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From serum to the mineral phase. The role of the odontoblast in calcium transport and mineral formation

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ABSTRACT Dentin may be considered as a calcified connective tissue and is in its composition as well as in its mode of formation closely related to bone. Dentin is formed by two simultaneous processes in which the odontoblasts are instrumental: the formation of the proteinaceous dentin matrix, and mineral crystal formation in this matrix. As part of this, the odontoblasts actively transport Ca^{2+} ions towards the site of mineral formation. The cells maintain a delicate intracellular Ca^{2+} ion balance by the concerted action of transmembraneous transport mechanisms, including Ca-ATPase, Na^+/Ca^{2+} exchangers and calcium channels of the L-type, and possibly intracellular Ca^{2+}-binding proteins. The net effect of this is a maintenance of a cytoplasmic sub-micromolar Ca^{2+} activity and an extracellular accumulation of Ca^{2+} ions at the mineralization front. In addition to the major matrix constituent, collagen, non-collagenous macromolecules, such as dentin phosphoprotein (phosphophoryn), dentin sialoprotein, and proteoglycan, are synthesized by the odontoblasts and deposited in the matrix. Such polyanionic macromolecules are presumably responsible for the extracellular induction of hydroxyapatite crystals, but may also function to inhibit mineral growth and to regulate crystal size. Accordingly, it can be concluded that dentinogenesis comprises an interplay between several factors in the tissue, cellular as well as extracellular.

KEY WORDS: *dentinogenesis, physiologic calcification, odontoblasts, phosphoproteins, calcium pump*

Introduction

When morphogenesis and differentiation of the tooth germ have been established through a series of delicate and complicated interactive processes, the dentin- and enamel-forming cells are set to embark on conceiving their respective mineralized tissue products. This review concentrates on the formation of dentin, dentinogenesis, with the focus set on the important role of the odontoblast and its specific activities concerning calcium ion transport and mineral induction.

The processes leading to the fully differentiated tooth germ are the subjects of other reviews in this volume (Ruch *et al.*, 1995; Slavkin *et al.*, 1995), whereas the structure and formation of dentin have been recently reviewed by Linde (1992) and Linde and Goldberg (1993). For a review, focusing on the organic matrix of dentin, the reader is referred to the paper by Butler and Ritchie (1995) in this issue. A recent conference proceedings (Bronckers *et al.*, 1994), where several papers discuss aspects of dentinogenesis, can also be recommended.

Dentinogenesis

The formation of calcified tissues is a highly regulated and controlled process, in which several contributing factors are of importance. Formation of dentin is – for obvious reasons – interesting in its own right, but one should also be aware that there are other, and perhaps more important, reasons for investigating the formation of this mineralized tissue. When Nature has established a general principle for performing a task, the same principle is often adapted to suit other, but similar, applications. A detailed knowledge about how dentin is formed may thus, to varying extents, apply to and explain biomineralization in other tissues as well.

Specifically, dentin and dentinogenesis share many similarities with the structure and formation of another mineralized connective tissue, bone, but is in certain aspects to be preferred from an experimental point of view (Linde and Goldberg, 1993). An advantage in the study of dentinogenesis is the relative simplicity by which it is possible to obtain dentinogenically active odontoblasts for analysis without having to rely on cell culture techniques (Linde, 1972). One has thus at hand a highly specialized cell type with a well-defined function, and whose origin and differentiation are reasonably well-characterized. From the point of view of cell

Abbreviations used in this paper: BSP, bone sialoprotein; Ca-ATPase, Ca^{2+}-activated ATPase; DPP, highly phosphorylated dentin phosphoprotein; DSP, dentin sialoprotein; GAGs, glycosaminoglycans; Gla, γ-carboxyglutamate; HAP, hydroxyapatite; NCPs, non-collagenous proteins; pCa, negative logarithm of the Ca^{2+} ion activity; PGs, proteoglycans.

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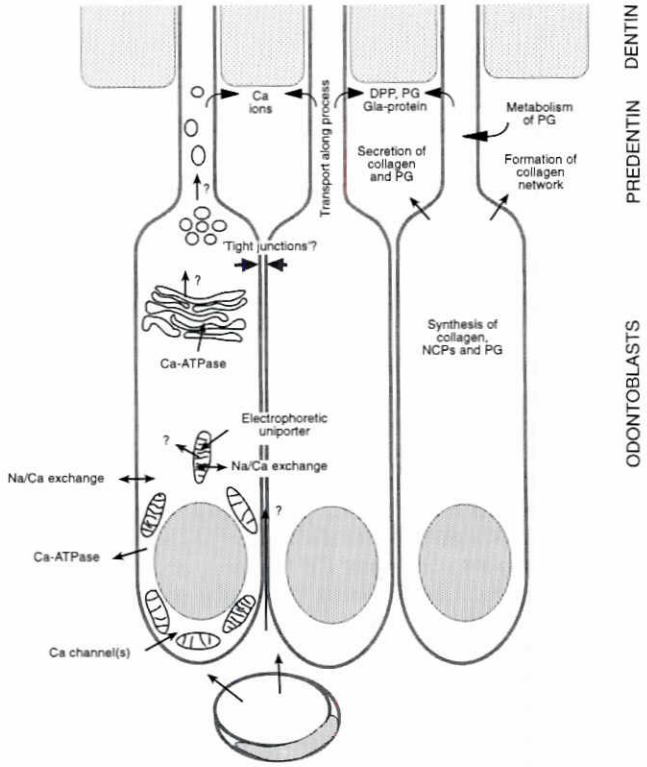

Fig. 1. Schematic drawing of fully differentiated, dentinogenically active odontoblasts showing different Ca^{2+} ion transport mechanisms (left part of figure) as well as putative transport routes for the dentin matrix macromolecules (right part of figure). *Ca^{2+} ions are transferred from serum across the odontoblast cell layer in direction of the predentin/dentin interface (the "mineralization front"). A major portion of this flow is transcellular, but the possibility exists that some Ca^{2+} is transported along an intercellular route, even though there seems to be some type of junctional complex between the odontoblasts that would prevent this. Several transmembraneous Ca^{2+} transport mechanisms have been identified in the odontoblast plasma membrane (left) and also in membranes of intracellular organelles, such as mitochondria and the endoplasmic reticulum. Still, several details are unknown with regard to the transcellular transport mechanisms, a major question being by which mechanism Ca^{2+} ions are extruded from the odontoblasts to the site of mineral formation. The proteins destined for the organic matrix (with, possibly, a few exceptions) are synthesized within the odontoblast cell bodies (right). Collagen and one pool of proteoglycan (PG) are secreted proximally to the cell bodies to form predentin. In the predentin zone, collagen fibrillogenesis occurs, and the collagenous network matures. There seems also to be a metabolism of the predentin PG pool. A major portion of the non-collagenous macromolecules, including phosphoprotein (DPP), Gla-protein and a second PG pool, is putatively transported along the odontoblast process and exocytosed just in advance of the mineralization front. Some of these may function as heterogeneous nucleators for hydroxyapatite mineral formation, but may also act as regulators of mineral crystal size and formation rate.*

biology, it is thus possible to make fruitful and detailed comparisons with other cell types from non-calcifying tissues. Another important point is that dentin does not participate in calcium homeostasis in the organism, and gross resorption of dentin by cells does not occur (except during deciduous tooth shedding). Still another advantage for dentinogenesis as an experimental system is the distinct morphology and the high rate of primary dentinogenesis.

During dentinogenesis, calcium and phosphate ions are transferred from the vascular bed proximally to, and to a certain extent between, the odontoblasts into the organic matrix of dentin in order to be incorporated into dentin mineral (Fig. 1). This *mineral phase* comprises, in principle, crystals of *hydroxyapatite* (HAP) $[Ca_{10}(OH)_2(PO_4)_6]$. The mineral has, however, a non-perfect crystallinity, is somewhat calcium-deficient, and contains other ions such as carbonate and fluoride (Posner and Tannenbaum, 1984). The mineral crystals in dentin are associated with the collagenous matrix, in that they are largely arranged with their c-axes parallel with the collagen fibers.

Hence, the role of odontoblasts in the transport of calcium ions during dentin formation and the detailed mechanisms involved will be the focus of review, as will the putative mechanisms for mineral induction.

Mineral formation in mantle dentin

During mineralization of most calcified tissues, a layer of cells forms an organic matrix in which an inorganic calcium phosphate is deposited in the form of mineral crystals. Dentin is produced by *odontoblasts*, which line the pulpal aspect of dentin during its formation. *Primary dentin* constitutes the predominant quantity of the dentin mass, and is the tissue formed at a relatively high rate during odontogenesis. Its most peripheral layer, the *mantle dentin*, is the zone beneath the enamel where dentin mineralization is initially started. In general, *de novo* mineralization, when there is no prior mineral in the tissue, seems to require specific utensils, and several observations indicate that the mechanisms involved in mantle dentin formation differ from those at later stages.

During mantle dentinogenesis the odontoblasts have not yet attained their full degree of differentiation and are not yet closely apposed (Jones and Boyde, 1984). Ultrastructural evidence indicates that initial crystal formation during dentinogenesis takes place inside membrane-bound so-called *matrix vesicles*, 0.1-0.2 μm in diameter, derived from odontoblasts (Bonucci, 1984). Only after this stage can mineral be seen in association with the collagen fibers in the tissue. Thus, matrix constituents seem to be of a limited functional importance at this stage and, even though only scant information can be found in the literature that specifically attempts at describing the constituents of the organic matrix of mantle dentin, its composition seems to differ from that of circumpulpal dentin. Specifically, the highly phosphorylated *dentin phosphoprotein* (referred to as DPP, PP-H, HP, or phosphophoryn by different authors) is reportedly absent from mantle dentin (Nakamura *et al.*, 1985; Takagi and Sasaki, 1986; Rahima *et al.*, 1988).

Whereas matrix vesicles have been observed in rather small numbers at the initial stages of dentin and bone formation, similar vesicles are abundant during cartilage calcification. The mechanisms for formation of mineral within such vesicles is not understood yet, but it is generally held that these vesicles may provide a protected micro-environment for crystal induction, and that they may function as vehicles for nucleating macromolecules, enzymes, and other essential components (Bonucci, 1984). The experimental difficulties associated with investigating such vesicles have contributed to the fact that their importance in dentin formation is not undisputed. At later stages, during circumpulpal dentin formation, such vesicles are not seen.

Circumpulpal dentin formation

As far as is known, the odontoblasts are alone responsible for the further formation of dentin, circumpulpal dentinogenesis, including the production of its constituents (with the exception of a few minor protein components derived from serum) and controlling its mineralization.

During this process, a layer of *predentin* is present between the odontoblasts and the mineralized dentin (Fig. 1). This comprises a non-mineralized organic matrix, primarily collagenous in nature and 10-40 μm in width. Circumpulpal dentinogenesis, like osteogenesis, occurs by two simultaneous processes: formation of the organic predentin, adjacent to the odontoblasts, and its subsequent mineralization at the so-called *mineralization front* (Linde, 1985). The time-lag between the two, morphologically displayed as the width of predentin, represents the time needed for arranging the collagen molecules in the form of a fibrous web. Predentin is thus a zone for the formation and maturation of the collagen scaffolding of the dentin matrix. Predentin differs in composition from the dentin matrix, showing that some components are added at or just in advance of the mineralization front, whereas others are metabolized (Linde, 1989).

The dentinogenically active odontoblasts are columnar with the characteristics of actively synthesizing and secreting cells; they have a well-developed granular endoplasmic reticulum, many mitochondria, a prominent Golgi apparatus, and numerous secretory vesicles derived from the Golgi (Jones and Boyde, 1984). Odontoblast processes, penetrating the predentin into dentin, are maintained within dentinal tubules by the centripetally migrating odontoblasts upon their production of dentin. These processes lack major organelles but have an abundance of longitudinally arranged filaments and microtubuli (Garant, 1972). The odontoblasts are attached to one another by junctional complexes, especially pronounced at the distal end of the cell body close to the predentin (Boyde *et al.*, 1978; Jones and Boyde, 1984). The presence of gap junctions between adjacent odontoblasts implies an intercellular communicatory capacity throughout the odontoblast cell layer.

Calcium distribution and transport

During dentin formation, Ca^{2+} ions are transferred from the vascular network across the odontoblast cell layer to be incorporated in the mineral phase at the interface between the non-mineralized predentin and the mineralized dentin (Fig. 1). Until quite recently, little detailed knowledge existed about this process. Now, it seems clear that odontoblasts play a central role in this ion transport, in that the Ca^{2+} ions are transported through the cells themselves by different transmembraneous ion-transporting mechanisms. Still, the relative importance at the cellular level of these mechanisms is not known, nor are the putative regulatory systems involved understood.

One important effect of the central role of the odontoblasts in this respect is that these cells seem to be in control – at least to a large extent – of the extracellular ionic milieu in the predentin zone. When contemplating mechanisms for mineral induction and growth during dentinogenesis, it is thus important to realize that the mineralization front is most probably not in a direct ionic equilibrium with the other body fluids, including serum.

The presence of calcium in odontoblasts and predentin has in the past mainly been displayed by techniques which quantify total calcium. Calcium is accumulated in the distal cell body as well as in the odontoblast process, and deposits of calcium within organelles have been shown at these sites (Reith, 1976; Boyde and Reith, 1977; Appleton and Morris, 1979). In rat incisor predentin, an extracellular enrichment of calcium and phosphorous has been demonstrated, indicative of their active accumulation, and it was suggested that the calcium is complexed to some predentinal macromolecule (Nicholson *et al.*, 1977). Systematic measurements along the dentin mineralization front revealed regions with a considerably higher calcium content.

When interpreting this type of data, one should realize that they describe the local accumulation of total calcium. To understand the dynamic handling of Ca^{2+} ions in the tissue, and when discussing mineral inductory mechanisms, *Ca^{2+} ion activity* is a more relevant parameter.

By the use of calcium-specific micro-electrode methodology, the Ca^{2+} ion activity extracellularly in predentin, as measured *in situ* in rat incisors, has been shown to be significantly higher than in the dental pulp (pCa 2.9 compared to pCa 3.4) from the same teeth (Lundgren *et al.*, 1992). The three times higher calcium activity in predentin shows that Ca^{2+} ions are, in fact, concentrated across the odontoblast layer in the direction of the mineralization front.

To obtain a dynamic picture of calcium influx during dentin formation, several authors have injected isotopes of calcium that can be detected in tissue sections after different time intervals. Earlier results from autoradiography in combination with $^{45}Ca^{2+}$ have given quite conflicting results as to the time it takes for Ca^{2+} ions to migrate from serum into dentin mineral, and evidence for both an intracellular and an intercellular transport route through the odontoblast layer were found (for review, see Linde and Lundgren, 1990). It seems clear, though, that the difference in results may – at least to a large extent – be explained by the use of techniques that could not adequately prevent a redistribution of the small Ca^{2+} ions in the tissue during processing.

More recent experiments, taking this problem into account, support a Ca^{2+} transport route through the odontoblasts and along

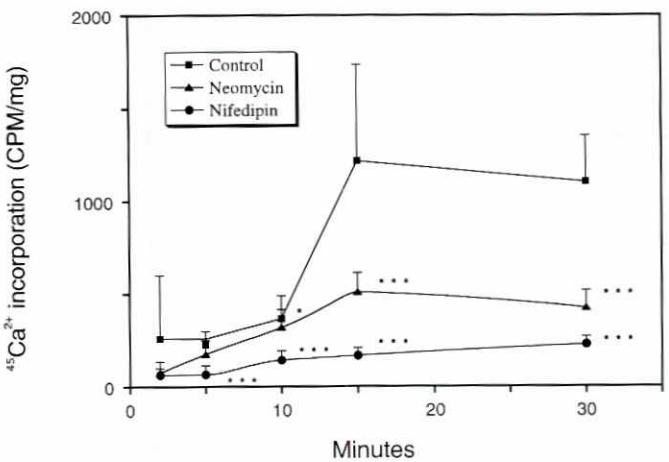

Fig. 2. Rate of incorporation of calcium into the rat incisor dentin mineral phase. *The time it takes after an i.v. injection of $^{45}Ca^{2+}$ for the isotope to reach dentin is approximately 10 min (■). When rats were treated with the calcium channel blockers nifedipine (●) or neomycin (▲) for 4 d, the radioactive incorporation was significantly reduced, showing the importance of calcium channels of the L-type for ion transport to the dentin mineral phase. Mean values ± S.D. (n= 4). From Lundgren and Linde (1992).*

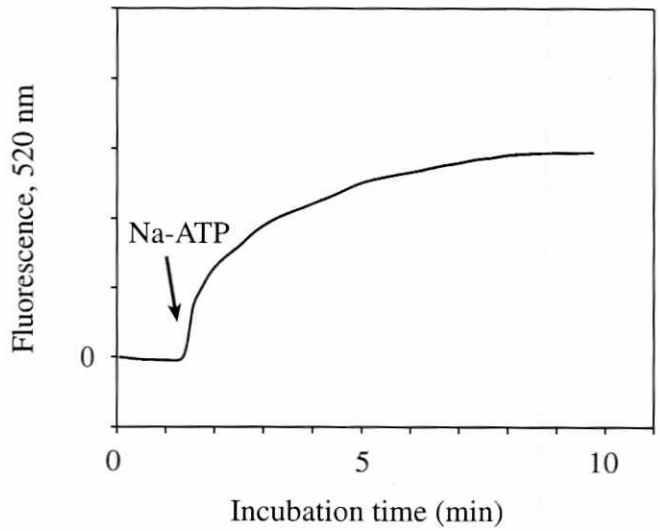

Fig. 3. Extrusion of Ca^{2+} ions from dissected rat incisor odontoblasts. *When Na^+ ions and ATP were simultaneously added in vitro (arrow) to odontoblasts, pre-loaded with calcium, the cells extruded Ca^{2+} ions at a rapid rate, due to the presence of both Na^+/Ca^{2+} antiports and a Ca^{2+}-activated ATPase in the plasma membrane. Fluorescence measurement, using chlorotetracycline as a calcium probe. The fluorescence, expressed in arbitrary units, was measured at 520 nm with an excitation wavelength of 320 nm. From Lundgren and Linde (1988).*

their processes to the mineralization front (Fig. 1). These studies, where ^{45}Ca has been assayed by autoradiography or liquid scintillation, and the non-radioactive isotope ^{44}Ca by secondary ion mass spectroscopy, showed that the time needed for mass calcium ion transfer from blood into mineralized dentin is approximately 10 min (Fig. 2) (Lundgren and Linde, 1992; Lundgren *et al.*, 1994).

The odontoblasts form a continuous cell layer, but views differ whether the intercellular spaces in this layer are freely permeable to mineral ions or not. La^{3+} ions and larger molecules have been used as tracers in combination with transmission electron microscopy to resolve this question (Tanaka, 1980; Bishop, 1985; Martineau-Doizé *et al.*, 1986; Uchida *et al.*, 1987; Bishop and Yoshida, 1992). It seems probable that the irreconcilable results obtained by different investigators may be explained by technical factors, for example fixation and demineralization methodologies. It is thus possible that Ca^{2+} ions may be transported both transcellularly through the odontoblasts into predentin as well as between them, even though the former seems to be quantitatively predominant (Lundgren and Linde, 1992). It seems clear, though, that mechanisms exist for concentrating Ca^{2+} in the odontoblast-predentin region prior to extracellular mineral formation.

Odontoblast Ca^{2+} ion chaperonage

A large number of studies have been devoted to the handling of Ca^{2+} ions in different cell types. Many of these discuss Ca^{2+} as a second messenger, while studies of Ca^{2+}-transporting systems in cells have primarily dealt with mechanisms to maintain a low, steady-state Ca^{2+} ion activity in the cytosol. Thus, a number of membrane-associated Ca^{2+}-translocating systems with different properties have been identified and characterized in the plasma membrane as well as in the membranes of cell organelles. Some of these systems are designed for slower movements of bulk amounts of Ca^{2+}, whereas others are capable of a precise and rapid adjustment of Ca^{2+} concentrations. Little is, however, known about how a unidirectional, transcellular bulk flow of Ca^{2+} ions occurs, as would be needed in odontoblasts during dentinogenesis.

The total calcium concentration in extracellular fluids, including blood plasma, is around 3 mM, about half of which or less is in ionized form. Cytoplasmic Ca^{2+} activities are regularly in the micromolar range or below. A Ca^{2+} concentration gradient, more than three orders of magnitude, thus exists over the plasma membrane. Even though this is the case, cells usually do not let Ca^{2+} ions diffuse freely across their plasma membrane. Instead, Ca^{2+} ion influx is monitored through so-called Ca^{2+} channels.

By means of fluorescence spectrometry, uptake of Ca^{2+} in dissected odontoblasts was found to be enhanced by the dihydropyridine BAYK-8644 as well as by plasma membrane depolarization, indicating the presence of *Ca^{2+} channels of the L-type* (Lundgren and Linde, 1995). Furthermore, dissected rat incisor odontoblasts exhibited a pronounced fluorescence, when incubated with a fluorescently labeled dihydropyridine, specific for this type of Ca^{2+} channels; this binding was competitively abolished by nifedipine. The presence of Ca^{2+} channels of the L-type in odontoblasts has also been demonstrated by immunohistochemical technique (Seux *et al.*, 1994).

The pivotal importance of odontoblast Ca^{2+} ion transporting channels during mineral formation *in vivo* was shown by the finding that the transport of Ca^{2+} ions into the mineral phase of dentin in rats was strongly impaired by specific blocking of L-type channels, using nifedipine and neomycin (Fig. 2) (Lundgren and Linde, 1992). From these studies it can be concluded that Ca^{2+} flux into dentinogenically active odontoblasts is principally gated through specific Ca^{2+} channels of the L-type, but that also other Ca^{2+} channel types may be present.

In order to maintain a low cytosolic Ca^{2+} ion activity, the odontoblast plasma membrane also bears transport mechanisms designed to extrude Ca^{2+} ions, as do other cell types. The high activity of a Ca^{2+}-activated ATPase (Ca-ATPase), distinct from non-specific alkaline phosphatase, was demonstrated two decades ago in rat incisor odontoblasts (Linde and Magnusson, 1975; Granström and Linde, 1976; Granström *et al.*, 1979), and this

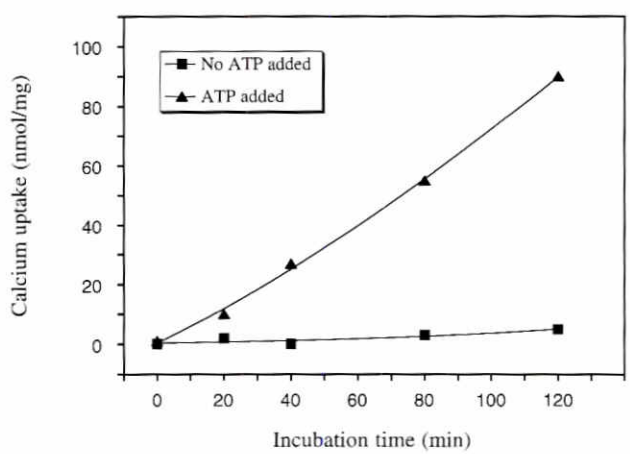

Fig. 4. Uptake of Ca^{2+} ions with time in microsomal fraction of dissected rat incisor odontoblasts. *Addition of ATP strongly increased the rate of uptake, demonstrating the presence of a membrane-bound Ca^{2+}-activated ATPase ion pumping mechanism. From Granström and Linde (1981).*

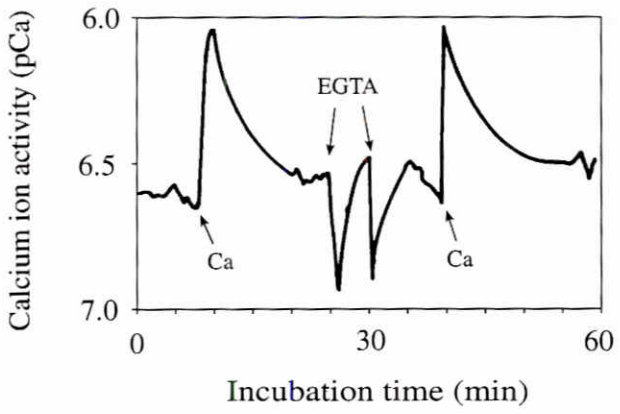

Fig. 5. Incubation of whole, dissected odontoblasts in the presence of digitonin. *This substance permeabilizes the plasma membrane to Ca^{2+} ions, thus equilibrating the Ca^{2+} ion activities intracellularly with those extracellularly. Ca^{2+} activity measurements outside the cells thus reflect the cytoplasmic activity. The cells were incubated in a 37°C measurement chamber of 500 µl volume, and Ca^{2+} activity was measured with an ion-specific mini-electrode. It can be seen that the intracellular organelles — in this case without the assistance of the plasma membrane transport systems — stabilizes the cytosolic Ca^{2+} activity at a pCa around 6.6. This level was restored even after repeated additions of Ca^{2+} ions or removal of Ca^{2+} by additions of EGTA. From Lundgren and Linde (1987).*

enzyme together with Na^+/Ca^{2+} antiports (Lundgren and Linde, 1988), where 2 Na^+ ions are exchanged for 1 Ca^{2+} ion, collaborate to free the cells from Ca^{2+} ions (Fig. 3). Furthermore, intracellular organelles also have a role to play in the cytosolic buffering of Ca^{2+} activity. Rat odontoblast mitochondria, isolated by subcellular fractionation methodology, have an *electrophoretic uniporter* mechanism for their uptake of Ca^{2+} ions. A Na^+/Ca^{2+} exchanger, aimed at mitochondrial Ca^{2+} extrusion, has also been demonstrated (Lundgren and Linde, 1988). A Ca-ATPase seems to be located also in the membranes of intra-odontoblastic vesicles, derived from the Golgi and possibly being part of the endoplasmic reticulum (Granström *et al.*, 1978; Lundgren and Linde, 1988). An ATP-dependent intravesicular accumulation of Ca^{2+} ions has been demonstrated in rat incisor odontoblast microsomal fractions (Fig. 4), showing the involvement of a transport Ca-ATPase (Granström and Linde, 1981; Granström, 1984; Lundgren and Linde, 1987).

For the analysis of intracellular calcium homeostasis, measurements with ion-specific electrode technique as well as fluorescence measurements, primarily using Fura-2 as a calcium probe, have been performed. Using these techniques, it has been shown that odontoblasts, through the interplay between the different transmembraneous Ca^{2+} ion transport mechanisms discussed above (Fig. 1), are capable of maintaining an intracellular steady-state pCa level in the range 6.4-6.6 (Lundgren and Linde, 1987). Estimation of Ca^{2+} influx/efflux cycling has been possible to accomplish in suspensions of odontoblast microsomes and mitochondria, as well as in whole cells (Lundgren and Linde, 1987, 1988). The steady-state free Ca^{2+} activities maintained by mitochondria and microsomes were determined to pCa 6.2-6.4 and pCa 6.4-6.6, respectively, these levels being buffered upon repeated additions of Ca^{2+} and EGTA. Such a buffering capacity at these pCa levels was further demonstrated in incubations of whole odontoblasts (Fig. 5) (Lundgren and Linde, 1987).

The free, ionized Ca^{2+} (which is quantitated by *activity* measurements) constitutes the easily mobilized portion, the portion of importance for considerations about transport modes and biomineralization mechanisms. In cells in general, a large portion of the intracellular calcium is not in free form but is complexed. Little is known, however, about intracellular organic ligands, capable of complexing, storing, and perhaps shuttling, larger amounts of Ca^{2+} in odontoblasts.

Of the vitamin D-dependent Ca^{2+}-binding proteins, it seems clear that the *9 kDa calbindin* is absent from odontoblasts (Berdal *et al.*, 1991, 1992), whereas data on the *28 kDa calbindin* (calbindin-D_{28K}) are divergent. Some authors have not been able to demonstrate its presence in odontoblasts (Taylor 1984; Elms and Taylor, 1987; Berdal *et al.*, 1991), although other findings imply that it is in fact expressed in these cells (Magloire *et al.*, 1988; Berdal *et al.*, 1992). The Ca^{2+}-binding protein *parvalbumin* could not be demonstrated in odontoblasts by Celio *et al.* (1984), nor has it been possible to identify it in the dentin matrix (unpublished observation). On the other hand, more recent results indicate its presence in odontoblasts (Davideau *et al.*, 1993). Finally, some of the *annexins* seem to be present in odontoblasts (Goldberg *et al.*, 1990). It must be inferred that the exact role, if any, of such Ca^{2+}-binding proteins in dentinogenesis is not understood.

It can be concluded that the intracellular transport route for Ca^{2+} ions seems to be the predominant one during dentinogenesis, even though intercellular pathways may be of some importance too (Fig. 1). Ca^{2+} channels of the L-type apparently have a special role in the cellular uptake of Ca^{2+}. Several Ca^{2+}-transporting systems, known from other non-calcifying cell types, have been identified in odontoblasts, and these are of importance in controlling the intracellular Ca^{2+} homeostasis. A major question, and for which there is no answer at present, is where and by which mechanisms Ca^{2+} ions are extruded from the cells in the direction of the mineralization front.

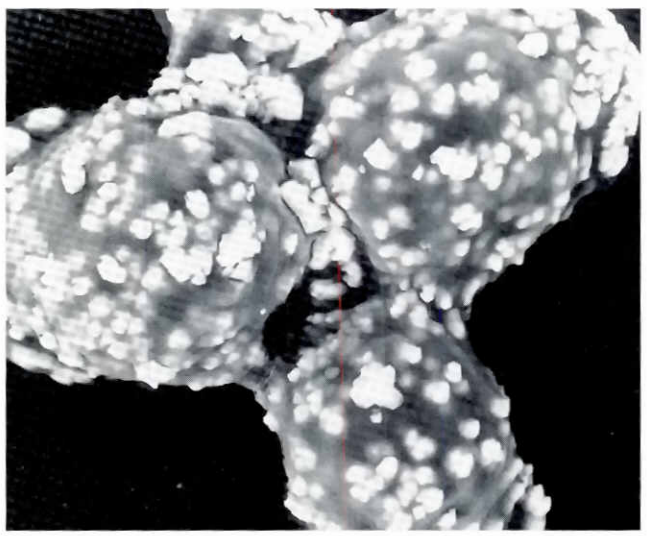

Fig. 6. Mineral induction by dentin phosphoprotein (DPP) at physiological ion concentrations *in vitro*. *DPP was immobilized on agarose beads (8 mg ligand/g dry beads) and incubated for 24 h at a [Ca]x[P] product of 1.4 mM^2. A strong induction of crystals can be seen (back-scattered electron imaging) both on and within the beads. The mineral can be identified as HAP by x-ray diffraction (Luusi et al., 1988).*

Extracellular mineral formation

The dentin matrix contains macromolecular constituents characteristic of many connective tissues and, in addition, some components specific for mineralized tissue, even for dentin itself (Linde, 1989). The organic matrix of circumpulpal dentin is synthesized and deposited extracellularly by the odontoblasts prior to mineral formation. Thus, a primary function of the organic matrix is to determine the three-dimensional morphology of dentin.

It is generally held that induction of the mineral crystals during biomineralization is facilitated by a mechanism referred to as *heterogeneous nucleation*. This term implies that there are extracellular matrix constituents which carry chemical groups with electrical charges or other properties that are arranged in a specific stereochemical way, mimicking certain crystal planes of the crystal phase to be formed. By this means, the energy barrier is lowered so that a solid phase, in the case of dentinogenesis, of calcium phosphate is formed from an otherwise stable solution. The mineral nuclei formed at these *nucleation sites* eventually grow and fuse to mineral crystals.

In addition, much evidence indicates that the mineralization inhibitory properties of molecules, and the removal of such molecules from the site of mineral formation, are of decisive importance. Such molecules may be the macromolecular constituents of the organic matrix, but smaller, phosphate-containing molecules have also been discussed in this context.

It is thus obvious that, in order to understand mineral formation in a tissue like dentin, a detailed knowledge of its matrix constituents is a prerequisite. Investigations during the last two decades have also provided us with much of this knowledge.

The dentin organic matrix

A general scheme during the formation of a calcified tissue is the prior existence of an extracellular macromolecular framework before mineral is formed. The organic matrix of circumpulpal dentin is, with the exception of a minor serum protein content, produced entirely by the odontoblasts; the connective tissue of the dental pulp is quite different in composition. As is the case in bone, almost 90% of the organic matrix in dentin is *collagen*, whereas the remainder consists of *non-collagenous proteins* (NCPs), including *proteoglycans* (PGs), with primarily an anionic character (Linde, 1989). In addition, some lipid-containing components exist in the dentin matrix, but details about their structure are scarce (Wuthier, 1984).

As it is not the aim of this review to describe the dentin organic matrix, but to provide a basis for the following discussion about mineral induction and growth, a brief summary will be given, and some matrix constituents which may be of specific importance will be mentioned. For a more in-depth treatise of the dentin organic matrix, the reader is referred to Linde (1989) and Butler *et al.* (1995).

A first important aspect is that in terms of dentin NCP constituents, there seems to be a clear demarcation line between two groups of dentin species, one comprising human and bovine teeth, the other comprising rodent teeth (Linde 1988, 1989). The former group seems, for example, to be totally devoid of γ-carboxyglutamate-containing proteins of the *osteocalcin* type, which constitute a major NCP fraction in rodent dentins (and in bone from all species). Such differences provide additional hints as to specific functions of the respective NCPs. Whether there are also differences in collagen composition between the two groups is not known. Another noteworthy observation is that the relative content of NCPs may differ within the same tooth (Takagi *et al.*, 1988; Steinfort *et al.*, 1989).

Dentin *collagen* is mainly of *type I*, as in bone, and it forms a dense, covalently cross-linked, insoluble network in which the mineral crystals are deposited. Odontoblasts are also known to synthesize *type V* collagen (Sodek and Mandell, 1982), and its presence in dentin has been demonstrated by immunohistochemical technique (Bronckers *et al.*, 1986). As to the presence of collagen types III and VI, the situation is not entirely clear. An as yet little understood observation is that a large amount of the collagen synthesized by rat and mouse odontoblasts seems to be of the *type I trimer*, $[\alpha 1(I)]_3$ (Munksgaard *et al.*, 1978; Lesot, 1981; Sodek and Mandell, 1982).

Operationally, the NCPs and PGs of dentin may be divided into three categories. In general, the NCPs and PGs in dentin are so strongly associated with the mineral phase in the tissue that they are extractable only after or simultaneously with demineralization (Linde *et al.*, 1980). A minor PG fraction is, however, extractable from the intact tissue. On the other hand, while the quantitatively predominant NCP portion is extracted upon demineralization, some components like the *matrix Gla protein* (MGP) and *bone morphogenetic proteins* (BMPs) need additional procedures to be solubilized.

The predominant NCP fraction in all dentin species comprises the highly phosphorylated *phosphoproteins* (variably denoted DPP, PP-H, HP or phosphophoryn), constituting half or more of the NCP fraction. This strongly polyanionic type of protein is dentin-specific and is the most acidic protein known (Jonsson *et al.*, 1978). This is caused by its very high phosphate content, 26% in rat and 20% in bovine PP-H, as well as by its unique amino acid composition; more than 80% of the amino acid residues carry negatively charged phosphate or carboxyl groups (Linde, 1988). In addition, there are several NCPs in dentin with a lower degree of phosphorylation.

Another major matrix constituent in dentin is *proteoglycan* (PG). The PGs in mineralized dentin belong to the class of small PGs with only one or two GAG side chains, referred to as *decorin* and *biglycan*, respectively (Rahemtulla *et al.*, 1984). The GAG side chains are primarily *chondroitin-4-sulfate* and *chondroitin-6-sulfate*. In addition, small amounts of a highly hybridized *dermatan sulfate* seem to be present (Hjerpe *et al.*, 1983).

The DPP and PGs seem at present to be the matrix constituents that are primarily implicated in mineral induction and growth regulation during dentinogenesis and for which there are experimental evidence. However, other NCPs, such as the *bone sialoprotein* (Chen *et al.*, 1993) and *dentin sialoprotein* (Butler *et al.*, 1992) may also have some role in this process. The possible role of *serum proteins*, such as albumin, present in the dentin matrix, should not be overlooked in this context.

Predentin vs. dentin

As we have seen above, the odontoblasts perform two simultaneous processes during circumpulpal dentin formation: the synthesis and secretion of the dentin organic matrix in the form of predentin just distal to the cell bodies, and the mineralization of this matrix at a certain distance away (Fig. 1).

The dentinogenesis experimental system provides an advan-

tage, in that it is possible to dissect out the predentin zone for biochemical analysis (Linde, 1973). This yields valuable data to add to the information obtained by immunohistochemical methodology. When such analyses are made, it turns out that some constituents of dentin are not present in predentin, and *vice versa*. In addition to collagen type I, predentin contains PG, a small quantity of protein with a low degree of phosphorylation and, possibly in some species, *osteonectin/SPARC*. Specifically, DPP and Gla-proteins of the osteocalcin type are absent from predentin (Jontell and Linde, 1983). Several sulfated PG fractions with different metabolic characteristics seem to exist, one pool being incorporated fairly rapidly into mineralized dentin, while the other pool remains in predentin and is presumably being metabolized there (Sundström, 1971) (Fig. 1).

A main reason for the fact that the composition of the mineralized dentin matrix is different from that of predentin thus seems to be that some macromolecular components, such as DPP, PG and Gla-protein, are added at or just in advance of the mineralization front, presumably subsequent to an intracellular transport along the odontoblast process followed by exocytosis (Linde, 1985) (Fig. 1). An obvious subject for research is thus the role of these NCPs in the main process occurring at this tissue level, the induction and growth of mineral crystals.

It is known that, in addition to inhibiting mineral formation, PGs may influence extracellular collagen fibrillogenesis. It may thus be that the predentin PG has some regulatory function in collagen fibril formation while at the same time preventing mineral to form in this zone. Since the highest concentrations of osteonectin/SPARC in non-mineralized tissues are observed in collagen-rich tissues, it may be that also this protein has some role in organizing the extracellular matrix.

Mechanisms of dentin mineralization

When calcium phosphate minerals form, the type of crystals and their rate of formation are highly dependent on variables such as pH, the concentrations of calcium, phosphate and other physiological ions, as well as on the presence of charged macromolecules.

Using ion-specific micro-electrode technique, pH at the dentin mineralization front within rat incisor predentin *in situ* was determined to 7.0 (Lundgren *et al.*, 1992). Thus, it seems safe to conclude that the conditions at the site of mineral formation in dentin are within the normal pH range.

Since HAP crystals in dentin and bone are seen in a specific relationship to collagen fibers, much interest has been focused on the properties of collagen from mineralized tissues in this respect. Chemically, these collagens differ very little from soft tissue ones, the major difference being in their crosslinking geometry (Mechanic *et al.*, 1987). It has thus been suggested that the non-reducible pyridinoline crosslinks are present exclusively in the tissue fraction of collagen that is not mineralized; mineralization would physically inhibit formation of pyridinoline and, conversely, pyridinoline may inhibit mineralization by firmly stabilizing a shortened intermolecular distance between the collagen chains (Mechanic *et al.*, 1985). The finding that there is a five times higher content of pyridinoline in mineralized dentin compared to predentin does not support this hypothesis (Linde and Robins, 1988).

Most authors seem to favor the notion that pure collagen in itself is not efficient as a mineral nucleator, even though the initial mineral nuclei during calcification seem to be formed on/in the collagen fibers, in the so-called hole zones (Höhling *et al.*, 1990). Collagen would rather function as a means for orientation and a stable support for other macromolecules, such as NCPs and PGs, and the mineral crystals (Glimcher, 1989).

Instead, the NCPs and PGs have been the focus of interest as candidates for the induction and regulation of mineral formation, the major reasons for this being their Ca^{2+} ion binding capacity and their specific chemical properties, enabling them to influence mineral induction and growth *in vitro*. Phosphorylated proteins have drawn attention for a long time, and lately interest has also been focused on polycarboxylated proteins.

As a matter of fact, direct evidence that dentin and bone NCPs and PGs have the capacity to function as heterogeneous nucleators for apatite at physiological ionic conditions has been provided only recently. On the other hand, many experiments in the past have shown NCPs and PGs to be inhibitory to mineral induction and growth. It turns out that polyanionic macromolecules such as dentin NCPs and PGs, in minute quantities, are capable of inducing apatite at physiological calcium and phosphate ion concentrations, provided these ligands are immobilized by a solid support, whereas when they are in solution, they are inhibitory (Lussi *et al.*, 1988; Linde *et al.*, 1989; Linde and Lussi, 1989; Lussi and Linde, 1993). *In vivo*, this support would presumably be the matrix collagen.

As pointed out above, DPP and some PG obviously bypass predentin and are transported directly to the site of mineral formation (Sundström, 1971; Weinstock and Leblond, 1973; Jontell and Linde, 1983). Gla-proteins of the osteocalcin type can be localized inside odontoblast processes, also suggesting a direct intracellular transport (Linde and Hansson, 1983; Gorter de Vries *et al.*, 1988). The finding that bovine dentin, in contrast to rat dentin, is virtually devoid of Gla-protein (Linde, 1988) makes it, however, difficult to envision any specific role for this protein in the mineralization process.

At relatively high concentrations in metastable solutions *in vitro*, DPP may influence the conversion of amorphous calcium phosphate to HAP and inhibits crystal growth (Nawrot *et al.*, 1976; Termine and Conn, 1976; Termine *et al.*, 1980). The strong affinity binding of Ca^{2+} ions to DPP, with the ions not localized but highly mobile on the surface of the molecule, as revealed by NMR technique, has been suggested to facilitate Ca^{2+} ion distribution at the mineralization front, which would be of significance for HAP formation (Cookson *et al.*, 1980; Linde, 1984).

The first experimental evidence that dentin NCPs can be inductory to mineral formation was the finding that PG and DPP, covalently linked to agarose beads, were capable of inducing an apatitic phase under *in vitro* conditions, similar to those *in vivo*, where spontaneous mineral precipitation would otherwise not occur (Fig. 6) (Lussi *et al.*, 1988; Linde *et al.*, 1989). To inhibit this, it took several orders of magnitude higher concentrations of DPP in solution. It was also shown that DPP and PG from rat incisor dentin, immobilized on agarose beads, can induce mineral under actual *in vivo* conditions (Lussi and Linde, 1993).

Experiments were also performed with collagenous matrices. Tendon collagen type I did not induce any mineral in the *in vitro* system, but when DPP was covalently coupled to the collagen, mineral formation occurred (Linde and Lussi, 1989). No mineral was found to be induced by dissected bovine predentin *in vitro* whereas, in contrast, thoroughly demineralized and extracted dentin (which still contains minute amounts of DPP) was capable of mineral induction. The same respective properties of predentin and dentin in relation to mineral induction were displayed when tested *in vivo* (Lussi and Linde, 1993).

Other authors have provided further information as to the mineral-inductive properties of DPP in various *in vitro* systems. It has been shown that DPP does not necessarily need to be immobilized by covalent attachment to a solid substratum in order to induce mineral. In a gelatin gel system, DPP at concentrations below 1 μg/ml was found to promote HAP formation, whereas high DPP concentrations were inhibitory (Boskey *et al.*, 1990). DPP, immobilized on agarose beads, has also been shown to be able to also induce an octacalcium phosphate-like phase (Doi *et al.*, 1993).

The anionic, phosphorylated *bone sialoprotein* (BSP) has been found to induce HAP in a steady-state agarose gel system (Hunter and Goldberg, 1993). It was shown that enzymatic dephosphorylation had no effect on this capacity, whereas chemical blocking of carboxyl groups was adverse. These findings may be of relevance also for dentin mineralization, since this tissue contains a protein related to the BSP, the 53 kDa *dentin sialoprotein* (DSP) (Butler *et al.*, 1992).

When interpreting the results of the experiments described above, it must, however, be observed that some of those seem to have been performed at [Ca]x[P] products higher than what may be within the physiological range.

Taken together, it can be concluded that polyanionic NCPs, such as DPP, may be instrumental in the nucleation of mineral during dentinogenesis. From a quantitative point of view, the minor fraction of DPP, strongly associated with dentin collagen, would suffice for this purpose. It may also be that the major DPP fraction, the one that is easily extracted upon demineralization of the tissue, is immobilized well enough by the collagen meshwork so as to attain a mineral-inductive capacity. Other candidates also exist as putative mineral inducers during dentinogenesis, such as PG and DSP.

An additional possibility is that polyanionic NCP molecules might function as regulators of mineralization rate and crystal size. It may be, for example, that the DPP first released into the matrix could induce the formation of the mineral crystals, while an additional accumulation of DPP could participate in the regulation of the extent of crystal formation (Boskey *et al.*, 1990). In this context, Hunter *et al.* (1994) have recently shown that the HAP crystal-modulating properties of *osteopontin* to a large extent reside in its phosphate groups, but that carboxyl groups are of importance too.

The fact that different strongly polyanionic proteins, for example *phosvitin*, are capable of mineral induction *in vitro* (Linde *et al.*, 1989), demonstrates that mineral induction by polyanions is a relatively non-specific process. In contrast, the results of Hunter *et al.* (1994) showed that inhibition of HAP formation is not a simple polyelectrolytre effect. In any case, the different findings in the literature imply that other regulating factors, such as the odontoblast-controlled transport of mineral ions, as well as a compartmentalization in the tissue, are of significance as well for the process of mineral formation during dentinogenesis.

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