Environmental and physiological conditions that led to the emergence of intracellular calcification

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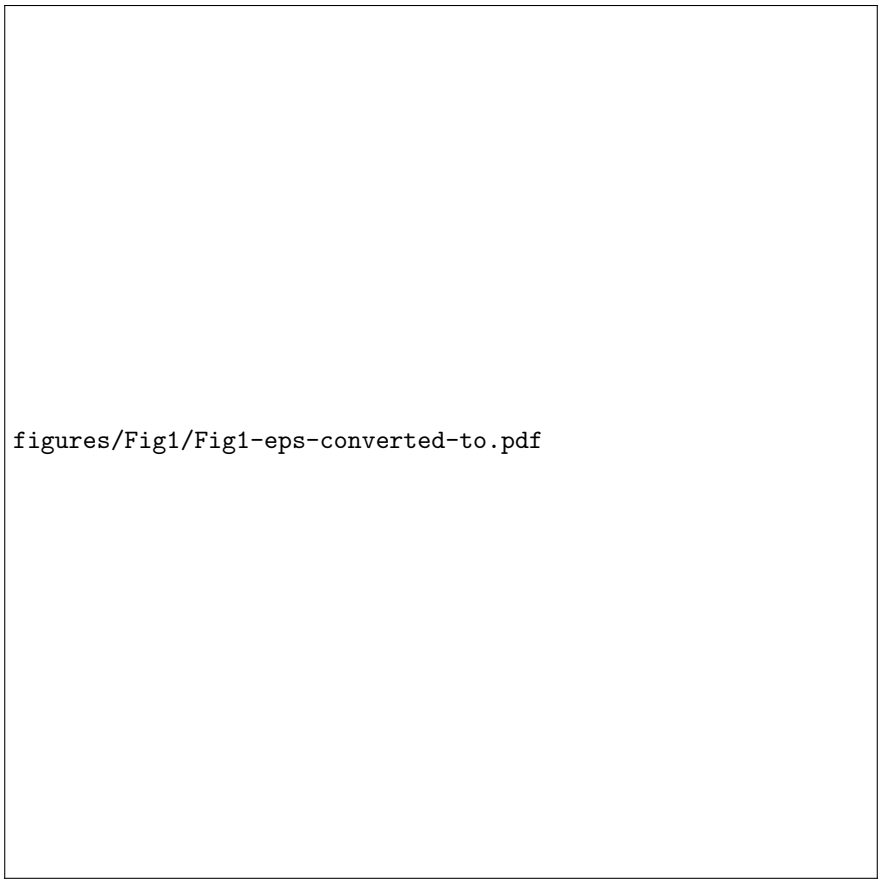
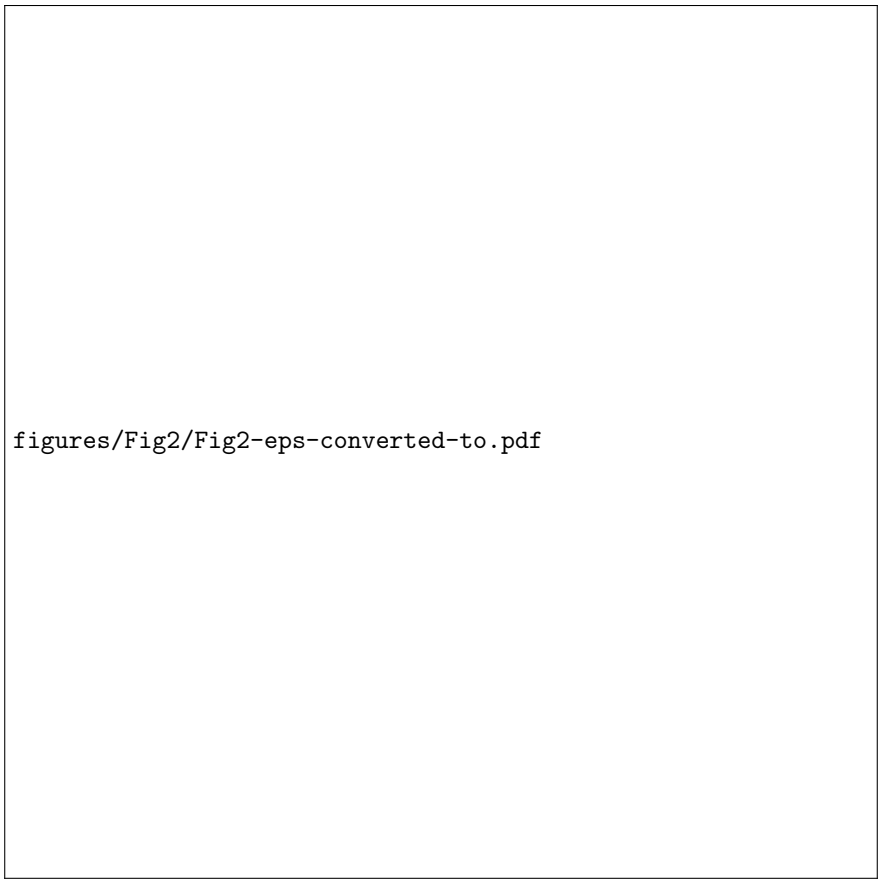
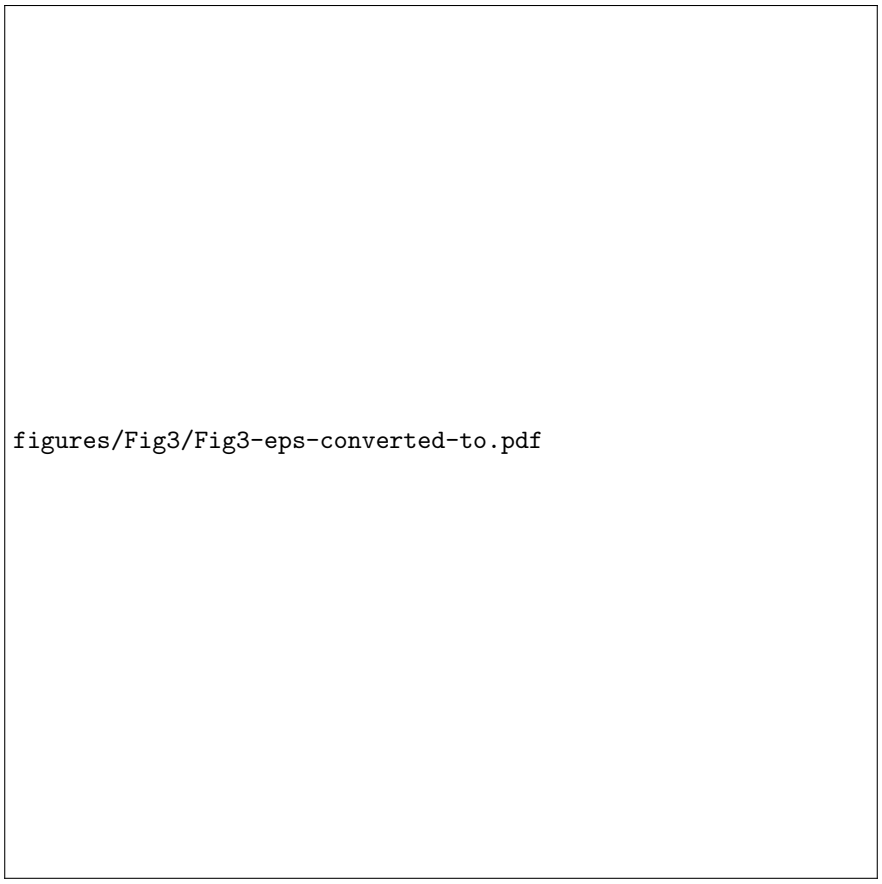
Abstract

The emergence of intracellular calcification among marine unicellular eukaryotes in the Late Triassic (237-201 Ma) had profound consequences for the carbonate buffering capacity of the ocean. Research on the appearance of calcification typically focuses on the reasons that made this process successful on a global scale. The underlying environmental and physiological conditions that led to its appearance, therefore, are still obscure. Using gene tree analysis, we show that the physiological machinery for calcification was already present in non-calcifying ancestor cells. Additionally, by modelling the energy demands for calcium transport in calcifying and non-calcifying cells, we demonstrate that intracellular calcification does not require additional energy investments. Since all eukaryotic cells export calcium across the plasma membrane, our findings indicate that the onset of intracellular calcification in Earth history only required the activation of calcium transport proteins during their passage to the plasma membrane. Our work sheds new light on the physiological and biogeochemical conditions that led to one of the most important evolutionary innovations of the Mesozoic era.







ORIGINAL RESEARCH

Environmental and physiological conditions that led to the emergence of intracellular calcification

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The emergence of intracellular calcification among marine unicellular eukaryotes in the Late Triassic (237 – 201 Ma) had profound consequences for the carbonate buffering capacity of the ocean. Research on the appearance of calcification typically focuses on the reasons that made this process successful on a global scale. The underlying environmental and physiological conditions that led to its appearance, therefore, are still obscure. Using gene tree analysis, we show that the physiological machinery for calcification was already present in non-calcifying ancestor cells. Additionally, by modelling the energy demands for calcium transport in calcifying and non-calcifying cells, we demonstrate that intracellular calcification does not require additional energy investments. Since all eukaryotic cells export calcium across the plasma membrane, our findings indicate that the onset of intracellular calcification in Earth history only required the activation of calcium transport proteins during their passage to the plasma membrane. Our work sheds new light on the physiological and biogeochemical conditions that led to one of the most important evolutionary innovations of the Mesozoic era.

KEYWORDS
calcareous nannoplankton, unicellular eukaryotes, calcium homeostasis, ion transport, Late Triassic

1 | INTRODUCTION

Rock-forming pelagic calcifiers appeared in the Late Triassic (Bralower *et al.*, 1991; Bown, 1998; Bown *et al.*, 2004; Onoue and Yoshida, 2010; Gardin *et al.*, 2012; Preto *et al.*, 2013b,a), Figure 1. The first were probably small calcareous dinoflagellates that produced simple calcispheres (on average about 10 μm in diameter) composed of sub-micron scale calcium carbonate crystals (Janofske, 1992; Onoue and Yoshida, 2010; Preto *et al.*, 2013b,a). Coccolithophores, which are haptophyte algae and secrete complex ornamented calcite plates (Young, 2003; Young *et al.*, 2005), appeared later in the latest Norian about 210 Ma (Gardin *et al.*, 2012). Before the emergence of pelagic calcification, the biological precipitation of calcium carbonate was dominated by metazoans and microbial mats that calcified extracellularly in a medium isolated from seawater (Lowenstam and Margulis, 1980; Kaźmierczak *et al.*, 1985; Knoll, 2003). In contrast, calcareous nannoplankton, such as calcispheres and coccolithophores, calcify inside intracellular compartments called vesicles and extrude the accreted crystals via exocytosis (Inouye and Pienaar, 1983; Paasche, 2002; Brownlee *et al.*, 2015). The emergence of intracellular calcification allowed biocalcification to expand into the surface waters of the open ocean (Zeebe and Westbroek, 2003; Ridgwell, 2005; de Vargas *et al.*, 2007). This expansion produced a much more efficient regulation of the global carbon cycle and changed the marine calcium carbonate saturation state, transforming the entire ocean carbonate chemistry (Zeebe and Westbroek, 2003; Ridgwell, 2005; Ridgwell and Zeebe, 2005). The emergence of pelagic calcification is thus one of the most important evolutionary innovations in Earth's history but how and why it originated is still unclear. The mechanisms driving evolutionary innovations are mutation and selection. Therefore, before identifying the benefits that could have selected intracellular calcification to make it a successful innovation at a global scale, it is important to determine the mutations required for its appearance.

While the evolution of extracellular calcification was likely an adaptive response to rising calcium concentrations in the ocean (Kaźmierczak *et al.*, 1985; Kaźmierczak and Kempe, 2004), the appearance of intracellular calcification does not clearly correlate with changes in ocean chemistry (Figure 1). For example, a causal link with a decline in atmospheric pCO_2 has been proposed but could not be proven (Goddéris *et al.*, 2008). Moreover, when intracellular calcification emerged, the concentration of calcium in the ocean was approximately 30 mM, dissolved inorganic carbon (DIC) was 5000 μM, and ocean pH was about 7.8 (Figure 1). These conditions would have led to a carbonate concentration of 160 μM and a calcite saturation state of 10. Although such chemical conditions would be favourable to calcification and could therefore positively select for this innovation, they alone are not sufficient to explain the appearance of intracellular calcification because the ocean experienced similar conditions 80 to 100 million years before the first evidence of pelagic calcification appears in the geological record (Late Triassic - Jurassic). The ocean experienced even higher calcium concentrations (from 50 mM to almost 60 mM) from the Cambrian to the Carboniferous and calcite saturation reached almost 15 in the Carboniferous and Permian. Therefore, the emergence of intracellular calcification was most likely a consequence of internal changes in cellular physiology rather than a response to external environmental factors such as changes in ocean carbonate chemistry. Using new energy budget calculations and gene analyses, we investigate the underlying mechanisms and prerequisites that allowed intracellular calcification to appear. We then discuss the factors that, after its appearance, led to the evolutionary success of calcifying phytoplankton on a global scale.

2 | MATERIALS AND METHODS

2.1 | Analysis of environmental data

We calculated the carbonate chemistry parameters from reconstructed historic ocean pH and DIC (Ridgwell, 2005) using the software CO2sys (Lewis and Wallace, 1998). Calculated ocean carbonate concentrations and reconstructed

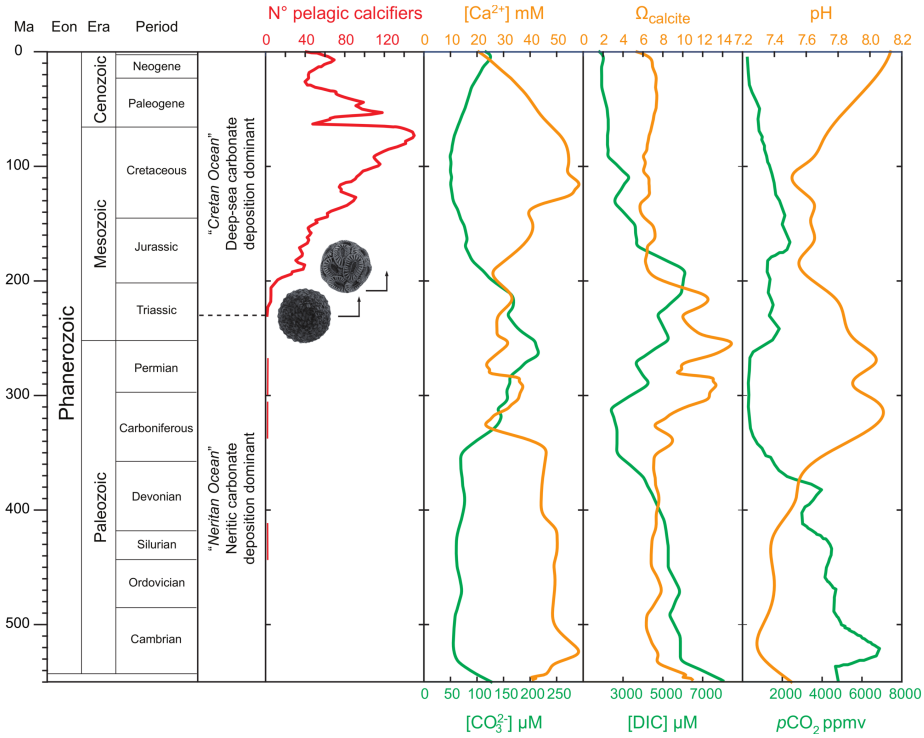

FIGURE 1 Fossil record of pelagic calcifiers and paleoceanographic carbonate chemistry over the past 550 Myr. The number of pelagic calcifiers is reconstructed by Bown *et al.* (2004). The concentration of calcium is taken from Stanley and Hardie (1998) and atmospheric pCO_2 from Berner and Kothavala (2001). Dissolved inorganic carbon (DIC) and ocean pH were reconstructed by Ridgwell (2005). The calcite saturation state ($\Omega_{calcite}$) was calculated from Ca^{2+} and CO_3^{2-} based on ocean temperature (Royer *et al.*, 2004). The emergence of intracellular calcification is marked by the appearance of calcareous dinoflagellates (Preto *et al.*, 2013b), which is indicated by the horizontal dashed line, and was followed by the appearance of coccolithophores. Unidentified calcispheres appeared previously in the Silurian, Devonian, and Carboniferous but are of uncertain origin (Kaźmierczak and Kremer, 2005).

calcium concentrations (Stanley and Hardie, 1998) were used to determine calcite saturation state. Atmospheric pCO_2 was obtained from previous reconstructions (Berner and Kothavala, 2001). We visually compared the carbonate chemistry parameters with the fossil record of pelagic calcifying nannoplankton (Bown *et al.*, 2004) to identify the environmental conditions under which intracellular calcification emerged (Figure 1).

2.2 | Calcium-transporter gene tree analysis

To examine the evolutionary history of calcification-related proteins, we identified proteins across lineages and created gene trees of relevant protein families. We downloaded protein databases from the National Center for Biotechnology Information (NCBI) genome browser for taxa represented in the phylogeny of Read *et al.* (2013), Supplementary Table 1. To account for differences in annotation completeness in each protein database, we used BLAST to identify homologs of *Emiliania huxleyi* proteins in each taxon. We searched the *E. huxleyi* protein database for annotated sequences in

each protein family implicated in calcification (Benner *et al.*, 2013) to use as query sequences (Supplementary Table 2). We then used pBLAST to identify all sequences in the protein database that matched these query sequences with probabilities less than e-6. After discarding duplicates, we aligned the BLAST results for each protein family in Geneious 11.1.3, (Kearse *et al.*, 2012) `http://www.geneious.com`, using the Geneious global alignment with cost matrix Blosum62, gap open penalty 12, and gap extension penalty 3. We created gene trees using Jukes-Cantor genetic distance module and the UPGMA tree building method in Geneious. We then inspected trees for phylogenetic representation and patterns of protein family expansion. Because the BLAST output results for calcium channels included both cation channels and calmodulin, we split these sequences into two subtrees for better visualization of the relationships among calmodulin-related proteins. Supplementary Figures S1 – S7 depict resulting gene trees.

2.3 | Calculation of energy invested into intracellular calcification

We calculated the energy required for calcium transport into golgi-derived vesicles as part of the energy invested to maintain calcium homeostasis. In order to achieve intracellular calcium homeostasis, total calcium transport out of the cytoplasm has to equal the passive influx via calcium channels. The passive, diffusive uptake via the calcium channels is driven by the difference between the calcium concentration at the cell surface (Ca_{surf}) and the calcium concentration in the cytoplasm (Ca_{cyt}). Thus, knowing the calcium gradient across the plasma membrane, the energy required to achieve calcium homeostasis (E_{ATP}) can be calculated by

$$E_{ATP} = P_{Ca} \cdot A_{cell} \cdot (Ca_{surf} - Ca_{cyt}) \cdot \Psi^{\frac{ATP}{Ca}}, \quad (1)$$

where P_{Ca} is the apparent membrane permeability per cell surface area (A_{cell}) and $\Psi^{\frac{ATP}{Ca}}$ is the amount of ATP required per calcium ion transported. The consumption of one ATP molecule for transporting two calcium ions through the membrane is a functional property of the reaction loop of the Ca-ATPase (Toyoshima and Inesi, 2004; Winther *et al.*, 2013) and is independent if transport is over the vesicle or plasma membrane. Without net calcium influx into the cell, Ca_{surf} is equal to the calcium concentration in the bulk medium (Ca_{out}). However, fluxes of calcium into intracellular compartments, such as vesicles, create a concentration gradient in the cell environment with $Ca_{surf} < Ca_{out}$. With the total flux of calcium into the vesicles ($Q_{vesicle}$), and thus into the cell, the integration of the spherical diffusion equation in the cell environment gives the following concentration at the cell surface:

$$Ca_{surf} = Ca_{out} - \frac{Q_{vesicle}}{4 \cdot \pi \cdot r_{cell} \cdot D_{Ca}}, \quad (2)$$

where D_{Ca} is the diffusion coefficient of calcium in seawater and r_{cell} is the cell radius. Here, we assume that the transport of calcium into vesicles represents a fixed fraction of total calcium transport (f_v) as compared to the transport over the plasma membrane ($1 - f_v$), i.e.:

$$Q_{vesicle} = f_v \cdot P_{Ca} \cdot A_{cell} \cdot (Ca_{surf} - Ca_{cyt}). \quad (3)$$

Inserting this expression for $Q_{vesicle}$ into Equation (2) and subtracting Ca_{cyt} from both sides of Equation (2) results in an implicit equation for the concentration difference Ca_{surf} - Ca_{cyt}, which has the solution:

$$\mathrm{Ca}_{surf} - \mathrm{Ca}_{cyt} = \frac{\mathrm{Ca}_{out} - \mathrm{Ca}_{cyt}}{1 + \frac{f_v \cdot P_{\mathrm{Ca}} \cdot A_{cell}}{4 \cdot \pi \cdot r_{cell} \cdot D_{Ca}}} \tag{4}$$

Replacing in Equation (1) the concentration difference $\mathrm{Ca}_{surf} - \mathrm{Ca}_{cyt}$ with Equation (4) gives E_{ATP} as a function of the relative fraction of calcium transport into the calcifying vesicles:

$$E_{ATP} = P_{\mathrm{Ca}} \cdot A_{cell} \cdot \frac{\mathrm{Ca}_{out} - \mathrm{Ca}_{cyt}}{1 + \frac{f_v \cdot P_{\mathrm{Ca}} \cdot A_{cell}}{4 \cdot \pi \cdot r_{cell} \cdot D_{Ca}}} \cdot \Psi^{\frac{ATP}{Ca}} \tag{5}$$

This equation shows that the higher f_v, the lower the absolute requirements for cellular energy. In order to quantify how much the cellular energy consumption can potentially be reduced by intracellular calcification, we calculate the energy requirements to maintain calcium homeostasis as a function of f_v from 0 (non-calcifying cell) to 1 (Supplementary Figure S8). The coccolithophore *Emiliania huxleyi* produces approximately 1 coccolith per hour (Paasche, 1962) and one coccolith contains around 22 fmol of $CaCO_3$ (Young and Ziveri, 2000). This results in a calcification flux ($Q_{vesicle}$) of about $6.11 \cdot 10^{-18}$ mol s^{-1} (Holtz *et al.*, 2013). Since the permeability of the plasma membrane for calcium ions is not known in coccolithophores, we estimate a permeability that is based on the conductance of calcium channels (Lux and Nagy, 1981) and measured densities of calcium channels in cell membranes of snail axons (Krishtal *et al.*, 1981; Tsien, 1983). Parameter values are reported in Supplementary Table 3.

2.4 | Calculation of intracellular calcium poisoning

The energy for the Ca^{2+} pumping ATPase is provided by the the hydrolysis of ATP at a high ATP/ADPxPi ratio in the cytosol with a free energy gain, ΔG, of 15 kcal/mol (Meldolesi and Pozzan, 1998). Cooperative binding of 2 Ca^{2+} per ATPase implies a Ca^{2+}/ATP coupling ratio of 2 (Inesi and Tadini-Buoninsegni, 2014). We assume an electroneutral $Ca^{2+}/2H^+$ cotransport for the Ca^{2+} pump and a physiological pH at both sides of the Ca^{2+} pump. If all of the free energy available from ATP hydrolysis is stored in the concentration gradient of Ca^{2+} between the cytosol and its environment, i.e.: $\Delta G = 2RT \cdot \ln(\mathrm{Ca}_{surf}/\mathrm{Ca}_{cyt})$, the calcium gradient can be up to 10^5. If the Ca-ATPases work near this thermodynamic limit, the cytosolic Ca^{2+} concentration of 0.1 μmol L^{-1} and the gradient of 10^5 yields a Ca^{2+} concentration of 10 mM at the cell surface, which, in fact, is exactly the Ca^{2+} concentration in today's ocean (Langer *et al.*, 2006). Ca^{2+} transport near the thermodynamic limit would have the advantages that: (1) pumping Ca^{2+} from the cytosol to the environment proceeds with a minimum dissipation of energy in ATP consumption, and (2) a steep Ca^{2+} gradient provides fast influx in cell signaling. We therefore postulate that a constant Ca^{2+}-gradient close to the thermodynamic limit represents a part of calcium homeostasis. Moreover, the Ca^{2+}-gradient across the plasma membrane should be quite independent of the bulk Ca^{2+} concentration in seawater.

Assuming that the Ca^{2+}-gradient across the plasma membrane is maintained at 10^5 by the Ca^{2+}-ATPase, the intracellular calcium concentration will respond to variations in extracellular calcium concentration as experienced during Earth's history (Stanley and Hardie, 1998), Figure 1. The intracellular concentration of calcium (Ca_{cyt}), however, generates a constraint on the intracellular phosphate concentration (PO_{cyt}) because calcium and phosphate precipitate to $Ca_3(PO_4)_2$ when the ion product exceeds the solubility product $K_{sp}^{Ca_3(PO_4)_2} = \mathrm{PO}_{cyt} \cdot \mathrm{Ca}_{cyt}$. We can therefore calculate the maximum possible intracellular phosphate concentration for any intracellular calcium concentration as follows:

$$PO_{cyt} = \sqrt{\frac{K_{sp}^{Ca_3(PO_4)_2}}{Ca_{cyt}}}. \quad (6)$$

3 | RESULTS AND DISCUSSION

3.1 | Calcium metabolism

Calcium ions play important roles in the physiology of eukaryotic cells, including control of DNA synthesis, chromosomal configuration, and signal transduction (Marcum *et al.*, 1978; Boynton *et al.*, 1980; Krishtal *et al.*, 1981; Lux and Nagy, 1981; Tsien, 1983; Poenie *et al.*, 1985; Carafoli, 1987; Steinhardt and Alderton, 1988; Hepler, 1994; Sanders *et al.*, 2002; Verret *et al.*, 2010). Calcium ions enter the cell passively via channels (Gussone *et al.*, 2006; Verret *et al.*, 2010) that allow for a diffusive influx driven by a steep concentration gradient across the plasma membrane (Langer *et al.*, 2006). Excess intracellular Ca^{2+} is then actively removed by calcium-binding proteins (e.g. calmodulin) and transport proteins (Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger) with different calcium affinities and maximum transport rates (Carafoli, 1987). The Ca^{2+} concentration inside the cell has to be tightly regulated (calcium homeostasis) at around 0.1 μmol L^{-1} (Brownlee *et al.*, 1995) for optimal cell functioning (Carafoli, 1987) and to avoid cell poisoning (Simkiss, 1977; Orrenius *et al.*, 1989; Müller *et al.*, 2015).

The same proteins that maintain intracellular calcium homeostasis in non-calcifying cells (Figure 2a) are implicated in intracellular calcification (Figure 2b). To determine whether an expansion of protein families coincides with the emergence of intracellular calcification, we compared all gene families involved in the precipitation of calcium carbonate (von Dassow *et al.*, 2009) in a large number of eukaryotes (Read *et al.*, 2013). These genes include Ca^{2+} transporting ATPases, HCO_3^- transporters, Ca^{2+} channels, calmodulin, carbonic anhydrase, Ca^{2+}/H^+ exchangers, Na^+/Ca^{2+} exchangers, and vacuolar H^+-ATPases. The presence of calcium transport proteins in all eukaryotes clearly indicates that these genes are not unique to intracellular calcification. We found no evidence that diversification of these gene families could explain the appearance of intracellular calcification (Supplementary Figures S1 – S7).

The most significant difference between calcifying and non-calcifying cells is that calcium carbonate precipitates inside golgi-derived vesicles. The vesicles fuse with the plasma membrane and the calcium carbonate is extruded outside the cell where it remains attached to the extracellular organic matrix. The same vesicular pathway is used in non-calcifying cells to produce new membrane material and to transport proteins to the plasma membrane (Figure 3). All ion transporters and channels that help establish calcium homeostasis in non-calcifying cells, therefore, reach the plasma membrane via golgi-derived transport vesicles (Pfeffer and Rothmann, 1987). However, the Ca^{2+} transporting ATPase initially occurs in its auto-inhibited form and becomes activated by the presence of calmodulin (Tidow *et al.*, 2012) at the plasma membrane. We thus argue that the emergence of intracellular calcification was triggered by the activation of calcium pumps during the passage of transport vesicles from the golgi apparatus to the plasma membrane, following the accumulation of calcium in the transport vesicles. This explanation is consistent with the simple structure of sub-micron scaled calcite crystals of the early calcispheres (Janofske, 1992; Preto *et al.*, 2013b). The activation of calcium transport inside the golgi apparatus would lead to the formation of more elaborated coccolith platelets due to the intrinsic shape of the endomembranous structure of the golgi apparatus (Figure 3). This is also in line with the finding that inhibition of the cytoskeleton and microtubule formation induces coccolith malformation (Langer *et al.*, 2010).

Such a change in timing of activation would not be detected in gene sequence comparison, as they require protein

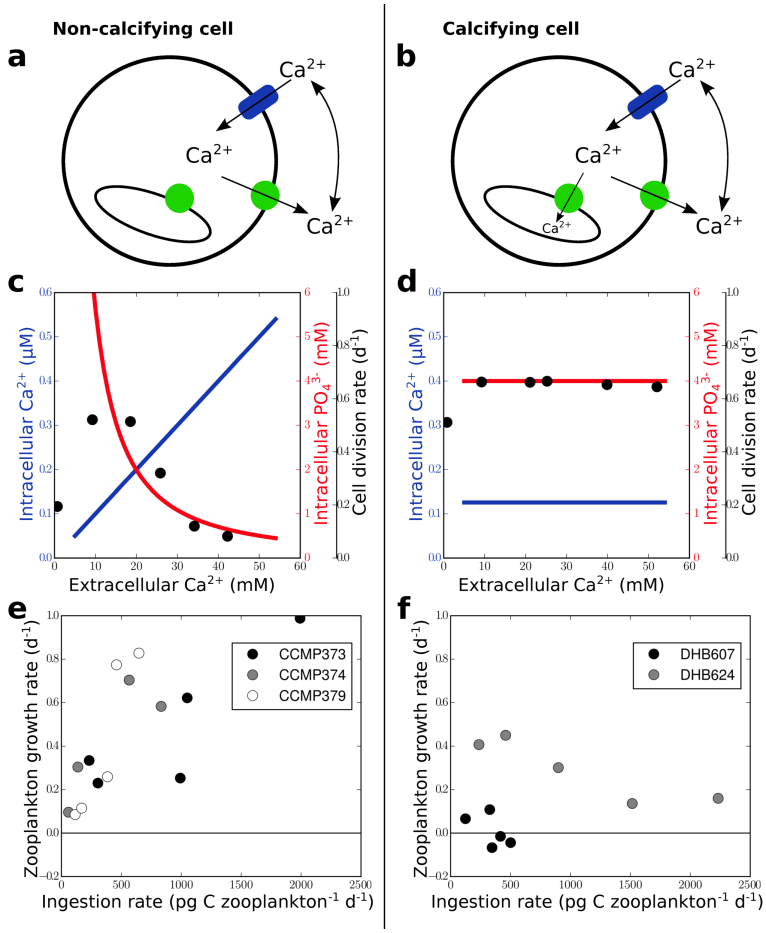

FIGURE 2 Physiological and ecological benefits of intracellular calcification. Calcifying cells (**b**) differ from non-calcifying cells (**a**) by the presence of intravesicular calcium accumulation in golgi-derived transport vesicles (green circles represent Ca^{2+} pump/ATPase; blue cylinder indicates calcium channel). Calcium transport into vesicles allows maintenance of calcium homeostasis under elevated extracellular calcium concentrations (**d**) and prevents drawdown of intracellular phosphate (**c**) due to precipitation of $Ca_3(PO_4)_2$ in the cytosol. Therefore, cell division rates, black dots in (**c**) and (**d**), redrawn from Müller *et al.* (2015), remain unaffected by extracellular calcium concentrations in calcifying cells contrary to non-calcifying cells. The growth rate of zooplankton fed with calcifying cells (**f**) is generally lower than that of zooplankton fed with non-calcifying cells (**e**), data after Harvey *et al.* (2015).

functional assays. We conclude, however, that all components necessary for the emergence of intracellular calcification were already present in non-calcifying ancestor cells and that no major modifications of cellular physiology and structure were required to induce intracellular calcification. An alternative hypothesis to the activation of calcium pumps could be that those pumps were already active but that precipitation of calcium carbonate in the vesicles was prevented by inhibitors, which were subsequently lost thus leading to the emergence of intracellular calcification. But also in this alternative case, the emergence of intracellular calcification would not require major innovations in cellular structure,

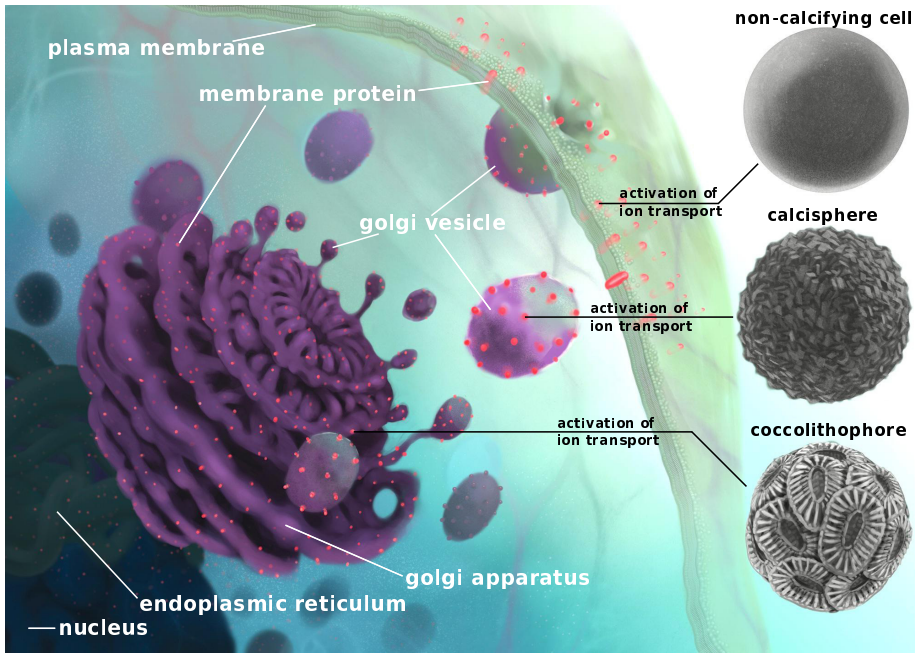

FIGURE 3 Biosynthesis and vesicular transport of membrane proteins. After transcription from DNA to mRNA in the nucleus, gene copies are translated into proteins in the endoplasmic reticulum. Proteins that are bound to the plasma membrane are incorporated into the ER membrane and are transported via vesicles to the golgi apparatus. Golgi-derived vesicles are then transported to the plasma membrane where the vesicles fuse and shed their content to the extracellular space. The vesicle membrane, including all membrane proteins, becomes part of the plasma membrane. We suggest that, in a non-calcifying cell, the activation of calcium transporters occurs at the plasma membrane. Earlier activation of calcium transporters in transport vesicles would allow precipitation of sub-micron scale crystals inside the vesicles that could lead to the formation of calcispheres. Activation of calcium transporters even earlier, in the more structurally complex endomembranous systems like the golgi apparatus, could explain the formation of delicate coccoliths and the emergence of coccolithophores. The chronological order of the appearance of calcispheres and coccolithophores does not necessarily represent direct evolutionary steps regarding the activation of calcium transporters but may represent parallel origins of early Ca^{2+} transport activation that happened independently (Meier *et al.*, 2007).

which is our main argument here.

3.2 | Energy requirements

Since ion transport requires energy, a previous study (Monteiro *et al.*, 2016) assumed that intracellular calcification is energetically costly, thus implying the presence of associated benefits to overcome natural selection. But because non-calcifying cells also export calcium from their cytoplasm, the energetic costs of calcification have to be quantified by comparing the costs required for ion transport into vesicles in calcifying cells versus those required by ion transport across the plasma membrane (to maintain calcium homeostasis) in both calcifying and non-calcifying cells. We therefore assessed the energetic costs of intracellular calcification by calculating the total energy required by cellular calcium

transport to maintain calcium homeostasis. Our calculations indicate that the precipitation of $CaCO_3$ inside the vesicle removes calcium from the solution and generates a sink that depletes calcium around the cell, Equation (2). Consequently, the gradient across the plasma membrane that drives the diffusive calcium influx through the channel is reduced with respect to non-calcifying cells, Equation (4), thus fewer ions have to be transported out of the cytoplasm in order to maintain calcium homeostasis, Equation (5). Since the energy required for active calcium transport out of a calcifying cell is negligibly lower than that required in non-calcifying cells (Supplementary Figure S8), we conclude that extra energy is not needed to transport calcium for intracellular calcification.

However, our energy calculations also show that the reduction in energy requirements is very small because the calcification flux is slow compared to extracellular calcium diffusion so that no significant diffusive boundary layer develops at the cell surface. If all calcium ions were transported into the vesicles and no calcium were exported through the plasma membrane, f_v = 1.0 in Equation (5), the energy requirements would only be 0.16 percent less than if no calcium were transported into vesicles and all ions were exported through the plasma membrane, f_v = 0.0 in Equation (5).

Besides calcium ions, also carbonate ions are needed in the vesicle to sustain calcification. The transport of carbon could thus be another process requiring energy. However, the transport of hydrogen ions that is coupled to calcium transport creates an alkaline medium inside the calcifying vesicle that shifts the equilibrium of the carbonate system towards carbonate ions and lowers the CO_2 concentration (Holtz *et al.*, 2013, 2015a). CO_2 can, therefore, passively diffuse over the vesicle membrane to replenish the carbonate ions consumed by calcification, thus sustaining calcification at moderate rates (see Figure 3 and Table 4 in Holtz *et al.*, 2013) without additional metabolic costs. Additional carbon transport into the vesicles may only be required to sustain higher calcification rates as observed in some coccolithophore species that appeared later in the geological records. Since the bicarbonate concentration in the calcifying vesicle is higher than in the cytoplasm, bicarbonate would then need to be transported actively under consumption of metabolic energy (Holtz *et al.*, 2015a). However, bicarbonate transport does not exceed calcium transport into the vesicles and is negligible compared to total cellular energy requirements.

3.3 | Evolutionary success

Because of its low metabolic requirements, intracellular calcification does not require secondary evolutionary benefits to emerge. Yet, a number of hypotheses in relation to potential benefits of intracellular calcification have been proposed to explain both emergence and success of coccolithophores. These hypotheses include the potential enhancement of photosynthesis via the removal of carbonate ions (Nielsen, 1966; Paasche, 2002), protection against photoinhibition, advantages in hydrodynamic control, and protection against viruses, bacteria, and grazing (Monteiro *et al.*, 2016). However, enhanced calcification does not enhance photosynthesis (Herfort *et al.*, 2004; Trimborn *et al.*, 2007) and non-calcifying coccolithophore cells can grow even faster than calcifying cells (Rost and Riebesell, 2004). Non-calcifying strains appear also not affected by photo-inhibition at high irradiances (Nanninga and Tyrrell, 1996). Additionally, coccolithophores use their non-calcifying haploid stages to escape viral infections of calcified diploid cells (Frada *et al.*, 2008), also questioning the relevance of calcification for viral protection. All these cases cast doubts on the assumption that calcification presents relevant physiological advantages.

Previous experiments (Müller *et al.*, 2015) showed that calcifying cells can grow faster than non-calcifying cells when exposed to elevated calcium concentrations, indicating that intracellular calcification may protect against calcium poisoning (Simkiss, 1977). Our calculations show that the Ca^{2+}-ATPase can transport ions against a gradient of up to 10^5 (see Supplementary Information). In non-calcifying cells, an increase of extracellular Ca^{2+} concentrations yields an increase of intracellular Ca^{2+} (Figure 2c). Since intracellular phosphate concentrations are usually around 4 -– 5 mM

(Auesukaree *et al.*, 2004), cells operate at calcium and phosphate concentrations just below the solubility product of $Ca_3(PO_4)_2$ (Chow, 2001). Increasing intracellular calcium concentrations, therefore, lead to precipitation with phosphate due to the low solubility of $Ca_3(PO_4)_2$ (Pytkowicz and Kester, 1967; Chow, 2001). The decrease of cellular phosphate impedes cell growth in non-calcifying cells (Figure 2c). In calcifying cells, calcium transport into intracellular vesicles can still occur under elevated extracellular calcium concentrations because the calcium concentration inside the vesicle (ranging from 0.5 to 10 mM Holtz *et al.*, 2013, 2015b) required for the precipitation of calcium carbonate is lower than outside the cell. The calcium gradient at the vesicle membrane therefore permits Ca^{2+}-ATPase activity and allows maintenance of calcium homeostasis even under elevated extracellular calcium concentrations (Figure 2d). Cell division of calcifying cells remains unaffected (Müller *et al.*, 2015). This physiological advantage of calcifying cells may explain the dominance of coccolithophores over non-calcifying phytoplankton during the Cretaceous when the calcium concentration in the ocean was 5 to 6 times higher than present (Stanley and Hardie, 1998).

Another benefit proposed for intracellular calcification is protection against zooplankton grazing (Jaya *et al.*, 2016; Monteiro *et al.*, 2016). However, coccolithophores are grazed by copepods without any significant sign of saturation of ingestion rates at increasing coccolithophore densities (Harris, 1994). This indicates that calcification and thus mechanical stability does not necessarily protect against copepod grazing. Calcification can, however, have an indirect negative effect on zooplankton grazing. Culture experiments (Harvey *et al.*, 2015) showed that ingestion of calcifying cells reduces the growth rate of the grazers (Figure 2f), whereas ingestion of non-calcifying cells leads to an incrase in zooplankton growth rate (Figure 2e). A reduction of coccolithophore grazing mortality would thus provide calcifying cells with a competitive advantage against non-calcifying cells (Monteiro *et al.*, 2016). Though not relevant for the emergence of pelagic calcification, the potential chemical protection against grazing may have contributed to its ecological success.

4 | CONCLUSIONS

In summary, our analyses indicate that the first appearance of intracellular calcification is intrinsically linked to cellular calcium metabolism and to the prevention of calcium accumulation in the cytoplasm. This process did not require major physiological mutations or extra energy but only a very minor change, that is the activation of calcium transport proteins during their passage from the endoplasmic reticulum to the plasma membrane. Therefore, this innovation could have occurred at any time in the geologic past because preconditions were favourable, in line with evidence suggesting unrelated appearances in different lineages (de Vargas *et al.*, 2007). Environmental factors, such as chemical conditions and ecological interactions, may subsequently have helped calcifying nannoplankton to become abundant at a global scale.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

SUPPORTING INFORMATION

Supplementary information is attached to this submission as separate file.

DATA ACCESSIBILITY STATEMENT

The number of pelagic calcifiers and the biogeochemical data are available from published literature (as detailed in the caption of Figure 1). The protein data are available from the protein sequence repository of the National Center for Biotechnology Information (`https://www.ncbi.nlm.nih.gov/home/proteins/`). Full details concerning the extraction of the protein data used in this research are provided in the main article and in the supplementary information file.

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