGRP, reaching a maximum 4.5fold increase in $10^{-6}$ M CGRP containing cultures, and progressively decreased over time reaching control values at 72 hours (Table 7). Addition of $10^{-15}$ M CGRP did not have any effect on PCS (Figure 14) and experimental cultures did not show any statistically significant difference from controls. The amount of CDP increased 24 hours after the addition of CGRP and progressively decreased, while in the control cultures CDP remained constant or slightly decreased at 72 hours (Figure 15). The amount of NCP decreased following addition of CGRP, while the amount of NCP in the controls remained at the same levels or slightly increased at 72 hours (Figure 16).

**Serum-free cultures:** Serum deprivation resulted in a significant reduction in PCS in cultures of human pulpal cells. CGRP at concentrations of $10^{-6}$ M, $10^{-9}$ M, $10^{-12}$ M was able to stimulate PCS in a dose response way (Figure 17). PCS showed a peak at 24 hours, reaching a maximum 4 fold increase from baseline values in $10^{-6}$ M CGRP containing cultures and progressively decreased over time. PCS values in the micromolar and nanomolar CGRP containing cultures were statistically different from the controls throughout the experimental period (Table 8). The amount of CDP increased in experimental cultures 24 hours following the addition of CGRP and returned to control values 48 and 72 hours later. The amount of CDP in control cultures increased over time (Figure 18). The amount of NCP slightly decreased following the addition of CGRP, while the amount of NCP in control cultures increased over time (Figure 19).
D. NORTHERN & DOT BLOT HYBRIDIZATION

Northern blot hybridization was used for determination of RNA sizes, and specificity of the probe and the hybridization conditions used for the $\alpha_1$ chain of type I procollagen mRNA. The probe for $\alpha_1$(I) procollagen hybridized to two mRNAs, a major species of 5.8 kb and a minor species of 7.2 kb (Figure 20).

Quantification of the relative amounts of $\alpha_1$(I) procollagen mRNA was done by the dot blot hybridization technique after normalization against poly A+ mRNA. A pool of mRNA from different experiments was used in order to account for possible variation in mRNA levels between experiments. The relative mRNA levels for $\alpha_1$(I) procollagen showed a progressive and continuous increase as a function of time, similar to the one noticed in PCS in the control cultures (Figure 21). Addition of $10^{-6}$ M CGRP to the cultures resulted in a drastic increase in the relative $\alpha_1$(I) mRNA procollagen levels 24 hours later and the mRNA levels progressively approached control values 72 hours later (Figure 22). Addition of $10^{-9}$ CGRP M to the cultures did not seem to alter the levels for $\alpha_1$(I) procollagen mRNA significantly.
DISCUSSION

The present study investigated the effects of calcitonin gene-related peptide (CGRP) on human pulpal cells in vitro. Explant pulpal cell cultures were established and second passage cells were used for the experimental studies. CGRP was added once during the midlog phase of growth and its effects were estimated 24, 48 and 72 hours later. We have demonstrated that CGRP was able to stimulate cell proliferation, alkaline phosphatase activity and collagen synthesis of human pulpal cells in vitro.

CGRP is a sensory neuropeptide that is widely distributed in the dental pulp (Fried, et al., 1989). CGRP is present in inflammed tissues and has been implicated in inflammatory reaction and tissue repair (Payan, et al., 1987b). CGRP immunoreactive nerve fibers have been demonstrated in close approximation with mast cells and CGRP receptors have been identified on mast cells (Abello, et al., 1990; Kvinnsland, et al., 1991). CGRP immunoreactivity has been shown to increase considerably in the dental pulp during pulp inflammation and tertiary dentin formation (Kimberly and Byers, 1988; Yamamura 1987; Kvinnsland and Kvinnsland, 1990). However, the reason for the observed increase and the possible role that CGRP may play in tertiary dentin formation remains unknown.

In our experiments we have demonstrated that CGRP was able to stimulate proliferation of human pulpal cells in vitro. The mitogenic effect of CGRP was concentration dependent for doses ranging between 10^{-6} M and 10^{-12} M and was abolished at ficomolar concentrations. Micromolar amounts of CGRP resulted in a 2.5 fold increase in cell number in the experimental cultures throughout the experimental period.
The magnitude of the mitogenic effect of CGRP was similar in serum-containing and in serum-free cultures, although cell number in the latter never reached baseline control levels. Serum supplementation is necessary for optimal growth of fibroblast-like cells in vitro (Ham, 1984a; Ham, 1984b). Deprivation of serum during the logarithmic phase of growth of human pulpal cells results in a sharp decrease in cell number. CGRP was able to counteract the growth inhibitory conditions but alone it could not substitute for serum. Previous investigators have reported that CGRP (10^{-6} M - 10^{-12} M) has a concentration dependent mitogenic effect on cultures of smooth muscle cell lines (Mitsuhashi and Payan, 1987) and human endothelial cells of the umbilical vein under serum-free conditions (Haegerstrand, et al., 1990). Active cell proliferation has been shown to occur during the initial stages of tertiary dentin formation when mesenchymal cells migrate to the injury site and deposit fibrodentin (Fitzerald, 1979; Fitzgerald et al., 1990). CGRP has the potential to stimulate cell proliferation and therefore, its presence in pulp inflammation may contribute to mesenchymal cell differentiation during tertiary dentin formation.

Alkaline phosphatase activity has been associated with connective tissue mineralization. Scientific evidence suggests that AP might mediate mineralization in a number of ways. AP may increase the levels of local phosphate ions, or help their transport to the mineralization site, remove inhibitors of mineral crystal growth or act as a calcium ion binding protein (DeBernard, et al., 1985; Neuman, et al., 1951; Register, et al., 1984; Wuthier and Register, 1985).

AP activity of cultured human pulpal cells declined to low levels during the logarithmic phase of growth and increased considerably after
the cells reached confluence. This pattern of AP activity in human pulpal cells is similar to cell cultures from other tissues that have inherent mineralization potential (Wuthier and Register, 1985). It has been reported that AP activity is enhanced under conditions that inhibit cell proliferation (Farley, et al., 1983). Accordingly, in our cultures AP activity per cell increased after serum removal during the logarithmic phase of cell growth.

CGRP was able to stimulate AP activity in cultures of human pulpal cells. The action of CGRP was independent of the presence of serum. AP activity increased almost 2 fold 24 hours following the addition of CGRP and then progressively declined. AP activity reached control levels at 48 hours and at 72 hours it was statistically lower than the controls. Growth factors, like fibroblast growth factor, have been shown to inhibit AP activity (Chin, 1984). The fact that CGRP can stimulate both cell proliferation and AP activity of human pulpal cells in vitro may reflect the existing heterogeneity of the cultured cells, in that a fraction of the non-actively proliferating cells may contribute to the observed increase in AP activity. However, AP activity declines over time either due to the end of the stimulatory action of CGRP, or as a result of the active cell proliferation.

AP activity has been shown to play an important role during dentin mineralization. AP levels are high during the period of active dentinogenesis and decrease by 30%-80% in mature teeth (Laikko and Larmas, 1980). However, AP activity increases by 2-10 fold when noxious stimuli insult the tooth triggering pulp inflammatory reaction and tertiary dentin formation (Karjalainen and LeBell, 1987; Karjalainen and MacKinen, 1977). CGRP can stimulate AP activity and may therefore
provide a rationale for its potential role in fibrodentin mineralization during tertiary dentin formation.

Cultures of human pulpal fibroblast-like cells are rich in collagen (Shuttleworth, et al., 1980). Synthesis of collagenous and non-collagenous proteins increases over time and the percentage of collagen synthesis reaches levels as high as 8-9% during the time that the cells become confluent. Similar levels of PCS have been reported for cell cultures of mineralizing connective tissue (Hurley, et al., 1989; Kream, et al., 1989). Serum deprivation during the midlog phase of growth resulted in a sharp decrease in PCS over time. This observation agrees with other investigators that have shown that protein secretion decreases under growth inhibitory conditions (Ham, 1984a). However, the amount of collagenous and non-collagenous proteins normalized versus the cell number shows a relative increase, probably reflecting the simultaneous dramatic decrease in cell number.

CGRP was able to stimulate collagen synthesis in the presence and absence of serum, although collagen levels peaked in serum-containing cultures. The CGRP effect was concentration dependent for doses ranging between $10^{-6}$ M and $10^{-12}$ M and was abolished at doses as low as $10^{-15}$ M. Addition of micromolar amounts of CGRP stimulated a 1.5-2 fold increase in the amount of collagenous digestible proteins 24 hours later, while the amount of non-collagenous proteins decreased. Therefore, PCS showed an almost 4 fold increase at 24 hours and declined over time. The decrease in the amount of non-collagenous proteins per cell may be due to an inhibitory action of CGRP on non-collagenous proteins synthesis or may simply reflect the very active cell proliferation.
The effect of CGRP on collagen synthesis of cultures of mineralized tissue has not been previously investigated. CGRP has been shown to increase the number and size of bone colonies in cultures of bone marrow cells implying an increase in collagen levels (Bernard and Shih, 1990), however, the amount of collagenous proteins was not quantitated. Casini et al., 1991 studied the effect of CGRP on collagen synthesis of fat storing cells. CGRP did not seem to affect significantly type I and III collagen levels, while it stimulated glycosaminoglycans synthesis. The different regulatory action of CGRP on collagen synthesis observed in rat fat-storing cells may be due to the different nature of the cells, their reduced total collagen levels and their lack of inherent mineralization potential. Collagen has been shown to constitute the primary organic component of tertiary dentin (Sela, et al., 1981; Schroeder, 1985). Therefore, CGRP may stimulate collagen synthesis of pulpal cells during tertiary dentin formation.

In order to further investigate the mechanism by which CGRP modulates collagen synthesis the effect of CGRP on the relative mRNA levels of the α₁ chain of type I procollagen was also examined. It has been shown that human pulpal cells secrete primarily type I and type III collagen along with smaller amounts of type V and type VI collagen (Andujar, et al., 1991; Lukimaa and Waltimo, 1992). Secretion of type I collagen is considered as one of the characteristic features of the differentiating odontoblastic cell (Magloire, et al., 1988). Besides, systemic hormones, parathyroid hormone, insulin, cortisol, and growth factors, transforming growth factor-β, insulin-like growth factor, have been shown to regulate type I collagen synthesis by regulating α₁(I) procollagen

Addition of micromolar amounts of CGRP to cultures of human pulpal cells resulted in a 1.5-1.8 fold increase in the relative \( \alpha_1 (I) \) mRNA levels 24 hours later and progressively decreased to control levels at 72 hours. The increase of the relative \( \alpha_1 (I) \) procollagen mRNA levels was similar to the increase in collagenous digestible proteins following addition of equimolar amounts of CGRP. Addition of \( 10^{-9} \) M CGRP to the cultures did not seem to alter significantly the relative \( \alpha_1 (I) \) mRNA levels and therefore, did not correlate with the observed increase in the amount of collagenous proteins.

These results suggest that CGRP may affect collagen synthesis by different mechanisms (i.e. transcriptional control, translational control). Addition of high doses of CGRP could result in an increase in the relative \( \alpha_1 (I) \) procollagen mRNA levels either by enhancing the transcription rate of mRNA, or by increasing mRNA stability. Low doses of CGRP do not seem to have an effect on \( \alpha_1 (I) \) procollagen levels and therefore CGRP could increase collagen synthesis by enhancing the translation rate of collagen mRNA. Alternatively, CGRP may affect both type I and type III collagen synthesis. Since, the probe used has been shown to be specific for \( \alpha_1 (I) \) and has no crossreactivity with \( \alpha_2 (I) \), or \( \alpha_1 (III) \) we were only able to quantitate changes in the \( \alpha_1 (I) \) collagen levels. Although it has been reported that the ratio of type I/type III in tertiary dentin is 88% / 12% respectively, it is not known at what ratio the two collagens are secreted during tertiary dentin deposition (Magloire, et al., 1988). Furthermore, type III collagen production has been shown to increase in cultures of mature fibroblast-like pulpal cells and account for 15% of total pepsin
digestible collagenous proteins (Shuttleworth, et al., 1980). The assay used to quantitate collagen synthesis accounted for all the collagenase digestible proteins and therefore our results may reflect an increase in both type I and type III collagen. Further investigation is required in order to clarify this issue.

The presence of CGRP has been associated with connective tissue deposition and mineralization. Increased CGRP immunoreactivity was demonstrated during tertiary dentin formation following pulp inflammation due to traumatic injury or tooth avulsion and subsequent reimplantation. In tooth reimplantation experiments intense CGRP immunoreactivity was associated with successful pulp healing, new odontoblast differentiation and tertiary dentin formation. In cases of unresolved pulp inflammation and subsequent bone deposition CGRP immunoreactive nerve fibers were sparse (Kvinnsland, et al., 1991). Besides, CGRP immunoreactive nerve fibers demonstrate increased sprouting following pulp injury (Kimberly and Byers, 1988). The increase of CGRP immunoreactivity is transient during the time that cells migrate to the injury site and fibrodentin is being deposited in an attempt to protect the pulp from the noxious agent (Taylor and Byers, 1990; Taylor, et al., 1988). We have demonstrated that CGRP has the potential to stimulate cell proliferation, alkaline phosphatase activity and collagen synthesis of mature human pulpal cells in vitro. These three parameters are intimately related to tertiary dentin formation thus, suggesting that the in vitro effects of CGRP may provide an explanation for its in vivo effects. Thus, we propose that CGRP may play a significant role in the response of dental pulp to injury and tertiary dentin formation.
CONCLUSIONS

- Second passage human pulpal cells reach confluence at 13 days and at midlog phase of growth have a doubling time of 35 hours.

- CGRP stimulates cell proliferation of human pulpal cells in a concentration dependent way for doses ranging between $10^{-6}$ M and $10^{-12}$ M in both serum-containing and serum-free cultures.

- CGRP stimulates alkaline phosphatase activity of human pulpal cells in both serum-containing and serum-free cultures.

- CGRP stimulates collagen synthesis of human pulpal cells in a concentration dependent manner for doses ranging between $10^{-6}$ M and $10^{-12}$ M CGRP in both serum-containing and serum-free cultures.

- CGRP stimulates an increase in the relative $\alpha_1$ (I) procollagen mRNA levels in cultures of human pulpal cells.

- CGRP has the potential to stimulate cell proliferation, collagen synthesis and alkaline phosphatase activity of human pulpal cells in vitro and may therefore play an important role in the healing response of human dental pulp to injury.
Table 1: Effect of 10-6 M, 10-9 M, 10-12 M and 10-15 M on cell proliferation in serum-containing cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=6).

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<td>119.30 (2.10)</td>
<td>116.78 (0.62)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>382.46 (3.26)**†</td>
<td>309.95 (1.64)**†</td>
<td>180.88 (1.98)*</td>
<td>154.10 (4.00)</td>
<td>154.33 (0.58)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
† Statistically significant difference between experimental groups p<.01
Table 2: Effect of 10-6 M, 10-9 M, 10-12 M and 10-15 M on cell proliferation in serum-free cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=6).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>45.16 (0.41)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>29.31 (0.48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>35.60 (0.74)*†</td>
<td>28.86 (0.70)*†</td>
<td>22.35 (0.87)</td>
<td>22.65 (0.75)</td>
<td>19.03 (0.63)</td>
</tr>
<tr>
<td>10</td>
<td>51.03 (0.64)*†</td>
<td>35.48 (1.25)*†</td>
<td>25.02 (0.80)*†</td>
<td>16.45 (1.75)</td>
<td>14.86 (0.79)</td>
</tr>
<tr>
<td>11</td>
<td>59.85 (0.62)*†</td>
<td>40.51 (0.77)*†</td>
<td>26.65 (0.63)*†</td>
<td>15.15 (0.85)</td>
<td>12.45 (0.45)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
† Statistically significant difference between experimental groups p<.01
Table 3: Effect of 10-6 M and 10-9 M CGRP on AP activity in serum-containing cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=12).

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>AP activity 10-6 M CGRP Means (SEM)</th>
<th>AP activity 10-9 M CGRP Means (SEM)</th>
<th>AP activity Controls Means (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>13.15 (1.46)</td>
<td>39.75 (4.58)**</td>
<td>19.20 (2.38)</td>
</tr>
<tr>
<td>9</td>
<td>52.65 (6.55)*</td>
<td>37.09 (3.90)**</td>
<td>20.90 (2.12)</td>
</tr>
<tr>
<td>10</td>
<td>45.47 (4.88)*</td>
<td>33.58 (2.95)**</td>
<td>22.46 (2.06)</td>
</tr>
<tr>
<td>11</td>
<td>39.65 (3.08)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
Table 4: Effect of 10-6 M and 10-9 M CGRP on AP activity per cell in serum-containing cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=12).

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>AP/CELL 10-6 M CGRP Means (SEM)</th>
<th>AP/CELL 10-9 M CGRP Means (SEM)</th>
<th>AP/CELL Controls Means (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>19.88 (2.21)</td>
</tr>
<tr>
<td>9</td>
<td>33.05 (4.10)**</td>
<td>32.41 (3.92)</td>
<td>22.82 (2.76)</td>
</tr>
<tr>
<td>10</td>
<td>17.41 (1.87)</td>
<td>17.79 (1.87)</td>
<td>17.97 (1.81)</td>
</tr>
<tr>
<td>11</td>
<td>10.37 (0.80)**</td>
<td>10.81 (0.93)**</td>
<td>14.57 (1.33)</td>
</tr>
</tbody>
</table>

** Statistically significant difference between experimental and control p<.05
Table 5: Effect of 10^{-6} M and 10^{-9} M CGRP on AP activity in serum-free cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=12).

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>AP activity 10^{-6} M CGRP Means (SEM)</th>
<th>AP activity 10^{-9} M CGRP Means (SEM)</th>
<th>AP activity Controls Means (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>13.35 (4.86)</td>
<td></td>
<td>13.12 (2.22)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>14.76 (1.84)</td>
</tr>
<tr>
<td>9</td>
<td>36.26 (3.90)*</td>
<td>27.85 (2.95)**</td>
<td>14.56 (1.65)</td>
</tr>
<tr>
<td>10</td>
<td>35.47 (3.31)*</td>
<td>26.27 (2.55)**</td>
<td>14.6 (1.65)</td>
</tr>
<tr>
<td>11</td>
<td>34.73 (3.38)*</td>
<td>27.57 (2.37)*</td>
<td>13.49 (1.49)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
† Statistically significant difference between experimental groups p<.05
Table 6: Effect of 10-6 M and 10-9 M CGRP on AP activity per cell in serum-free cultures of human pulpal cells. Values represent the means and SEM of three experiments (n=12).

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>AP/CELL</th>
<th>AP/CELL</th>
<th>AP/CELL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-6 M CGRP</td>
<td>10-9 M CGRP</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Means (SEM)</td>
<td>Means (SEM)</td>
<td>Means (SEM)</td>
</tr>
<tr>
<td>7</td>
<td>29.14 (10.62)</td>
<td>44.77 (7.57)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>103.36 (10.74)</td>
<td>98.25 (9.96)</td>
<td>77.59 (9.71)</td>
</tr>
<tr>
<td>9</td>
<td>95.44 (10.26)</td>
<td>77.77 (10.17)</td>
<td>73.78 (7.36)</td>
</tr>
<tr>
<td>10</td>
<td>58.02 (5.65)*</td>
<td>70.39 (5.38)**</td>
<td>106.05 (11.83)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
Table 7: Effect of 10-6 M, 10-9 M, 10-12 M and 10-15 M on percentage of collagen synthesis (PCS) in serum-containing cultures of human pulpal cells. Values represent the means and SEM of at least three experiments.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>PCS 10-6 M CGRP Means (SEM)</th>
<th>PCS 10-9 M CGRP Means (SEM)</th>
<th>PCS 10-12 M CGRP Means (SEM)</th>
<th>PCS 10-15 M CGRP Means (SEM)</th>
<th>PCS Controls Means (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.04 (0.43)</td>
</tr>
<tr>
<td>9</td>
<td>38.20 (5.24)**†</td>
<td>24.20 (1.74)**†</td>
<td>10.86 (1.25)**</td>
<td>7.01 (1.75)</td>
<td>7.18 (0.76)</td>
</tr>
<tr>
<td>10</td>
<td>31.47 (3.81)**†</td>
<td>17.13 (1.52)*</td>
<td>9.02 (1.44)</td>
<td>7.48 (1.20)</td>
<td>7.74 (0.77)</td>
</tr>
<tr>
<td>11</td>
<td>12.54 (1.13)**†</td>
<td>8.84 (0.95)</td>
<td>6.17 (1.03)</td>
<td>8.13 (1.79)</td>
<td>8.13 (0.89)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
† Statistically significant difference between experimental groups p<.01
Table 8: Effect of 10-6 M, 10-9 M, 10-12 M and 10-15 M on percentage of collagen synthesis (PCS) in serum-free cultures of human pulpal cells. Values represent the means and SEM of at least three experiments.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>PCS 10-6 M CGRP Means (SEM)</th>
<th>PCS 10-9 M CGRP Means (SEM)</th>
<th>PCS 10-12 M CGRP Means (SEM)</th>
<th>PCS 10-15 M CGRP Means (SEM)</th>
<th>PCS Controls Means (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.91 (0.67)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.02 (0.18)</td>
</tr>
<tr>
<td>9</td>
<td>27.50 (4.09)*†</td>
<td>15.37 (1.02)**†</td>
<td>10.86 (1.56)**</td>
<td>3.23 (1.10)</td>
<td>2.19 (0.27)</td>
</tr>
<tr>
<td>10</td>
<td>20.01 (4.09)*†</td>
<td>11.61 (1.08)**†</td>
<td>7.05 (1.61)*</td>
<td>2.12 (0.23)</td>
<td>1.34 (0.25)</td>
</tr>
<tr>
<td>11</td>
<td>10.03 (2.14)*†</td>
<td>6.79 (1.22)*</td>
<td>4.32 (0.39)*</td>
<td>1.79 (0.46)</td>
<td>0.76 (0.23)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between experimental and control p<.01
** Statistically significant difference between experimental and control p<.05
† Statistically significant difference between experimental groups p<.01
Figure 1: Phase contrast photomicrograph of pulp explants. Pulp tissue has attached on the culture dish and spindle shaped pulpal cells have started to grow out of the explant (x 90).
Figure 2: Composite growth curve of human dental pulp cells (T_2). Each data point represents the means ±SEM (x 10,000) of three experiments.
Figure 3:

a) Phase contrast photomicrograph of serum-containing cultures of human pulpal cells 24 hours following addition of $10^{-6}$ M CGRP. The cells have a spindle-shaped, elongated appearance and the formation of bundles is evident. One can appreciate the increased density of the monolayer compared to controls. (x 180)

b) Phase contrast photomicrograph of control serum-containing cultures of human pulpal cells. The cells have the typical fibroblast-like shape, although they appear to be wider than the experimental ones. (x 180)
Figure 4: Plot of cell number versus days following addition of $10^{-6}$ M, $10^{-9}$ M, $10^{-12}$ M and $10^{-15}$ M CGRP in serum-containing cultures of human pulpal cells. Each data point represents the mean ± SEM ($\times$ 10,000) of three experiments.
Figure 5:

a) Phase contrast photomicrograph of serum-free culture of human pulpal cells 24 hours following addition of $10^{-6}$ M CGRP. The cells have the typical fibroblast-like appearance. One can appreciate the increased density of the monolayer compared to controls. (x 180)

b) Phase contrast photomicrograph of serum-free control cultures of human pulpal cells. Fewer cells appear in the field. The cells have multiple elongated processes. (x 180).
Figure 6: Plot of cell number versus days following addition of $10^{-6}$ M, $10^{-9}$ M, $10^{-12}$ M and $10^{-15}$ M CGRP in serum-free cultures of human pulpal cells. Each data point represents the mean ± SEM ($\times$ 10,000) of three experiments.
Figure 7: Total alkaline phosphatase (AP) activity (μmole/minute) of human pulpal cells over time in culture. Each data point represents the mean ± SEM of three experiments.
Figure 8: Alkaline phosphatase (AP) activity per cell (nmole/minute/cell) of human pulpal cells over time in culture. Each data point represents the mean ± SEM of three experiments.
Figure 9: Alkaline phosphatase (AP) activity (μmole/minute) following addition of 10^{-6} M and 10^{-9} M CGRP in serum-containing cultures of human pulpal cells. Each column represents the mean ± SEM of three experiments.
Figure 10: Alkaline phosphatase (AP) activity per cell (nmole/minute/cell) following addition of $10^{-6}$ M and $10^{-9}$ M CGRP in serum-containing cultures of human pulpal cells. Each column represents the mean ± SEM of three experiments.
10-6 M CGRP
10-9 M CGRP
Controls

A.P. activity/cell

Days in culture

8 9 10 11
Figure 11: ALkaline phosphatase (AP) activity (µmole/minute) following addition of 10^{-6} M and 10^{-9} M CGRP in serum-free cultures of human pulpal cells. Each column represents the mean ± SEM of three experiments.
Figure 12: Alkaline phosphatase (AP) activity per cell (nmole/minute/cell) following addition of $10^{-6}$ M and $10^{-9}$ M CGRP in serum-free cultures of human pulpal cells. Each column represents the mean ± SEM of three experiments.
Figure 13: Percentage of collagen synthesis (PCS) of human pulpal cells over time in culture. Each column represents the mean ± SEM of two experiments.
Figure 14: Percentage of collagen synthesis (PCS) following addition of $10^{-6}$ M, $10^{-9}$ M, $10^{-12}$ M and $10^{-15}$ M CGRP in serum-containing cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Days in culture

% collagen synthesis (PCS)

- 10-6 M CGRP
- 10-9 M CGRP
- 10-12 M CGRP
- 10-15 M CGRP
- Controls

Days in culture
Figure 15: Collagenous digestible proteins (CDP) per cell (dpm/cell) following addition of $10^{-6}$ M and $10^{-9}$M CGRP in serum-containing cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Figure 16: Non-collagenous proteins (NCP) per cell (dpm/cell) following addition of $10^{-6}$ M and $10^{-9}$ M CGRP in serum-containing cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Days in culture

NCP/CPELL

controls
10-6 M CGRP
10-9 M CGRP
Figure 17: Percentage of collagen synthesis (PCS) following addition of $10^{-6}$ M, $10^{-9}$ M, $10^{-12}$ M and $10^{-15}$ M CGRP in serum-free cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Days in culture
Figure 18: Collagenous digestible proteins (CDP) per cell (dpm/cell) following addition of $10^{-6}$ M and $10^{-9}$M CGRP in serum-free cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Figure 19: Non-collagenous proteins (NCP) per cell (dpm/cell) following addition of $10^{-6}$ M and $10^{-9}$M CGRP in serum-free cultures of human pulpal cells. Each column represents the mean ± SEM of at least three experiments.
Figure 20: Northern-blot hybridization analysis for $\alpha_1$ (I) procollagen mRNA from cultures of human pulpal cells. Total RNA was electrophoresed, transferred to nitrocellulose and hybridized with $30 \times 10^6$ cpm of $^{32}$P cDNA probe (HF677). Lane A: 9 day unchallenged (control) cultures. Lane B: 9 day experimental cultures (24 hours following addition of $10^{-6}$ M CGRP). The Northern-blot were used for determination of RNA sizes and not for mRNA quantitation.
Figure 21: Relative levels of $\alpha_1$(I) procollagen mRNA of human pulpal cells over time in culture. A pool of mRNA from different cultures was used for each experiment. Each column represents the mean $\pm$ SEM of two experiments.
Figure 22: Effects of CGRP on the relative levels of \( \alpha_1(\text{I}) \) procollagen mRNA: \( 10^{-6} \) M and \( 10^{-9} \) M CGRP was added to cultures of human pulpal cells at day 8 and its effects were quantitated 24, 48 and 72 hours later. The mRNA levels in the experimental cultures are expressed as a percentage of the controls. A pool of mRNA from different cultures was used for each experiment. Each column represents the mean ± SEM of two experiments.
Hours after addition of CGRP

% of controls

- 10-6 M CGRP
- 10-9 M CGRP


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