Calbindin Independence of Calcium Transport in Developing Teeth Contradicts the Calcium Ferry Dogma*

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Mechanistically, active transport is considered in three steps: the entry of calcium to the cell, transit across it, and extrusion at the other side. The transit step has received the most attention over several decades, being considered rate-limiting and having key molecular players defined. However, recent molecular characterization of calcium entry channels has transformed the field by providing a new mechanistic focus for vitamin D-restricted transport (3, 4). With these advances re-igniting interest in therapeutic applications, it is important to revisit what happens following calcium entry.

The 30-year-old paradigm that calcium is ferried through cytosol by mobile calcium-binding proteins (calbindins) remains widely accepted (3–9). Calbindins are thought to facilitate the naturally poor diffusion of calcium in cytosol and simultaneously buffer calcium at safe concentrations. Comprehensive supporting this view, tight correlations between calbindin expression and vitamin D-dependent transport were found in intestine and kidney, and mathematical modeling verified that calbindin could boost the self-diffusion rate of calcium to the required extent (70-fold). This mechanism appears robust and flexible since all major calcium-transporting tissues express either the 28- or 9-kDa type of calbindin (1, 10).

Active calcium transport is crucial for normal production of tooth enamel, and it appeared that the calbindin ferry model would apply here too. Enamel, the most highly calcified of all tissues, is formed in two stages (secretion, maturation) involving low and high rates of calcium transport, respectively. Calbindin28kDa is expressed abundantly in enamel-forming cells in a vitamin D-dependent fashion, and calbindin9kDa is also expressed at trace levels (11). Unexpectedly, however, proteome analysis revealed that calbindin28kDa was down-regulated during maturation, whereas calcium-handling proteins from the endoplasmic reticulum (ER/Ca2+ stores)1 were elevated in parallel with calcium transport. These and other findings led us to postulate a “calcium transcytosis” mechanism whereby the ER serves as a transcellular pipeline for calcium (1, 12–14). Null mutant mice completely lacking calbindin28kDa (15) constitute a powerful tool to pursue this stimulating possibility further. These animals maintain serum calcium normally but have a distinctive neurological phenotype attributable to diminished calcium buffering in the cerebellar Purkinje cells (15, 16).

This study addressed the hypothesis that, should calbindin28kDa be crucial for active calcium transport, its absence in null mutants will either disrupt enamel mineralization or be compensated by overexpression of equivalent cytosolic buffers. Our approach involved comparative analysis of null

The active transport of calcium across cells holds widespread importance in medicine and biology, yet the underlying mechanisms remain unclear. Operating in many places (e.g. gut, kidney, placenta, teeth, bones, oviduct, lung, inner ear), active transport is used to control the amount of calcium in body fluids and so impacts on nutrition, mineralization, fertility, respiration, and hearing (1, 2). Superior control is achieved by passing calcium actively through cells rather than passively between them, but this comes at the risk of cytotoxicity should the ability to regulate intracellular calcium be overburdened.

Cytosolic calcium-binding proteins termed calbindins are widely regarded as a key component of the machinery used to transport calcium safely across cells. Acting as mobile buffers, calbindins are thought to ferry calcium in bulk and simultaneously protect against its potentially cytotoxic effects. Here, we contradict this dogma by showing that teeth and bones were produced normally in null mutant mice lacking calbindin28kDa. Structural analysis of dental enamel, the development of which depends critically on active calcium transport, showed that mineralization was unaffected in calbindin28kDa−/− null mutants. An unchanged rate of calcium transport was verified by measurements of 45Ca incorporation into developing teeth in vivo. In enamel-forming cells, the absence of calbindin28kDa was not compensated by other cytosolic calcium-binding proteins as detectable by 45Ca overlay, two-dimensional gel, and equilibrium binding analyses. Despite a 33% decrease in cytosolic buffer capacity, cytotoxicity was not evident in either the null mutant enamel or its formative cells. This is the first definitive evidence that calbindins are not required for active calcium transport, either as ferries or as facilitative buffers. Moreover, in challenging the broader notion of a cytosolic route for calcium, the findings support an alternative paradigm involving passage via calcium-tolerant organelles.

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1 The abbreviation used is: ER, endoplasmic reticulum.
Calbindin-independent Calcium Transport

Animals and Isolation of Tissues—All animal experimentation complied with national ethical and gene regulatory requirements. Transgenic mice were from a colony we established at Dunedin by crossing calbindin28kDa-null mutants (15) with local C57BL/6 wild types. Heterozygote crosses gave null mutant offspring at Mendelian frequency and with equivalent weights as wild type littermates (quantified at 5, 9, and 44 days old). Genotyping was done using a PCR procedure (19) and corroborated for all reported experiments by immunoblot analysis of brain samples using a mixture of antibodies against calbindin28kDa and calretinin (the latter providing a positive control for null mutants). Developing mandibular first molars (5 and 9 days postnatal for secretion and maturation, respectively) and soft tissues were isolated essentially as reported for rat (12, 20). For morphology, isolated hemimandibles were stripped of soft tissue using household bleach and then water-washed and air-dried thoroughly. An additional washing step with SDS was used to ensure delipidation of the whole skeletons used for chemical analysis.

Morphological and Chemical Characterization of Teeth and Bone—Hard tissues were examined just before the first molars erupted, when eruption was complete, and 1 week after weaning (days 12, 19, and 28, respectively). Macroscopic examinations were made under a dissecting microscope and by x-radiography (molybdenum source at 32 kV, Kodak Industrex SR5 film), and abrasion resistance was assessed with a dental probe and during preparation for scanning electron microscopy as for wild type. Null mutant and wild type hemimandibles were mounted pairwise in epoxy glue, and the erupted portion of both incisors was excised accurately at the bony margin using a diamond disc. The retained tooth faces were then polished (Aloxite-1200 slurry), etched (0.1% nitric acid, 3 × 10 s), coated, and examined essentially as before (21). Morphometric analysis was done on digital images (×150) using NIH Image software. For histology, 7-day-old hemimandibles were fixed by immersion in formalin, demineralized, and processed through wax (22). Skeletal chemistry was analyzed on samples (two 44-day-old males and females of each genotype) prepared for as above, powdered, weighed individually ("dry residue"), and then pooled for triplicated analyses of mineral ash, calcium, and phosphorus by a commercial laboratory (ChemSearch, Dunedin, New Zealand).

Calbindin-Independent Calcium Transport into Developing Teeth—The 45Ca transport assay was based on a novel procedure we have characterized extensively in rat, as will be reported elsewhere. In brief, mandibular first molars were isolated at various periods after intraperitoneal injection of 45Ca (20 μl of saline containing 3 μl of 45Ca; CES3 from Amersham Biosciences), and then the hard (enamel, dentine) and soft (enamel cells, pulp) tissue components were dissected apart and extracted twice under sonication with 0.2 ml HCl prior to liquid scintillation counting. Serum harvested at decapitation was extracted and quantified likewise. Since serum 45Ca decreased markedly over the first 60 min, 45Ca mineral values were normalized to a constant substrate activity (i.e. 1000 cpm 45Ca/μl serum), yielding linear progress curves for hard tissue as noted under "Results." Moreover the 3-fold increased rate seen at maturation (see Fig. G; data pooled from similar numbers of 8, 9, and 10 day olds) was close to that predicted (4-fold over secretion (1)), suggesting the 45Ca mineral was located mainly in enamel. In contrast, soft tissue uptake plateaued within 20 min, at which time it comprised ≤3% of the corresponding value for hard tissue. Protein Biochemistry—Procedures for immunoblotting, 45Ca overlay, two-dimensional PAGE, densitometric quantitation, and normalization of sample loadings by Coomassie Blue-stained SDS-PAGE were reported previously (23). Epithelial extracts from mandibular first molars were prepared in buffer containing 2% Triton X-100 as described (23) except that tissue was disrupted by repeated freeze-thawing and bath sonication. The non-denaturing 45Ca binding microassay was done on nuclease-treated EGTA-soluble extracts as before (12) using enriched cytosol released from semi-intact cells by five freeze-thaw cycles (25). Assays were done within the linear range (≤10 μl of extract, r > 0.98).

FIG. 1. Teeth and bone are structurally normal in calbindin28kDa-null mutants. Hemimandibles were examined at 28 days (A–E and G) and 7 days (F) postnatal. A–D, light microscopy (A and B) and x-radiography (C and D) showing equivalent gross morphologies and mineral densities in null mutants and wild types as indicated. Notably, enamel was radiographically normal when partially and fully mineralized (arrowheads and arrow, respectively). E, light microscopy showing normal enamel features (color, transluence, reflectiveness, unpitted surface) and crown morphologies in null mutant molars. F, null mutant incisor (cf. arrowhead in C) showing classical histological appearance of the secretory enamel epithelium (ameloblasts (AB) and stratum intermedium (SI)) and its extracellular enamel product (En). Notably, the ameloblasts exhibited intact apical extensions (Tomes' processes; asterisk) indicative of cellular health. Also visible are normal bone (Bo) surrounded by osteoblasts and dentine (De) with its formative odontoblasts (OB). During enamel mineralization, calcium passes from the highly vascularized papillary layer (PL) to be actively transported across ameloblasts into enamel. G, scanning electron microscopy showing normal microstructure in null mutant enamel, including the constituent crystals and higher order prism assemblages (corresponding to formative cells; two vertical rows shown). Scale bars are as follows: D, 2 mm; F, 50 μm; G, 3 μm.
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The molecular and perhaps morphological levels (1).

To address these possibilities, we initially compared enamel formation in calbindin28kDa-null mutants and wild type controls using a variety of conventional structural approaches (Fig. 1). Null mutant teeth (n = 12) were found to be morphologically normal by visual inspection and x-radiography (Fig. 1, A–D). Enamel in null mutants exhibited the same physical properties as controls, including high radiographic density (Fig. 1, C and D), color, translucence and reflectiveness (Fig. 1E), and abrasion resistance. Standard enamel microstructure, comprising highly ordered assemblies of mineral crystals and prisms, was also evident by scanning electron microscopy (Fig. 1G). Morphometric analysis indicated that normal total amounts of enamel mineral were produced in null mutants (≥99 ± 1% versus wild types; ± S.E., n = 4), whether being nursed on milk or consuming a solid diet (Fig. 2A). The developmental rate of mineralization was also unaltered, as evident from the incompletely formed enamel at the necks of unerupted molars (not shown). Finally, histological examination of 7-day-old hemimandibles (n = 5) showed that null mutant enamel cells retained the classical features of a columnar epithelium during secretion (Fig. 1F) and maturation. No signs of cellular distress were visible either histologically (e.g. gross cellular debris, fragmented Tomes’ processes) or in the enamel (e.g. discoloration, pitting). Collectively, by showing that mineralization was unaltered both qualitatively and quantitatively, these results implied that calcium was transported normally into null mutant enamel.

Like enamel, dentine and bone were indistinguishable when compared in null mutants and wild types using microscopy, x-ray analysis, scanning electron microscopy, and histology (Fig. 1). Moreover, chemical analysis of the skeletons showed equal contents of total mineral, calcium, and phosphate (≥98 ± 1%; n = 4), indicating that null mutants produced and retained bone mass normally (Fig. 2B).

Calcium Is Transported Normally into Developing Teeth of Calbindin28kDa-Null Mutants—To test calcium transport more directly, we developed a radiotracer assay to measure the rate at which calcium is added to developing tooth mineral in vivo. Initially, a linear incorporation rate consistent with the expected steady progression of calcium transport and mineralization was verified by introducing 45Ca to the systemic circulation and measuring its uptake into molar teeth after various intervals. Subject to normalization of serum 45Ca (i.e. substrate) levels as described under “Experimental Procedures,” 45Ca mineral accumulated linearly for over 60 min (r > 0.97; not shown). Using this novel assay, null mutants were found to have the same calcium transport rate as heterozygotes and wild type controls (≥94 ± 6% versus wild types; n = 15–32), during both maturation and secretion, where calcium transport and calbindin28kDa expression levels are highest, respectively (Fig. 3). These results endorsed the structural evidence that calcium transport was unaffected in null mutants, raising the potential for functional compensation.

Fig. 2. Teeth and bone are produced normally in calbindin28kDa-null mutants. A, morphometric analysis of mature incisor enamel, showing equal amounts (thickness, cross-sectional area) in null mutants and controls both before and after weaning (19 and 28 days, respectively). B, comparative chemical analysis of young adult skeletons (bones, teeth), showing that null mutants had equivalent amounts of dry residue (percentage of live mass), mineral ash, calcium, and phosphorus as wild types. All data are mean ± S.E.

Fig. 3. Calcium is transported at the normal rate into developing teeth of calbindin28kDa-null mutants. Uptake of 45Ca into dental hard tissue was measured 30 min after systemic administration and found to be independent of genotype as indicated (mean ± S.E.).
DISCUSSION

According to widely accepted models, the removal of calbindins from calcium-transporting tissues should either curtail active transport or be compensated by functionally similar “calcium ferries.” Contradicting this dogma, we found that active calcium transport proceeded normally during enamel formation in null mutant mice lacking calbindin_{28kDa} despite a major loss of cytosolic calcium binding capacity. These findings constitute the first definitive evidence that calbindins are not a crucial component of the machinery used to transport calcium across enamel cells and also undermine the associated doctrine that bulk calcium is conveyed through cytosol by a facilitated diffusion mechanism.

Our results showed that calbindin_{28kDa} is a predominant calcium buffer in enamel cells but, strikingly, its absence in null mutants was not corrected by detectable overexpression of other cytosolic calcium-binding proteins. Comprising about 1% of soluble protein, calbindin_{28kDa} is a major constituent of the proteome (Fig. 4A) (20), and so a significant demand for compensation might be expected in null mutants. However, no such compensation was found either at the molecular expression or at the buffer activity levels. Molecular evidence included the targeted assessment of self-rectification in heterozygotes and replacement in null mutants by other calmodulin superfamily members and also a more global analysis covering acidic proteins that could potentially buffer calcium. The additional two possibilities of restoration by undetectably small contributions from several other buffers or by calcium-binding proteins that did not survive the \(^{45}\text{Ca} \) overlay procedure were precluded by the equilibrium binding results (Fig. 4C). Here, the finding that calbindin_{28kDa} contributed 33% of the total capacity\(^2\) endorsed its assignment as the main cytosolic buffer besides calmodulin (12, 20). This overt lack of compensation mirrors the situation in null mutant brain (15, 27), although in that case the molecular findings were not verified by binding analysis, and the overall reduction in buffer capacity was not quantified. A strong likelihood that null mutant enamel cells have altered calcium dynamics like null mutant Purkinje neurons (16) follows because calbindin_{28kDa} is expressed at similar levels in cerebellum and enamel epithelium (20). Having established that null mutant enamel cells were practically free of calbindins and did not appear to overexpress analogous proteins in their place, this experimental system could be used confidently to address the specific contributions of calbindin_{28kDa} to calcium transport.

Despite the predominance of calbindin_{28kDa} as a cytosolic buffer, our results did not support the tenet that calcium transport would be disrupted in null mutants due to shortage of calcium ferries. Instead, the calcium-dependent mineralization process was found to be unaffected both qualitatively and quantitatively. Evidence of normal mineral production came from \(^{45}\text{Ca} \) incorporation and morphometric data, which comprised dynamic and static measures over short and long periods, respectively. The normal optical properties and microstructure of null mutant enamel also implied production by healthy cells maintaining strict control over calcium transport as usual. Together these results established that calcium could be conveyed actively in the practical absence of 28- and 9-kDa calbindins, nullifying their postulated transport roles as a ferry or otherwise facilitatory buffer. It is remarkable that calbindin-independent transport was maintained at the same high rate as normal (Fig. 3), approximating the active fluxes achieved maximally in gut and kidney (1). That this was accomplished with a 33% reduction in cytosolic buffering, and with no cytotoxic overload apparent, casts serious doubt on facilitated diffusion as the underpinning mechanism. The possibilities remain that baseline buffer capacity vastly exceeds that needed for routine transport demands and that calcium is conveyed by non-calbindin ferries or some other cytosolic mechanism. However, another possibility with more substance is that calcium traverses enamel cells via a non-cytosolic route.
An alternative paradigm whereby calcium is routed through organelles instead of the cytosol does fit our observations that bulk calcium can be transported safely in the absence of calbindins. Calcium transcytosis via an ER/Ca\(^{2+}\) pipeline is conceptually attractive since it embraces free calcium levels that are normally 3 orders higher than in cytosol, averting the cytotoxic threat and need for assisted diffusion. Indeed, it was calculated that to maintain calcium flux during enamel mineralization, ER/Ca\(^{2+}\) stores would need to be exchanged once every 4 s, in striking contrast to the 150 exchanges/s required for cytosolic calcium (1). As a calbindin-independent pathway, calcium transcytosis is consistent not only with the null mutant results obtained here but also with the unexpected 4-fold down-regulation of calbindin\(_{28\text{kDa}}\) during maturation (20), evidence that a substantial proportion of calbindin\(_{28\text{kDa}}\) is not freely mobile (20, 26), and with the surprising resilience of enamel formation to vitamin D-dependent rickets (28). On the other hand, the 3-fold increased transport rate at maturation, quantified here for the first time (Fig. 3), closely matches the up-regulation of ER/Ca\(^{2+}\) store machinery (12, 14) and associated capacity for sequestering \(^{44}\)Ca\(^{3}\). Collectively, the results of this study warrant further investigation of the calcium transcytosis paradigm in enamel cells.

Do these findings hold broader significance beyond teeth? In general terms, calcium handling processes appear to be largely conserved across different animal tissues, albeit each system is adapted to its particular physiological circumstances. Enamel cells conform in this regard, having been found to express conventional calcium machinery in similarly high abundance as brain, a particularly calcium-centric tissue (12, 20). Calcium transcytosis componentry is generally available and, as such, ER/Ca\(^{2+}\) stores are known to be capable of translocating calcium at least partly across cells during calcium signaling (1, 29). Although the calbindin ferry concept was founded on highly credible correlations between calbindins and vitamin D-dependent transport, various departures from this relationship have emerged in the gut, kidney, and elsewhere (1, 30–34). More research is needed to determine whether such exceptions reflect occasional variation from a normal dependence on calbindins or instead represent informative glimpses of what actually is a calbindin-independent transit process. Null mutant models should be invaluable in this regard and, provocatively, current evidence hints that calbindin\(_{28\text{kDa}}\) has substantially less impact on calcium transport in the kidney than does the calcium entry channel, TRPV5. Although TRPV5-null mutants exhibited a major, constitutive loss of renal calcium compensation from intestine (36, 37) or bone (Fig. 2), the possibilities of compensation by other calcium-binding proteins or by passive transport processes remain to be addressed in the kidney and gut of calbindin\(_{28\text{kDa}}\)-null mutants however. Notably, being regulated by vitamin D (3, 4), TRPV5 also provides an alternative explanation for the vitamin D dependence of calcium transport previously attributed to calbindins.

In conclusion, this study brings to the fore a fundamentally important question with broad biomedical significance: is bulk calcium transported across cells via the cytosol or organelles? Answers are needed if active calcium transport is to be targeted accurately with a view to developing new therapeutics in key areas such as nutrition, biomineralization, and fertility. Until now, calbindins have stood as prime targets in the transit step of active calcium transport. This no longer seems appropriate, however, at least in the tooth, where this study has rendered the calbindin ferry concept untenable.

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