Endoplasmic reticulum Ca^{2+}-ATPase pump is up-regulated in calcium-transporting dental enamel cells: a non-housekeeping role for SERCA2b

Isobel K. FRANKLIN, Robert A. WINZ and Michael J. HUBBARD[1]

Department of Biochemistry, University of Otago, PO Box 56, 710 Cumberland Street, Dunedin, New Zealand

Dental enamel-forming cells face a major challenge to avoid the cytotoxic effects of excess calcium. We have characterized sarcoplasmic/endoplasmic reticulum calcium-ATPase pumps (SERCA) in rat enamel cells to address the proposal that non-mitochondrial calcium stores play a dominant role in transcellular calcium transport. A single major isoform, SERCA2b, was detected during the protein-secretory and calcium-transport stages of enamel formation using reverse-transcriptase PCR, cDNA cloning, Northern analysis and immunoblotting. Most importantly, SERCA2b exhibited a specific 3-fold up-regulation to high expression levels during calcium transport, as determined by quantitative immunoblotting and ATPase assays. Sensitivity of the calcium-dependent ATPase to thapsigargin and three other SERCA inhibitors was characterized. These findings indicate that enamel cells are well-equipped to sequester calcium in endoplasmic reticulum stores and so protect against calcium toxicity, associate SERCA with transcellular calcium transport for the first time, and establish SERCA2b as a molecular and pharmacological target for future investigations of calcium transcytosis. The observed physiological regulation in enamel cells contradicts the widespread perception that SERCA2b is restricted to general housekeeping duties.

Key words: calcium store, calcium homoeostasis, calcium toxicity, cytotoxicity, ameloblast.

INTRODUCTION

The cytotoxic effects of excess calcium are widely recognised, but causal relationships between calcium dysregulation and disease remain a conundrum [1,2]. Much attention has focused on neurons which contain many calcium homoeostasis proteins in high abundance. Yet, despite detailed understanding of individual protein components, it has proven difficult to establish robust correlations between pathophysiological responses and the complement of calcium-handling machinery expressed in different types of neuron [3,4]. Previously we hypothesized that dental enamel cells would provide useful insights to the calcium toxicity problem since they handle large amounts of calcium in a developmentally programmed manner [5,6]. Evidence that common calcium-handling proteins were expressed at similarly high levels as in brain, and in a developmental pattern that varied with cell function, substantiated the utility of the rat enamel cell model.

Dental enamel is formed in two stages termed secretion and maturation, each associated with distinctive enamel cell morphologies and functions. First, enamel epithelial cells (ameloblasts) secrete a protein-rich and partially mineralized extracellular matrix. Second, maturation ameloblasts hypermineralize and deproteinate the matrix to yield mature enamel that comprises 96 % mineral (hydroxylapatite, 40 % of which is calcium) [7]. In rat, the developmental switch into maturation is accompanied by a 3-fold increase in the rate of enamel calcification and a 10-fold decrease in secretory protein synthesis [7,8]. We found previously that the cellular content of calcium-binding proteins increased markedly during maturation, indicative of an increased calcium burden associated with transcellular calcium transport [5,6]. Surprisingly, none of the major cytosolic calcium-binding proteins was up-regulated at this stage, heightening our interest in how enamel cells can transport calcium in bulk without succumbing to its toxic effects.

Active calcium transport across epithelial cells is widely believed to employ a cytosolic route, but our recent findings have raised the possibility of an organelle-based mechanism that we refer to as calcium transcytosis [8]. Numerous investigations of calcium transport in intestine and kidney have favoured a mechanism that uses cytosolic calcium-ferrying proteins (calbindins) in conjunction with plasmalemmal calcium channels and pumps that regulate the influx and extrusion of calcium [9,10]. Computer modelling indicated that mobile calbindins can preserve cytosolic calcium at non-toxic levels and concomitantly boost the rate of calcium diffusion, as needed to account for the rapid fluxes observed *in vivo*. However, this proposed cytosolic mechanism is dependent on high expression levels of calbindins [11]. Such a role for calbindins seems unlikely in enamel cells since we found that calbindin$_{28\,kDa}$ was highly expressed in secretory enamel cells but down-regulated 4-fold during maturation. Instead, internal calcium stores were implicated in a dominant calcium-handling role. Calreticulin and endoplasmin, the major calcium buffers in endoplasmic reticulum (ER), were both up-regulated 2-fold during maturation and an ER calcium-release channel (inositol 1,4,5-trisphosphate receptor) was also expressed at unusually high levels [5,6]. With supporting evidence including parallel up-regulation of mitochondrial ATP synthase [12], we proposed a mechanism (calcium transcytosis) whereby ER-associated calcium stores serve as a transcellular conduit for calcium [6,8]. In brief, transcellular transport of serosal calcium into developing enamel was envisaged to involve entry through basolateral membrane channels, uptake and rapid diffusion through the calcium-rich ER lumen, and active extrusion at the

Abbreviations used: Ca^{2+}-ATPase, calcium-dependent ATPase; ER, endoplasmic reticulum; RT, reverse transcriptase; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; tbuBHQ, 2,5-di(tert-butyl)-1,4-benzohydroquinone.

[1] To whom correspondence should be addressed (e-mail mike.hubbard@stonebow.otago.ac.nz).

The nucleotide sequence data reported appears in the GenBank® Nucleotide Sequence Database under the accession number AF043106.

secretory pole. Ameloblasts contain an extensive tubulovesicular network that might represent such a transcellular pathway. Consequently, a picture is emerging that maturation enamel cells use capacious internal stores to transport calcium vectorially and so avoid exposure to extreme amounts of cytosolic calcium [8].

This study focused on the sarcoplasmic/ER calcium-dependent ATPase (SERCA) which we hypothesized would play a key role in calcium transcytosis. SERCAs reside in the ER/nuclear membrane and harness a calcium-dependent ATPase activity (Ca^{2+}-ATPase) to translocate cytosolic calcium into the ER lumen [13]. In common with other P-type ATPases, the ER Ca^{2+}-ATPase forms a characteristic acid-stable phosphoenzyme intermediate that is sensitive to vanadate and lanthanum. Selective targeting of SERCA activity can be accomplished with several well-characterized inhibitors including thapsigargin, cyclopiazonic acid and 2,5-di(tert-butyl-1,4-benzohydroquinone (tbuBHQ) [14–16]. SERCAs are encoded by three distinct genes (*SERCAs 1–3*) that produce a total of seven different proteins due to alternative splicing [17,18]. The 115-kDa isoform, SERCA2b, is expressed in all non-muscle cells and has a relatively high affinity for calcium whereas the other isoforms (approx. 108–112 kDa) have limited distributions and lower calcium affinities. Developmental regulation and experimentally induced isoform-switching patterns have been reported for these latter SERCAs, but not for SERCA2b which is widely regarded as a constitutively expressed housekeeping isoform [13,19,20]. A central role for SERCA in calcium transcytosis seemed likely given its established importance in filling ER-calcium stores, sequestering cytosolic calcium and protecting against cell death [13,21,22]. No biochemical characterization of enamel cell SERCA has been reported to date although high levels of Ca^{2+}-ATPase were detected in enamel cell extracts [23,24].

Here we report our molecular and functional characterization of SERCA in rat enamel cells. The principal goals were to use expression profiling to seek clues about SERCA function, and to define SERCA as a target at the molecular and pharmacological levels. Our results indicate that enamel cells express a single major isoform of SERCA that is strongly up-regulated during the calcium-intensive phase of enamel development. Consistent with the ER having a dominant calcium-handling role in enamel cells, this finding establishes SERCA as a strategic target for future investigations of calcium transcytosis. Surprisingly, our results contradict the widespread perception that SERCA2b is restricted to general housekeeping duties.

EXPERIMENTAL

Tissue isolation and extracts

Enamel epithelium was rapidly microdissected from rat mandibular first molars at secretion and maturation phases (5- and 9–10-day-old pups respectively) as described previously [5]. For microsome preparations, freshly isolated epithelia were homogenized by hand with 1 tissue volume (typically 30 μl for 10 epithelia) of ice-cold homogenization buffer (20 mM Tris/HCl, pH 8.0, at 20 °C, 150 mM NaCl, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM aminoethylbenzenesulphonyl fluoride, 1 mM benzamidine, 5 μg/ml leupeptin and 5 μg/ml pepstatin with 1 mM PMSF added just before use). Post-mitochondrial supernatants (15000 ***g*** for 5 min at 4 °C) were ultracentrifuged in a Beckman Airfuge (150000 ***g*** for 6 min at 4 °C), the particulate fraction (termed microsomes) resuspended in homogenization buffer (3–5 mg of protein/ml) and stored at −80 °C.

Other rat tissues were dissected from 6–8-week-old animals and homogenized mechanically (Tissue Tearor; Biospec, Bartlesville, OK, U.S.A.) in 3 tissue volumes (approx. 10 ml) of homogenization buffer. After centrifuging twice at 20000 ***g*** for 30 min at 4 °C, the supernatant was ultracentrifuged at 125000 ***g*** for 60 min. The resultant particulate fraction (microsomes) was washed by two cycles of ultracentrifugation and resuspension in homogenization buffer and stored as described above.

Molecular biology procedures

Total RNA was isolated from enamel epithelium and other rat tissues (stored at −80 °C) using Trizol (Life Technologies) according to the manufacturer's instructions. Yields from enamel epithelium (approx. 80 μg of total RNA from 10 epithelia) were double those obtained previously with the guanidinium acid–phenol–chloroform method [25].

For reverse-transcriptase (RT)-PCR, random-primed cDNA template was generated from total RNA using Expand reagents (reverse transcriptase, hexanucleotide mix, RNase inhibitor) from Roche Molecular Biochemicals. Degenerate primers designed to amplify a 333-bp region (e.g. nucleotides 2620–2952 in rat brain SERCA2b; GenBank® accession number J04022) that is highly conserved (approx. 80% nucleotide identity) in all known isoforms of mammalian SERCA were as follows: forward, 5′-GA(C/T)GC(G/A)CC(C/A)GCTCTGAAGA-3′; reverse, 5′-CATGATGTCCAGATCTGG(A/T)GGATTGAA-3′. PCR products were amplified with *Taq* polymerase (Roche) using 100 ng of DNA template and the following thermal-cycling profile (35 cycles): 20 s at 94 °C, 30 s at 53 °C, 60 s at 72 °C. Restriction endonuclease digests (*Hin*fI and *Bgl*I; New England Biolabs) were carried out according to the manufacturer's instructions, and the products subjected to 2% agarose gel electrophoresis with ethidium bromide staining. Spleen was used as a positive control [20] for co-detection of SERCA3 and SERCA2b transcripts (i.e. PCR products that were labile to *Bgl*I and *Hin*fI, respectively; results not shown).

To isolate the SERCA clone pRnS2b(2)′, a secretory enamel cell cDNA library was screened with ^{32}P-labelled probes randomly primed from the 333-bp SERCA PCR product (Rediprime; Amersham Pharmacia Biotech), as described previously for pRnERp29 [25]. Clone pRnS2b(2)′ was expanded in XL-1 Blue cells and plasmids were purified on silica mini-columns (Plasmid mini kit; Qiagen). Automated DNA sequencing (ABI Model 373 sequencer; Centre for Gene Research, University of Otago, New Zealand) of pRnS2b(2)′ was done in one direction, using external (vector) and internal primer sites (designed from GenBank® accession number J04022). The 333-bp PCR product was also cloned (pCR-Script kit; Stratagene) and sequenced in both directions. For Southern analysis, the pRnS2b(2)′ insert was excised with *Eco*RI and *Xho*I and then hybridized with ^{32}P-labelled 333-bp PCR product using standard procedures [26].

Northern analysis was done as described previously, but with Church and Gilbert reagent [25,26]. The 2.1-kb *SERCA2* fragment was excised from pRnS2b(2)′ with *Eco*RI and *Xho*I, gel purified, and used to prepare randomly primed ^{32}P-labelled probes as described above. Blots from 1% agarose gels were stringency washed to 0.1 × 0.15 M NaCl/0.015 M sodium citrate and 0.4% SDS at 65 °C for 15 min, then the RNA revealed by autoradiography.

Protein gel electrophoresis procedures

SDS/PAGE (Laemmli discontinuous buffer system) and immunoblotting were carried out as described previously [5,6]. Immunoblots were developed using an avidin–biotin-amplified

alkaline phosphatase detection system (Vectastain ABC kit; Vector) and quantified by imaging densitometry under linear conditions.

Ca^{2+}-ATPase and phosphoenzyme assays

To assay Ca^{2+}-ATPase, microsomes were pre-incubated in 25 μl of Ca^{2+}-ATPase buffer (50 mM Tris/HCl, pH 7.2, and 100 mM KCl) with 0–1 mM $CaCl_2$ added as indicated, for 3 min at 20 °C. The reaction was started by adding 1 μl of ^{32}P-labelled MgATP [100 μM MgATP adjusted to pH 7 with NaOH, spiked with 0.01 volume of [γ-^{32}P]ATP (370 MBq·ml^{-1}) (Amersham)] and stopped with 1-volume ice-cold 20 % trichloroacetic acid. After precipitation (for 15 min on ice) and centrifuging (17000 ***g*** for 10 min at 4 °C), ^{32}P was extracted from the supernatant using the acidic molybdate procedure [27] and quantified by Cerenkov counting. Samples were assayed under linear conditions with < 3 % substrate consumption, and calcium-independent activity (i.e. from samples with excess EGTA) was subtracted. Where indicated, ATPase inhibitors were added at the beginning of the preincubation step from 100-fold concentrated stocks in DMSO (controls contained an equivalent amount of DMSO).

The acid-stable SERCA phosphoenzyme intermediate was assayed essentially as described previously [28]. Control experiments verified that the observed ^{32}P-phosphoenzyme bands (most intense at 90 kDa, but extending variably from 115–70 kDa) exhibited characteristic properties including: rapid turnover during a cold-ATP chase; sensitivity to EGTA, vanadate and lanthanum; exclusive localization in the particulate fraction (results not shown).

Other methods and materials

Wistar-derived rats were given unlimited access to a standard pellet chow and maintained as described previously [5]. Rabbit antiserum C4 which recognizes SERCA isoforms 1, 2a, 2b and 3 from rat [18,29] was the generous gift of Dr J. Lytton (Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Canada). Rabbit antiserum against protein disulphide isomerase from rat liver was prepared in this laboratory by Dr S. Shnyder. Protein was quantified by dye-binding microassay with BSA as standard [5]. Statistical comparisons of paired mean values were done with Student's *t*-test (two-tailed, homoscedastic). Cyclopiazonic acid, clotrimazole, oligomycin and lanthanum were obtained from Sigma, thapsigargin from Alomone (Jerusalem, Israel), tbuBHQ from Biomol and vanadate from Aldrich. Other materials were as described previously [6,30].

RESULTS

RT-PCR analysis of SERCA isoforms

To evaluate SERCA transcript expression in rat enamel cells, RT-PCR was performed with degenerate primers for a 333-bp region that is highly conserved in all known *SERCA* isoforms. A PCR product of the expected mass was obtained from enamel cell cDNA (Figure 1). This product was fully cleaved by *Hin*fI, an endonuclease specific for SERCA2, but completely resistant to the SERCA3-specific nuclease *Bgl*I (results not shown). Equivalent results were obtained with liver, a tissue that predominantly expresses SERCA2b (Figure 1). Accordingly, enamel cells appeared to express a single major type of *SERCA* transcript derived from the *SERCA2* gene.

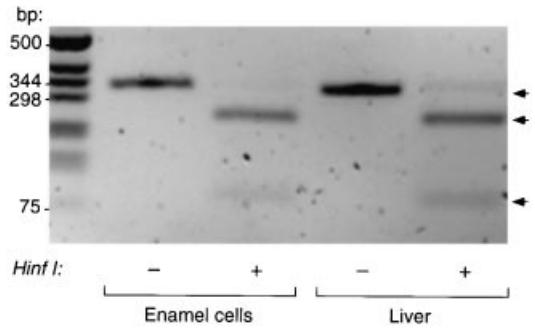

Figure 1 Restriction analysis of SERCA RT-PCR products

Total RNA from rat enamel cells and liver was subjected to RT-PCR analysis using pan-specific *SERCA* primers. After digestion with *Hin*fI as indicated, PCR products were resolved by 2% agarose gel electrophoresis with ethidium bromide staining (molecular mass markers are shown). The major 333-bp PCR product was completely cleaved into 243-bp and 90-bp fragments by *Hin*fI in both tissues (arrowheads), indicating a predominance of SERCA*2* transcript. No PCR product was detected in controls with RT omitted (results not shown). This result was obtained in three independent experiments.

Isolation of a SERCA2b clone

An enamel cell cDNA library was screened for SERCA clones using the 333-bp PCR product as probe. The single SERCA-like clone isolated from 200000 plaques screened was positively identified by Southern analysis. Complete sequence analysis of this 3.4-kb clone, here named pRnS2b(2)′, revealed near-identity with SERCA2b cDNAs isolated from rat brain and kidney [31]. By analogy, pRnS2b(2)′ comprised 72 % of the coding sequence followed by 822 nt of 3′ untranslated region and a short poly(A) tail (results not shown; GenBank® accession number AF043106). A single coding-nt mismatch did not alter the encoded amino acid, leucine (i.e. nucleotide T911 in enamel cell sequence versus C1938 for brain). The 3′-untranslated region was identical to that of the short SERCA2b transcript from kidney (class 2; clone RK7-12 [31]) except that pRnS2b(2)′ extended for a further 7 nt before the poly(A) tail. Consequently, pRnS2b(2)′ appeared to represent a variant class-2 SERCA2b transcript of approx. 4.5 kb. Inspection of the longer transcript from brain (class 4, GenBank® accession number J04022) revealed a single upstream polyadenylation motif (nucleotides 4430–4435) [31] and two consensus downstream elements for pre-mRNA cleavage [32] (nt 4467–4474 and 4498–4505), which may account for the cleavage site heterogeneity evident between the enamel cell and kidney transcripts. Equivalent heterogeneity in the corresponding transcripts from human and mouse was revealed by analysis of the expressed sequence tag database, but any functional significance of this previously unreported cleavage pattern remains unclear.

Northern analysis

Four classes of SERCA2 transcript have been characterized in rat tissues by cDNA cloning and Northern blotting [31,33,34]. Major (approx. 4.5 kb) and minor (approx. 8 kb) transcript bands were revealed by Northern analysis of enamel cell RNA (Figure 2). These two transcripts coincided with the class-2 and -3 SERCA2b transcripts in liver (approx. 4.5 kb and 8 kb, respectively), and were clearly distinguished from the class-4 SERCA2b (5.6 kb) transcript in brain. No SERCA2a transcript band (5.0 kb, class 1) was detected in enamel cells, although it was readily discerned in heart (Figure 2). The observed expression in enamel cells of class-2 $\gg$ class-3 transcripts, but not the

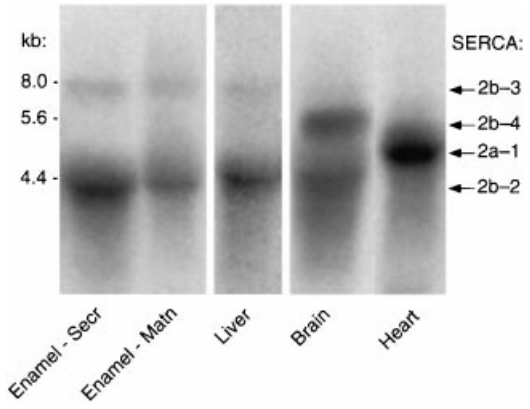

Figure 2 Northern analysis of SERCA transcripts

Total RNA (20 μg) blots were hybridized with ^{32}P-labelled probes randomly primed from a 2.1-kb fragment of SERCA2 cDNA, and then revealed by autoradiography. Secretory (Secr) and maturation (Matn) enamel cells gave a major 4.4-kb band that corresponded with class-2 SERCA2b transcripts in liver and brain, and a weaker 8-kb band that matched class-3 SERCA2b transcript in liver. Enamel cells lacked both the class-4 SERCA2b transcript (5.6 kb) expressed in brain, and the class-1 SERCA2a transcript (5 kb) detected in heart. All lanes are from the same blot and radiographic exposure (6 h), except for liver which was exposed for 14 h. This result is representative of three independent experiments.

neuronal class-4 transcript, is consistent with reports for other nonexcitable tissues [20,33,34]. Together with the RT-PCR results, these findings suggested that SERCA2b is the major isoform expressed in enamel cells during secretion and maturation, and identified pRnS2b(2)′ as a clone of the predominant class-2 SERCA2b transcript.

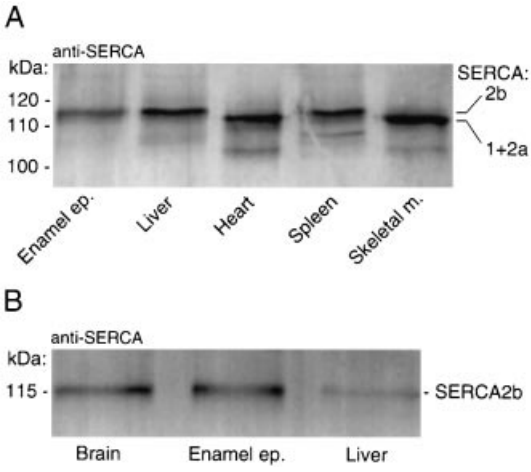

Figure 3 Immunoblot analysis of SERCA expression

Microsomal fractions were resolved by long-run SDS/PAGE (7.5% total acrylamide) and immunoblotted with pan-specific SERCA antibodies. (**A**) SERCA immunoreactivity in enamel epithelium coincided with SERCA2b (115 kDa) in liver and spleen. Marginally faster mobilities were exhibited by SERCA2a in heart and SERCA-1 and -2a in skeletal muscle as indicated (approx. 112 kDa). Samples (0.5–5 μg protein) were loaded to give similar amounts of immunoreactivity, and the indicated mass calibration was from Amido Black-stained standards in an adjacent lane. The minor immunoreactive bands of faster mobility remain unidentified, but could represent SERCA3 (108 kDa band in spleen) or proteolytic degradation (105 kDa band in muscle). (**B**) Specific abundance of SERCA2b in maturation enamel cells was similar to that in brain, and approx. 3-fold higher than in liver. Sample loads (3 μg protein) were equated by SDS/PAGE with Coomassie Blue staining, as illustrated in Figure 4(A). Results are representative of at least four and three separate experiments in A and B, respectively.

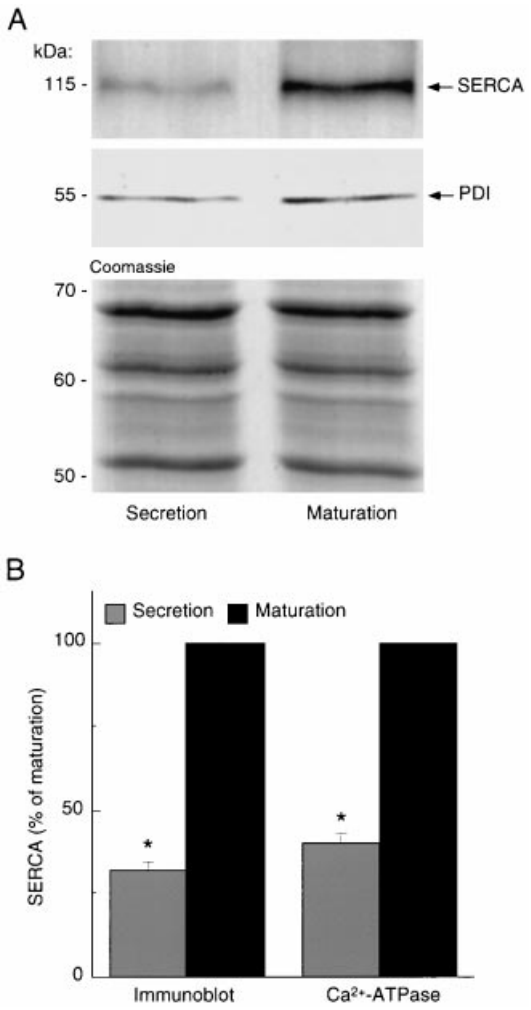

Figure 4 Developmental regulation of SERCA in enamel cells

(**A**) Secretion- and maturation-phase microsomes (1.6 μg protein) were immunoprobed on the same blot with anti-SERCA and anti-(protein disulphide isomerase) (PDI) (upper panel). Parallel samples were stained with Coomassie Blue (lower panel). The specific abundance of SERCA2b was markedly higher at maturation, unlike that of protein disulphide isomerase. (**B**) Comparative analysis of SERCA2b immunoreactivity [Figure 4A (upper panel)] and nonmitochondrial Ca^{2+}-ATPase activity (e.g. Figure 5A) during secretion and maturation. An equivalent up-regulation at maturation (approx. 3-fold) was evident from both assays. Immunoblot and Ca^{2+}-ATPase results, expressed as means $\pm$ S.E.M., are from six and three separate experiments respectively, normalized to the maturation values. Significant differences for pairwise comparisons between secretion and maturation are indicated ($^*P < 0.01$).

Immunoblot analysis of SERCA expression

To characterize SERCA expression at the protein level, membrane fractions were immunoblotted with a pan-specific SERCA antibody [18,29]. As shown in Figure 3(A), the single 115-kDa band detected in enamel cells coincided with SERCA2b in liver and spleen, and was marginally resolved from SERCA2a in heart and SERCA1/2a in skeletal muscle (approx. 112 kDa) when subjected to electrophoresis under optimized conditions. A slightly lower mobility of enamel cell and liver SERCA2b, relative to heart SERCA2a, was confirmed in sample mixing experiments (results not shown). The specific abundance of SERCA2b in enamel cells was similar to that in brain, and about 3-fold higher than in liver (Figure 3B).

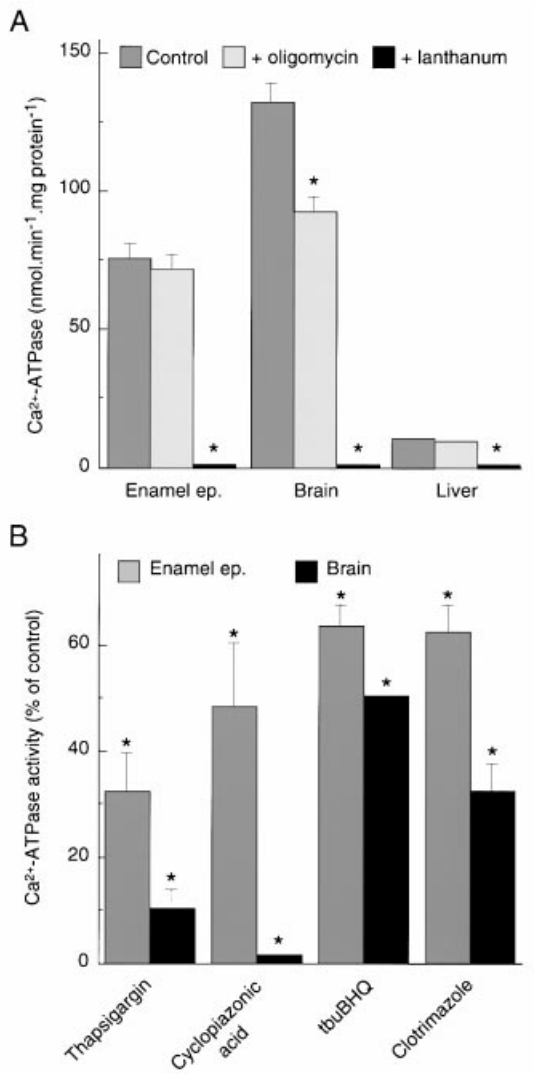

Figure 5 Characterization of SERCA activity in enamel cells

Microsomes were assayed for calcium-dependent ATPase (Ca^{2+}-ATPase) activity during 6 min period at 20 °C. The assay buffer contained 0.1 mM $CaCl_2$, and sample protein loads were 0.6 μg, 0.3 μg and 1.1 μg for maturation enamel epithelium (Enamel ep), brain and liver, respectively. (**A**) Ca^{2+}-ATPase activity without (Control) and with oligomycin (10 μM) or lanthanum (500 μM), as indicated. (**B**) Inhibition of Ca^{2+}-ATPase by thapsigargin (100 μM), cyclopiazonic acid (1 mM), tbuBHQ (1 μM) and clotrimazole (200 μM), as indicated. All inhibitor concentrations were the minimum required for maximum effect on enamel cell Ca^{2+}-ATPase (results not shown). Results, expressed as mean $\pm$ S.E.M., are from at least three separate experiments, except for cyclopiazonic acid and tbuBHQ in brain where two experiments were performed. Significant differences for pairwise comparisons with inhibitor-free controls are indicated (**A**, $^*P < 0.05$; **B**, $^*P < 0.01$). Enamel secretion-phase samples exhibited equivalent sensitivity to thapsigargin (results not shown).

During enamel maturation, SERCA2b levels were substantially higher than at secretion (Figure 4A). Densitometry gave an increase of 3.1-fold when normalized against Coomassie-stained protein (Figure 4B). Unlike SERCA2b, protein disulphide isomerase (another ER resident protein) was expressed at similar levels during secretion and maturation (Figure 4A), as described previously [6]. These findings showed that SERCA2b is specifically up-regulated to high levels during enamel maturation, and suggested that SERCA2b is the major isoform expressed in enamel cells, consistent with the results from the transcript experiments.

ATPase activity and inhibitor sensitivity of enamel cell SERCA

Functional characteristics of enamel cell SERCA were investigated using a conventional Ca^{2+}-ATPase assay (Figure 5). Oligomycin was used to inhibit any mitochondrial ATPase activity present in the microsomal fractions. A relatively strong oligomycin-insensitive Ca^{2+}-ATPase activity (approx. 75 nmol/min per mg of protein) was detected in maturation enamel cells, and lanthanum gave complete inhibition (Figure 5A). This non-mitochondrial Ca^{2+}-ATPase activity was 3-fold lower in secretion cells, in parallel with SERCA immunoreactivity (Figure 4B). Qualitatively similar activities were detected in liver and brain microsomes, although significant mitochondrial contamination was evident in the latter.

As shown in Figure 5(B), enamel cell Ca^{2+}-ATPase was inhibited markedly by thapsigargin (approx. 70 %), and to a lesser extent by cyclopiazonic acid and tbuBHQ (55 % and 35 % respectively). Clotrimazole, recently characterized as a SERCA inhibitor [35], also gave partial inhibition equivalent to tbuBHQ. A similar pattern was obtained for brain microsomes, but the inhibition was more profound in all cases (Figure 5). We undertook several experiments to investigate the apparently modest efficacy of thapsigargin against the enamel cell Ca^{2+}-ATPase (high micromolar concentrations were required for maximum inhibition). First, thapsigargin sensitivity was found to vary inversely with amount of sample assayed, for both enamel cells and brain. For example, when enamel cell membranes were assayed as in Figure 5(B), inhibition values were 68 % and 45 % for 0.6 μg and 1.0 μg protein loads respectively. Consequently, the higher sensitivity of brain SERCA appeared at least partially due to the smaller amount of sample assayed (0.3 μg protein, Figure 5B). Second, the effects of varied calcium concentration (0–1 mM) were evaluated since high calcium can protect SERCA against thapsigargin [36,37]. Thapsigargin sensitivity was independent of calcium up to 0.2 mM, but decreased at higher levels. Likewise, addition of a calcium ionophore (0.1 μM A23187) did not affect thapsigargin sensitivity in enamel cells, although a significant benefit was observed in brain. Third, phosphoenzyme analysis suggested that enamel cell SERCA2b was particularly susceptible to proteolysis, since ^{32}P-phosphoenzyme bands were spread diffusely between 115 kDa and 70 kDa. In contrast, a more distinct major ^{32}P-band was observed near 115 kDa in liver and brain (results not shown). Together, these functional characteristics supported the molecular evidence that SERCA2b is abundant and up-regulated in maturation enamel cells, and established that enamel cell SERCA activity can be discerned using a variety of well-characterized inhibitors.

DISCUSSION

We have found that SERCA2b is up-regulated to high expression levels during enamel maturation consistent with a central role in the transport of calcium across enamel cells. Our results associate SERCA with transcellular calcium transport for the first time, and establish SERCA2b as a molecular and pharmacological target to evaluate ER function in the proposed calcium transcytosis mechanism. It can be inferred that enamel cells are well-equipped to sequester calcium into ER stores and thereby avoid the cytotoxic effects of excess cytosolic calcium. The physiological regulation of SERCA2b in enamel cells contrasts strikingly with the constitutive expression patterns reported in other tissues, and so indicates that SERCA2b is not functionally restricted to a general housekeeping role.

Evidence that enamel cells express a single major SERCA isoform was obtained at both the transcript and protein levels (Figures 1–3). Moreover, comparison of secretion and maturation phases indicated that the change in enamel cell function was not accompanied by a switch of dominant SERCA isoform. While only SERCA2b was detected, the possibility remains that other SERCAs were expressed at lower levels or during the presecretory stage of enamel development not examined here. The finding that enamel cells and brain express the same dominant SERCA isoform at comparable abundance levels (Figure 3) extends our earlier evidence that these tissues have a similar complement of calcium-handling machinery, both qualitatively and quantitatively [5,6].

The proposal that ER-calcium stores mediate vectorial, transcellular calcium transport (calcium transcytosis) is supported by our findings that SERCA is expressed abundantly in enamel cells, and SERCA levels increased in parallel with calcium transport. SERCA expression in maturation enamel cells is uncommonly high for a nonexcitable tissue, as evident from comparison with reference tissues that express SERCA at high and moderate levels (brain and liver respectively [20,29]; Figures 3 and 5). Such high abundance of SERCA conforms with the implicit need of enamel cells to handle calcium in bulk and with our previous evidence that other calcium store proteins are expressed at unusually high levels [6]. The rate of calcium transport is estimated to be 3-fold higher during maturation than secretion of enamel in rat [8] which matches the observed 2–3-fold up-regulation of SERCA, calreticulin and endoplasmin (Figure 4 and [6]). Accordingly, the developmental regulation of SERCA parallels calcium transport both temporally and quantitatively. We note that up-regulation of *SERCA* was not evident at the mRNA level, despite the striking change in protein levels detected by Ca^{2+}-ATPase and immunoblot analysis (Figures 2 and 4). A similarly poor correspondence between transcript and protein expression has been observed by others and ascribed to post-transcriptional regulation [38,39]. This finding reinforces the value of extending SERCA analyses through to the protein level.

To our knowledge, SERCA has not been associated previously with transcellular calcium transport, and so any functional involvement remains to be established. We anticipate that SERCA might be involved in maintaining a vectorial flux of bulk calcium, in addition to its accepted role in loading calcium stores. Indeed, SERCA does have a well established role in vectorial movement of calcium across cells, albeit for the relatively small fluxes associated with calcium signalling. The calcium-induced calcium-release mechanism used to propagate calcium signals in heart and other electrically excitable tissues relies on SERCA for calcium re-uptake [21]. Similarly, in an electrically nonexcitable tissue (pancreatic acinar cells), it was found that serosal calcium followed a thapsigargin-sensitive transcellular route during replenishment of calcium stores at the secretory pole [40]. It seems plausible that an up-scaled version of such mechanisms could be used to translocate calcium in bulk across enamel cells, given the SERCA expression pattern observed here.

Our results presented here justify the use of SERCA2b as a molecular marker to delineate ER-calcium stores in enamel cells. SERCA can now also be adopted as a strategic target to test the calcium transcytosis hypothesis functionally, once suitable assays have been developed. For example, calcium transport might be compromised by thapsigargin-induced depletion of calcium stores. Sensitivity to thapsigargin and other SERCA inhibitors was established (Figure 5), but the relatively low potencies observed in enamel cell preparations raise some potential concerns. Thapsigargin is considered to be highly selective for SERCA, subject to use at low nanomolar concentrations [16]. However, many reports of poor sensitivity to thapsigargin appear in the literature and several contributing factors have been recognized including non-specific adsorption to bulk tissue and plastic surfaces, and indirect competition by calcium [36,37,41,42]. Our finding that the large amount of membranes assayed contributed to the low efficacy of thapsigargin was supported by recent investigations (I. K. Franklin and M. J. Hubbard, unpublished work) of permeabilized enamel cells, where thapsigargin was effective at 20-fold lower concentrations than in this study. The particular susceptibility of enamel cell SERCA2b to proteolysis, as revealed by phosphoenzyme analysis, might also have contributed to the low thapsigargin sensitivity although a mechanistic basis for this is not known. Increased confidence about inhibitor specificity can be gained by comparing the effects of several such compounds (Figure 5), since each has distinctive pharmacological properties [14–16,35]. It is plausible that our microsome preparations also contained significant amounts of thapsigargin-insensitive Ca^{2+}-ATPases (i.e. non-SERCA), such as those associated with plasmalemma, the secretory pathway and other cellular fractions [43,44].

The striking developmental regulation of SERCA2b in enamel cells contradicts the widespread impression that this isoform fulfills only 'housekeeping duties'. Recognition of SERCA2b as a generic housekeeper followed evidence of universal cellular distribution, and constitutive expression during a variety of developmental and pathophysiological situations [19,20,28]. The production of *SERCA2*-knockout mice established that SERCA2b is essential for life, consistent with a fundamental housekeeping function [45]. Conversely, *SERCA3*-null mutant mice were viable [45], and SERCA-1, -2a and -3 isoforms are expressed in relatively few cell types and subject to regulation *in vivo* [13,20,46]. SERCA2b expression was recently shown to be regulated in a cell-type-specific manner following drug-induced activation of lymphoblastoid and myeloid cell lines [47]. A 2-fold increase in SERCA2b was also observed after induction of the ER-stress response in a neuroendocrine cell line, PC12 [38]. Together these studies indicate that SERCA2b is indeed subject to regulatory control, at least *in vitro*. The present finding in enamel cells constitutes the first report of SERCA2b regulation during a normal physiological process *in vivo*, as far as we are aware.

Calcium transcytosis uncertainties aside, we infer that SERCA2b has a specialist role to handle the large fluxes of calcium associated with enamel mineralization, in addition to general calcium homoeostatic duties. SERCA2b is distinguished by a relatively high affinity for calcium, but low turnover rate [17,18]. In the context of enamel cells, these properties would help avoid calcium toxicity by maximizing the ability to maintain cytosolic calcium at low levels. The trade-off in the reduction of transport rate is apparently countered by high expression levels of SERCA2b during the calcium-transport phase. Another beneficial characteristic of SERCA2b might be the potential for regulation by calreticulin and calnexin, a property not shared by SERCA2a [48]. Interestingly, SERCA2b is the predominant isoform in other calcium-transporting tissues (e.g. kidney, small intestine) where it is also expressed at relatively high levels [20]. SERCA3 was proposed to have a specialist role handling bulk calcium since it exhibited substantially lower affinities for calcium [17,18]. However, we found no evidence that SERCA3 is expressed at high levels in enamel cells (Figures 1 and 3).

In conclusion, this study has provided new evidence that enamel cells have a strong capability to sequester calcium in non-mitochondrial stores. The principal components of ER-calcium stores (i.e. calcium pump, calcium buffers and calcium-release

channel) have now all been found expressed at unusually high levels (present study and [6]), suggesting that calcium protection is a dominant ER function in enamel cells. Intriguingly, the up-regulation of SERCA2b during maturation opposes the down-regulation of ERp29, an ER resident apparently involved in secretory protein synthesis and not calcium handling [30]. It therefore appears that the ER undergoes a developmental switch in its primary role, from the production of secretory proteins to a calcium store. Questions now follow about the identities of other ER proteins (e.g. calcium-release channels) in enamel cells and how they function collectively as calcium stores during transcellular calcium transport. It is anticipated that further investigations of these exceptionally calcium-orientated cells will provide useful insights into calcium regulation and the calcium toxicity problem.

We thank Nicky McHugh for skilled technical assistance, Jerome Demmer for advice on cDNA screening, and Jon Mangum for information about pre-mRNA processing. Jonathan Lytton and Steve Shnyder are thanked for providing antibodies. Thanks also to our colleagues and J. Lytton for constructive critique of the manuscript and support. This work was funded by the Health Research Council (New Zealand), New Zealand Lottery Health Research and the University of Otago (Postgraduate Scholarship to I.K.F.). M.J.H. is a Senior Research Fellow of the Health Research Council (New Zealand).

REFERENCES

1 Berridge, M. J. (1994) The biology and medicine of calcium signalling. Mol. Cell. Endocrinol. **98**, 119–124
2 Trump, B. F. and Berezesky, I. K. (1995) Calcium-mediated cell injury and cell death. FASEB J. **9**, 219–228
3 Klapstein, G. J., Vietla, S., Lieberman, D. N., Gray, P. A., Airaksinen, M. S., Thoenen, H., Meyer, M. and Mody, I. (1998) Calbindin-$D_{28\,k}$ fails to protect hippocampal neurons against ischemia in spite of its cytoplasmic buffering properties: evidence from calbindin-$D_{28\,k}$ knockout mice. Neuroscience **85**, 361–373
4 Bouilleret, V., Schwaller, B., Schurmans, S., Celio, M. R. and Fritschy, J. M. (2000) Neurodegenerative and morphogenic changes in a mouse model of temporal lobe epilepsy do not depend on the expression of the calcium-binding proteins parvalbumin, calbindin or calretinin. Neuroscience **97**, 47–58
5 Hubbard, M. J. (1995) Calbindin$_{28\,kDa}$ and calmodulin are hyperabundant in rat dental enamel cells: Identification of the protein phosphatase calcineurin as a principal calmodulin target and of a secretion-related role for calbindin$_{28\,kDa}$. Eur. J. Biochem. **230**, 68–79
6 Hubbard, M. J. (1996) Abundant calcium homeostasis machinery in rat dental enamel cells. Up-regulation of calcium store proteins during enamel hypermineralization implicates the endoplasmic reticulum in calcium transcytosis. Eur. J. Biochem. **239**, 611–623
7 Smith, C. E. (1998) Cellular and chemical events during enamel maturation. Crit. Rev. Oral Biol. Med. **9**, 128–161
8 Hubbard, M. J. (2000) Calcium transport across the dental enamel epithelium. Crit. Rev. Oral Biol. Med. **11**, 437–466
9 Bronner, F. (1991) Calcium transport across epithelia. Int. Rev. Cytol. **131**, 169–212
10 Peng, J. B., Chen, X. Z., Berger, U. V., Vassilev, P. M., Brown, E. M. and Hediger, M. A. (2000) A rat kidney-specific calcium transporter in the distal nephron. J. Biol. Chem. **275**, 28186–28194
11 Feher, J. J., Fullmer, C. S. and Wasserman, R. H. (1992) Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am. J. Physiol. **262**, C517–C526
12 Hubbard, M. J. and McHugh, N. J. (1996) Mitochondrial ATP synthase F_1-β-subunit is a calcium-binding protein. FEBS Lett. **391**, 323–329
13 Misquitta, C. M., Mack, D. P. and Grover, A. K. (1999) Sarco/endoplasmic reticulum Ca^{2+} (SERCA)-pumps: link to heart beats and calcium waves. Cell Calcium **25**, 277–290
14 Khan, Y. M., Wictome, M., East, J. M. and Lee, A. G. (1995) Interactions of dihydroxybenzenes with the Ca^{2+}ATPase: separate binding sites for dihydroxybenzenes and sesquiterpene lactones. Biochemistry **34**, 14385–14393
15 Soler, F., Plenge-Tellechea, F., Fortea, I. and Fernandez-Belda, F. (1998) Cyclopiazonic acid effect on Ca^{2+}-dependent conformational states of the sarcoplasmic reticulum ATPase. Implication for enzyme turnover. Biochemistry **37**, 4266–4274
16 Tremain, M., Caspersen, C. and Christensen, S. B. (1998) A tool coming of age. Thapsigargin as an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases. Trends Pharmacol. Sci. **19**, 131–135
17 Dode, L., de Greef, C., Mountian, I., Attard, M., Town, M. M., Casteels, R. and Wuytack, F. (1998) Structure of the human sarco/endoplasmic reticulum Ca^{2+}-ATPase 3 gene. J. Biol. Chem. **273**, 13982–13994
18 Poch, E., Leach, S., Snape, S., Cacic, T., MacLennan, D. H. and Lytton, J. (1998) Functional characterization of alternatively spliced human SERCA3 transcripts. Am. J. Physiol. **275**, C1449–C1458
19 Anger, M., Samuel, J. L., Marotte, F., Wuytack, F., Rappaport, L. and Lompre, A. M. (1994) In situ mRNA distribution of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoforms during ontogeny in the rat. J. Mol. Cell. Cardiol. **26**, 539–550
20 Wu, K. D., Lee, W. S., Wey, J., Bungard, D. and Lytton, J. (1995) Localization and quantification of endoplasmic reticulum Ca^{2+}-ATPase isoform transcripts. Am. J. Physiol. **269**, C775–C784
21 Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. Nature (London) **361**, 315–325
22 Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol. Rev. **74**, 595–636
23 Mornstad, H. (1978) Calcium-stimulated ATPase activity in homogenates of the secretory enamel organ in the rat. Scand. J. Dent. Res. **86**, 1–11
24 Yoshimura, F. and Suzuki, T. (1975) Calcium-stimulated adenosine triphosphatase in the microsomal fraction of tooth germs from porcine fetus. Biochim. Biophys. Acta **410**, 167–177
25 Demmer, J., Zhou, C. M. and Hubbard, M. J. (1997) Molecular cloning of ERp29, a novel and widely expressed resident of the endoplasmic reticulum. FEBS Lett. **402**, 145–150
26 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn.,, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27 Chan, K. M., Delfert, D. and Junger, K. D. (1986) A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. **157**, 375–380
28 Magnier, C., Bredoux, R., Kovacs, T., Quarck, R., Papp, B., Corvazier, E., de Gunzburg, J. and Enouf, J. (1994) Correlated expression of the 97 kDa sarcoendoplasmic reticulum Ca^{2+}-ATPase and Rap1B in platelets and various cell lines. Biochem. J. **297**, 343–350
29 Lytton, J. and MacLennan, D. H. (1988) Molecular cloning of cDNAs from human kidney coding for two alternatively spliced products of the cardiac Ca^{2+}-ATPase gene. J. Biol. Chem. **263**, 15024–15031
30 Hubbard, M. J., McHugh, N. J. and Carne, D. L. (2000) Isolation of ERp29, a novel endoplasmic reticulum protein, from rat enamel cells: Evidence for a unique role in secretory-protein synthesis. Eur. J. Biochem. **267**, 1945–1956
31 Gunteski-Hamblin, A. M., Greeb, J. and Shull, G. E. (1988) A novel Ca^{2+} pump expressed in brain, kidney and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca-ATPase gene. J. Biol. Chem. **263**, 15032–15040
32 Zhao, J., Hyman, L. and Moore, C. (1999) Formation of mRNA 3′ ends in eukaryotes. Mechanism, regulation and interrelationships with other steps in mRNA synthesis. Microbiol. Mol. Biol. Rev. **63**, 405–445
33 Burk, S., Lytton, J., MacLennan, D. H. and Shull, G. E. (1989) cDNA cloning, functional expression and mRNA tissue distribution of a third organellar calcium pump. J. Biol. Chem. **264**, 18561–18568
34 Wuytack, F., Raeymaekers, L., de Smedt, H., Eggermont, J. A., Missiaen, L., van den Bosch, L., de Jaegere, S., Verboomen, H., Plessers, L. and Casteels, R. (1992) Ca^{2+}-transport ATPases and their regulation in muscle and brain. Ann. N.Y. Acad. Sci. **671**, 82–91
35 Snajdrova, L., Xu, A. and Narayanan, N. (1998) Clotrimazole, an antimycotic drug, inhibits the sarcoplasmic reticulum calcium pump and contractile function in heart muscle. J. Biol. Chem. **273**, 28032–28039
36 Wells, K. M. and Abercrombie, R. F. (1998) Luminal Ca^{2+} protects against thapsigargin inhibition in neuronal endoplasmic reticulum. J. Biol. Chem. **273**, 5020–5025
37 Caspersen, C. and Treiman, M. (1995) Thapsigargin discriminates strongly between Ca^{2+}-ATPase phosphorylated intermediates with different subcellular distributions in bovine adrenal chromaffin cells. FEBS Lett. **377**, 31–36
38 Caspersen, C., Pedersen, P. S. and Treiman, M. (2000) The sarco/endoplasmic reticulum calcium-ATPase 2b is an endoplasmic reticulum stress-inducible protein. J. Biol. Chem. **275**, 22363–22372
39 Misquitta, C. M., Sing, A. and Grover, A. K. (1999) Control of sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump expression in cardiac and smooth muscle. Biochem. J. **338**, 167–173
40 Mogami, H., Nakano, K., Tepikin, A. V. and Petersen, O. H. (1997) Ca^{2+} flow via tunnels in polarized cells. Recharging of apical Ca^{2+} stores by focal Ca^{2+} entry through basal membrane patch. Cell **88**, 49–55

41 Baudet, S., Shaoulian, R. and Bers, D. M. (1993) Effects of thapsigargin and cyclopiazonic acid on twitch force and sarcoplasmic reticulum Ca^{2+} content of rabbit ventricular muscle. Circ. Res. **73**, 813–819
42 Du, G. G., Ashley, C. C. and Lea, T. J. (1994) Effects of thapsigargin and cyclopiazonic acid on the sarcoplasmic reticulum Ca^{2+} pump of skinned fibres from frog skeletal muscle. Eur. J. Physiol. **429**, 169–175
43 Pizzo, P., Fasolato, C. and Pozzan, T. (1997) Dynamic properties of an inositol 1,4,5-trisphosphate- and thapsigargin-insensitive calcium pool in mammalian cell lines. J. Cell Biol. **136**, 355–366
44 Sorin, A., Rosas, G. and Rao, R. (1997) PMR1, a Ca^{2+}-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. J. Biol. Chem. **272**, 9895–9901
45 Shull, G. E. (2000) Gene knockout studies of Ca^{2+}-transporting ATPases. Eur. J. Biochem. **267**, 5284–5290
46 Lacabaratz-Porret, C., Corvazier, E., Kovacs, T., Bobe, R., Bredoux, R., Launay, S., Papp, B. and Enouf, J. (1998) Platelet sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 3b and Rap 1b. Interrelation and regulation in physiopathology. Biochem. J. **332**, 173–181
47 Launay, S., Gianni, M., Kovacs, T., Bredoux, R., Bruel, A., Gelebart, P., Zassadowski, F., Chomienne, C., Enouf, J. and Papp, B. (1999) Lineage-specific modulation of calcium pump expression during myeloid differentiation. Blood **93**, 4395–4405
48 Roderick, H. L., Lechleiter, J. D. and Camacho, P. (2000) Cytosolic phosphorylation of calnexin controls intracellular Ca^{2+} oscillations via an interaction with SERCA2b. J. Cell Biol. **149**, 1235–1247

Received 25 January 2001/18 April 2001; accepted 4 May 2001