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Modulation of endoplasmic reticulum calcium pump expression during lung cancer cell differentiation

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Running title : ER calcium pumps in lung cancer

Keywords: SERCA, lung cancer, differentiation, calcium, endoplasmic reticulum

Abstract

Cellular calcium signaling plays important roles in several signal transduction pathways that control proliferation, differentiation and apoptosis. In epithelial cells calcium signaling is initiated mainly by calcium release from endoplasmic reticulum-associated intracellular calcium pools. Because calcium is accumulated in the endoplasmic reticulum by Sarco/Endoplasmic Reticulum Calcium ATPases (SERCA), these enzymes play a critical role in the control of calcium-dependent cell activation, growth and survival. We investigated the modulation of SERCA expression and function in human lung adenocarcinoma cells. In addition to the ubiquitous SERCA2 enzyme, the SERCA3 isoform was also expressed at variable levels. SERCA3 expression was selectively enhanced during cell differentiation in lung cancer cells, and marked SERCA3 expression was found in fully differentiated normal bronchial epithelium. As studied by using a recombinant fluorescent calcium probe, induction of the expression of SERCA3, a lower calcium affinity pump, was associated with decreased intracellular calcium storage, whereas the amplitude of capacitative calcium influx remained unchanged. Our observations indicate that the calcium homeostasis of the endoplasmic reticulum in lung adenocarcinoma cells presents a functional defect due to decreased SERCA3 expression that is corrected during pharmacologically induced differentiation. The data presented in this work show, for the first time, that endoplasmic reticulum calcium storage is anomalous in lung cancer cells, and suggest that SERCA3 may serve as a useful new phenotypic marker for the study of lung epithelial differentiation.

Introduction

Non-small cell lung cancer is currently the most frequent fatal malignancy worldwide with more than a million deaths occurring annually [1, 2]. Although much is known about risk factors such as tobacco smoking, or regarding molecular and genetic events that occur during lung tumorigenesis, the overall survival rate of lung cancer could not be significantly improved during the last thirty years [2]. This indicates that currently available knowledge of the molecular biological behavior of lung carcinoma cells is insufficient for the development of efficient therapy. In particular, knowledge about calcium signaling in lung cancer cells is scarce, although calcium signaling is an essential component of several regulatory networks involved in the control of cell growth, differentiation and apoptosis.

Calcium-dependent cell activation is initiated by the release of calcium ions stored in the endoplasmic reticulum (ER) into the cytosol through inositol-1,4,5-*tris*phosphate receptor calcium channels [3]. In addition, calcium in the ER lumen is required for the function of various ER located chaperones such as calreticulin or calnexin involved in the conformational maturation and posttranslational modification of newly synthesized proteins that transit through the organelle [4-6]. Abnormal endoplasmic reticulum calcium homeostasis can lead to cellular stress responses, growth arrest and apoptosis [4, 7-9].

Sarco/Endoplasmic Reticulum Calcium ATPases (SERCA) enzymes are essential for the dynamic equilibrium of ER calcium homeostasis. Located in the ER membrane, these enzymes sequester calcium ions from the cytosol into the ER lumen by ATP-dependent active ion transport, and thus generate a steep calcium concentration gradient between the cytosol (approximately 50-100 nM) and the ER lumen (approximately 0.1 mM) [10]. SERCA proteins are encoded by three genes (ATP2A1, 2 and 3), that can give rise to several protein

isoforms by alternative splicing [11-15]. SERCA expression is tissue dependent and developmentally regulated. SERCA1 is expressed in skeletal muscle, and the SERCA2a isoform is mainly expressed in cardiomyocytes. Whereas SERCA2b is a ubiquitously expressed isoform thought to be involved in "housekeeping" functions, in several cell types such as cells of hematopoietic origin, gastric and colonic epithelial cells, β-cells of islets of Langerhans or vascular endothelium, SERCA2b has been shown to be co-expressed with SERCA3 [15-26]. Because SERCA2b and SERCA3 enzymes display distinct calcium affinities [27-32], their co-expression is involved in the fine-tuning of the parameters of ER calcium sequestration according to the specific calcium storage and release requirements of the ER of a given cell type.

By controlling intra-ER calcium levels, SERCA enzymes are involved in the control of cell growth [33-36]. Small molecular SERCA inhibitors are tumor promoters [37, 38], and SERCA mutations have been identified in cancer [39-43]. In this context we have previously reported that the expression of SERCA enzymes is anomalous in breast, colon, as well as gastric carcinoma due to the loss of expression of SERCA3 [18, 44, 45]. It has also been shown that SERCA3 expression is induced during histone-deacetylase inhibitor-induced differentiation of gastric and colon carcinoma cells [18], as well as following the inhibition of the APC/β-catenin/TCF4 axis [44], and that normal colonic and gastric epithelium express SERCA3 abundantly [44]. In addition, the pharmacological modulation of SERCA activity has also been shown to enhance cell differentiation in colon carcinoma [44] and acute promyelocytic leukemia [46] cells. Taken together, these observations indicate that the functional maturation of ER calcium homeostasis that occurs during normal cell differentiation is blocked due to deficient SERCA3 expression in malignant cells, and that this phenomenon is involved in the maintenance of the neoplastic phenotype [16, 47].

In order to establish whether defects of ER calcium homeostasis can be found in pulmonary neoplasia, in this work we investigated SERCA expression in normal lung *in situ* and in lung adenocarcinoma cell lines undergoing pharmacologically induced cell differentiation *in vitro*, and investigated functional calcium storage capacity by confocal microscopic calcium fluorimetry using a green fluorescent protein-based calcium probe in differentiated and control cells. Our results indicate that SERCA3 is abundantly expressed in normal fully differentiated bronchial epithelium, whereas expression is highly heterogeneous in carcinoma tissue. We also show that SERCA3 expression is induced during the pharmacologically induced differentiation of lung adenocarcinoma cell lines, and that differentiation induction leads to decreased ER calcium storage.

These observations show that similarly to other types of neoplasia, lung cancer cell calcium homeostasis can also be remodeled during drug-induced cell differentiation. This indicates that defects of SERCA3 expression in cancer may be a widespread phenomenon, and show, for the first time that endoplasmic reticulum calcium homeostatic defects are involved in shaping the lung cancer phenotype.

Results

SERCA expression in lung tumor cell lines and primary tumor tissue

Western immunoblot analysis performed on equal amounts of proteins obtained from total cell lysates from various non-small cell lung carcinoma cell lines indicates that the SERCA expression pattern of these cells is heterogeneous. As shown in **Figure 1 Panel A**, whereas all cell lines express the ubiquitous SERCA2 isoenzyme, SERCA3 expression displays a marked heterogeneity: whereas in cell lines such as HCC-827 or NCI-H441, SERCA3 protein expression is barely detectable or absent, in other cell lines such as Calu-3,

A-427, NCI-H23, A549, Cha-Go K-1, NCI-H1650, NCI-H358 or NCI-H460, SERCA3 protein is highly expressed and expression levels vary depending on cell line.

When investigated *in situ* by immunohistochemistry using a SERCA3-specific monoclonal antibody, SERCA3 expression could be detected in normal ciliated bronchial epithelium and, to a variable degree also in primary human lung adenocarcinoma tissue. As shown in **Figure 1 Panel B**, whereas bronchial epithelial cells were markedly labeled (++, Photograph 2, black arrow) in a highly reproducible manner for SERCA3, staining in lung adenocarcinomas was heterogeneous, and varied between weak (+/-, Photograph 6), moderate (+; Photographs 4 and 5) and marked (++, Photograph 3). SERCA3 labeling in normal lung parenchyma (Photograph 1) was readily detectable in alveolar macrophages (black arrowhead), and normal infiltrating lymphocytes displayed strong SERCA3 labeling (+++, white arrowheads) in normal (Photograph 2), as well as tumor tissue (Photographs 3-7).

These observations taken together show that SERCA3 expression is part of the normal differentiation program of lung epithelium, and that SERCA3 expression may be decreased or lost in lung adenocarcinoma. In addition, these observations indicate that heterogeneous SERCA3 expression in lung cancer cell lines *in vitro* reflects a pathophysiologically relevant phenomenon, because this is also observed in tumour tissue *in situ*.

Induction of SERCA expression by short chain fatty acids

When various non-small cell lung carcinoma cell lines were subjected to treatments with short chain fatty acids and analogs, a selective induction of SERCA3 expression could be observed. As shown in **Figure 2**, treatment with 5 mM phenylbutyrate for 5 days resulted in a marked and selective up-regulation of the expression of the SERCA3 isoenzyme, whereas the expression of SERCA2 was not modified significantly or was decreased. SERCA3 induction could also be obtained by *n*-butyrate or *n*-valerate as well (not shown). As studied in the A549

lung adenocarcinoma cell line, induction of SERCA3 expression was concentration-dependent in the low millimolar range, and reached a plateau above 2 mM *n*-butyrate or 4-phenylbutyrate concentrations (**Figure 3**, **Panels A** and **B**). SERCA3 expression reached a plateau of four to six-fold without significant toxicity when compared to untreated cells over a period of 4-7 days in cells treated with 3 mM butyrate or phenylbutyrate, respectively (**Supplemental Figure 1**, **Panels A** and **B**). Induction of SERCA3 expression could be obtained by other short chain fatty acid analogs as well. As shown in **Supplemental Figure 2**, when A549 cells were treated with short chain fatty acids of increasing chain length (from acetate to caproate), maximal induction could be obtained by butyrate and valerate. Among the tested branched chain fatty acid analogs, valproate was also active. Aryl-substituted analogs such as 3-phenylpropionate, 4-phenylbutyrate or 5-phenylvalerate also induced SERCA3 expression, with maximal activity observed in the case of phenylbutyrate.

In parallel with SERCA2 and SERCA3, the expression of gelsolin and $p21^{CIP1/WAF1}$, two general markers of lung cancer cell differentiation [48, 49] has been investigated by Western blotting in several lung adenocarcinoma cell lines treated with various short chain fatty acids for 5 days. As shown in **Figure 4**, the selective induction of SERCA3 expression of A549, NCI-H358, NCI-H1650, Cha-Go K1 and HCC-827 cells with butyrate, valerate or phenylbutyrate was accompanied, in all investigated configurations, by increased gelsolin and $p21^{CIP1/WAF1}$ expression. This indicates, that the treatments induce phenotypic differentiation of the cells, and that the induction of SERCA3 expression is part of this process in all investigated model systems.

Induction of SERCA3 expression during the spontaneous differentiation of Calu-3 cells in post-confluent culture

Calu-3 lung adenocarcinoma cells have been shown to spontaneously undergo phenotypic differentiation towards a ciliated bronchial epithelial phenotype in post-confluent

cultures [50]. The cells form a tight, polarized monolayer that displays trans-epithelial electrical resistance, barrier function, vectorial solute transport, as reflected by the formation of domes when grown on plastic, and express apical microvilli and tight junctional complexes [51]. Post-confluent Calu-3 cells are widely used in pharmacological research as an *in vitro* model for bronchial epithelium [52]. As shown in **Figure 5**, the expression of SERCA3 is markedly increased in Calu-3 cells after confluency, whereas SERCA2 expression is not modified significantly. Interestingly, short-chain fatty acid treatment of confluent Calu-3 led to a further increase of SERCA3 expression. Phenylbutyrate has already been shown to enhance CFTR protein expression in post-confluent, differentiated Calu-3 cells [52]. These data, when taken together indicate that the induction of SERCA3 expression takes place during the spontaneous differentiation of post-confluent Calu-3 cells, a process that can be further enhanced by phenylbutyrate.

Endoplasmic reticulum calcium storage in short-chain fatty acid differentiated cells

In order to investigate ER calcium storage capacity in lung carcinoma cells, we established eleven clonal cell lines (BWV543 cells) derived from A549 lung adenocarcinoma, that stably express the GCaMP2 recombinant fluorescent calcium probe in the cytosol, and performed confocal microscopic measurements of fluorescence on cells treated with 5 mM phenylbutyrate for 5 days, and on untreated control cells. Treatment of all clones led to increased SERCA3 expression similarly to the parental A549 cell line. ER calcium storage capacity was tested by confocal microscopy on two randomly selected clones (BWV543 clones 4 and 2.4). As shown in **Figure 6**, when clone 4 cells incubated in the absence of extracellular calcium were treated with thapsigargin, a significant calcium release from the ER to the cytosol could be observed in control cells, whereas thapsigargin-induced calcium release in cells in which SERCA3 expression was induced by phenylbutyrate was

significantly smaller. After the completion of thapsigargin-induced calcium release measured in the absence of extracellular calcium, 2 mM Ca^{2+} was added to the extracellular medium in order to observe capacitative calcium influx. Despite the marked decrease of thapsigargin-induced calcium release in phenylbutyrate treated cells, capacitative calcium influx was of essentially identical amplitude in untreated and phenylbutyrate treated cells. Identical results were obtained on BWV543 clone 2.4 cells as well.

Discussion

Because calcium uptake into the ER is performed exclusively by SERCA enzymes, SERCA function constitutes a key nodal point in cellular calcium homeostasis and signaling. SERCA activity is required for calcium accumulation in a resting cell for intra-ER calcium dependent chaperone functions, as well as for calcium accumulation in the ER for inositol-1,4,5-*tris*phosphate-induced calcium release during cell activation. Moreover, because SERCA enzymes take up calcium from the cytosol even during an inositol-1,4,5-*tris*phosphate-induced calcium-release event, they can shape the amplitude and the duration of calcium peaks, or modulate the spatiotemporal characteristics of calcium oscillations in the cytosol. SERCA activity can therefore critically modulate the state of activation of a cell, and can influence the type and the amplitude of the response given by a cell to calcium-dependent stimuli. This has important consequences for tumor biology: direct pharmacological SERCA inhibitors such as thapsigargin or 2,5-di-*tert*-butyl-1,4-benzohidroquinone are known tumor promoters, and $SERCA2^{+/-}$ knock-out is known to lead to squamous tumorigenesis with a long incubation time. In addition, sequencing of the SERCA2 and SERCA3 genes in various tumors including lung cancer and corresponding normal tissue suggests that inactivating mutations occur in these genes in a proportion of tumors [40, 41], and it has thus been suggested that SERCA3 haploinsufficiency may predispose to cancer development, or may

promote tumor progression [41, 42]. In this context the induction of SERCA3 expression, as observed in our work, may therefore exert anti-oncogenic effects in lung epithelial cells.

The observations presented in this work show, for the first time, that the expression of SERCA-type calcium pumps in lung cancer cells is dynamic, as various cell differentiation-inducing stimuli led the selective induction of the expression of the SERCA3 isoenzyme in all investigated cell lines. In addition, strong SERCA3 expression was found in fully differentiated bronchial epithelium. These data, when taken together, indicate that SERCA3 expression is part of the differentiation program of normal bronchial epithelium, and that this phenomenon can be recapitulated at various degrees during the differentiation of all investigated cell lines.

SERCA3 induction in A549 cells, a widely studied lung adenocarcinoma line, was associated with a decreased calcium release signal upon SERCA inhibition. The calcium affinity of SERCA3 is inferior (approximately 1.2 μM) to that of the co-expressed SERCA2b isoform (approximately 0.2 μM). The decreased calcium release signal observed in phenylbutyrate treated A549 cells cannot be attributed exclusively to SERCA3 induction due to the interconnectedness of calcium homeostatic mechanisms that operate simultaneously in a cell. Decreased calcium release upon SERCA inhibition is, however, compatible with the notion of the formation of a leakier or lower-affinity, SERCA3-associated intracellular calcium pool, similarly to that observed previously in butyrate treated KATO-III gastric carcinoma cells [18].

The normal lung contains several distinct and specialized epithelial cell types. In addition, lung carcinoma as a group consists of several histological tumor types that can display different degrees of histological differentiation. The ontogenic relationship between normal and tumoral cell types in the lung is not established in sufficient detail [1], and the molecular signaling mechanisms involved in the induction and the maintenance of tumoral

phenotypes of increasing malignancy are not sufficiently known in the lung. Consequently, lung cancer is currently not amenable to successful pharmacological intervention. The observation that lung cancer cell calcium ER homeostasis is remodeled during differentiation is important for cancer biology because this indicates that lung adenocarcinoma cells may display calcium homeostatic defects similar to those observed in colon, gastric and breast carcinoma, as well as acute promyelocytic leukemia. In those systems the cross-talk of ER calcium homeostasis with other mechanisms of control of cell differentiation permits to induce and potentiate pharmacologically induced cell differentiation and to overcome certain forms of resistance to differentiation-induction therapy *in vitro* [44, 46]. In addition, SERCA3 expression has also been shown to be a useful new immunohistochemical marker for the study of the state of differentiation and degree of malignancy of colon and breast tumors [44, 45]. Data presented in this paper indicate, for the first time, that ER calcium biology is connected to cell differentiation also in pulmonary epithelium, and shows that ER calcium homeostasis presents defects in lung cancer cells, which can be overcome by pharmacologically induced cell differentiation. Although the precise correlation of the loss of SERCA3 expression with lung adenocarcinoma histological type, grade, molecular subtypes or clinical parameters requires further work, the demonstration of calcium homeostatic anomalies in lung cancer may help identify new calcium-dependent targets for the therapy of the disease. In addition, our observations suggest that SERCA3 may prove useful for the immunohistochemical analysis of lung tumors and for the study of bronchic epithelial differentiation.

Materials and Methods

Chemicals

n-butyric and n-valeric, 4-phenylbutyric acid, as well as sodium acetate, sodium caproate, sodium valproate, isobutyric, 4-methylvaleric, as well as phenylacetic, 3-

phenylpropionic and 5-phenylvaleric acids were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Free acids were dissolved to 300 mM final concentration in 300 mM sterile sodium bicarbonate solution, filtered through 0.2 µm membranes and stored at -25°C. Sodium salts were dissolved at 300 mM concentration in sterile water and filtered through 0.2 µm membranes.

Cell lines

The Calu-3, NCI-H23, NCI-H358, NCI-H441, NCI-H460, NCI-H596, NCI-H1299 and NCI-H1650, as well as the HCC-827 cell lines were obtained from ATCC (ATCC-LGC Standards Sarl, Molsheim, France). A549 and A427 cells were from DSMZ (Braunschweig, Germany), and Cha-Go K1 was purchased from ECACC (Porton Down, UK). The various cell lines were cultured according to the instructions of the cell line depository of origin. RPMI-based media contained Ultraglutamine-I (Lonza, Verviers, Belgium) in addition to 2 mM glutamine.

Treatments

Exponentially growing cells were trypsinized and plated into 60cm² cell culture grade Petri dishes in new medium. When cultures reached 40-60% confluency as examined by light microscopy, medium was renewed and drugs were added from concentrated stock solutions. Following treatments as indicated in Figures, cells were washed with ice-cold 150 mM NaCl and precipitated with 5% trichloroacetic acid (TCA). TCA pellet was quantified and dissolved in modified Laëmmli-type sample buffer exactly as described earlier [17], and 50 µg total cellular protein was deposited for SDS-polyacrylamide gel electrophoresis per well [17]. Following electrophoresis and transfer to nitrocellulose membranes, equal loading of samples was controlled prior immunoblotting by Ponceau red staining of total protein deposited on the

membranes. Ponceau red stained blots were scanned and equal protein loading was controlled by densitometry using the ScionImage software (Scion Corp. CA) as described earlier in detail [17].

Western blotting

The PLIM430 anti-SERCA3 mouse monoclonal antibody was used for Western immunblotting as hybridoma supernatant as described earlier in detail [17, 18, 44]. Purified mouse anti-gelsolin antibody (Cat # 610412, BD Transduction Laboratories, San José, CA, dissolved in TBS-milk at 0.05 μg/ml final concentration), and the mouse monoclonal anti-p21 antibody SC-71811 (Santa Cruz Biotechnologies, CA, dissolved in TBS-milk at 0.2 μg/ml final concentration) were used on heat treated samples (100°C for 12 min) run in 8% polyacrylamide gels. Antibodies were revealed with an anti-mouse Ig-horseradish peroxydase conjugate (Jackson Immuno-Research, Newmarket, Suffolk, UK) and the Enhanced Chemiluminescence system of Amersham (Courtaboeuf, France) as described earlier [17, 18, 44]. Non-saturated luminograms were scanned and densitometric analysis was done with the ScionImage software [11-15].

Immunohistochemistry

Formalin fixed paraffin embedded lung cancer tissue microarray slides were purchased from BioChain Institute, Hayward, CA (T8235724, T8235732, Z7020066, Z7020067, CliniSciences, Montrouge, France) and from US Biomax, Rockville, MD (BC04015, BC041114, BC041115, BC04119b, LC20810, Euromedex, Souffelweyersheim, France). Staining for SERCA3 was performed using the clone 2H3 monoclonal mouse anti-SERCA3 antibody (IgG2a kappa) from Abnova (Tebu-bio, Le Perray en Yvelines, France) raised against a GST-tagged peptide sequence encompassing the 501-621 amino acid fragment of

SERCA3, at 3 µg/ml final concentration diluted in Dako REAL™ antibody diluent (Dako France S.A.S., Trappes, France) as described in [45]. Briefly, after inhibition of endogenous peroxydase and antigen retrieval of deparaffinized sections by a *tris*-hidroxymethyl-aminomethane-based reagent (CC1 solution, Ventana Medical Systems, Illkirch, France) at 95-100°C for 12 minutes, slides were incubated for 30 minutes at 37°C with the antibody, and staining was revealed using the Ventana I-View Biotin-Ig-streptavidin-biotin-horseradish peroxydase system with blocking of endogenous biotin activity and copper enhancement, according to the instructions of the manufacturer. As negative control, isotype matched irrelevant antibody was used at the same concentration, and this gave no staining. As internal positive control, lymphocytes and vascular endothelial cells present in the samples and which express high levels of SERCA3 [17, 24], were used. Slides were counterstained with hematoxylin and bluing agent (Ventana). SERCA3 immunostaining of the samples was evaluated semi-quantitatively using a four grade scale (0 to 3+). Photomicrographs were taken with a Zeiss Axio Scope.A1 microscope equipped with a Zeiss N-Achroplan 40x/0.65 objective and an AxioCam ICc1 camera, using the Axiovision 4.8.2 software. Tissue microarray slides were also processed using the ImmPRESS™ biotin-free polymerized enzyme staining system (Vector Laboratories, Clinisciences SAS, Nanterre, France) according to the instructions of the manufacturer, and SERCA3 staining with this system gave identical results.

Confocal fluorescent microscopic calcium measurements

In order to generate the SB-CAG-GCaMP2 fluorescent calcium probe, a DNA fragment encoding GCaMP2 was first generated by PCR using the pN1-GCaMP2 plasmid, a generous gift of Junichi Nakai, RIKEN Brain Science Institute, Saitama, Japan [53]. Primer sequences were as follows: forward, 5'-CTACCGGTCTCGCCACCAATG-3'; and reverse,

5’-AGATCTCCGCTCACTTCGCTGTC-3’. The forward primer adds an AgeI restriction site, the reverse primer adds a BglII restriction site. The complete GCaMP2 DNA-fragment was cloned via *Age*I(5')/*Bgl*II(3') into a SB-CAG-AmaxaGFP vector from which Amaxa GFP was excised with the same enzyme pair. The GCaMP2 fragment is framed in the resulting vector by two left inverted repeat-direct repeat (IRDR) regions, which are the recognition motifs of *Sleeping Beauty* (SB) transposase [54-57], and possesses a CAG [58] promoter. The efficiency of such a symmetrical transposon was proven by T. Orbán (personal communication). This construct was used for stable transfection using the SB transposon delivery system as described earlier in detail [54-57, 59]. For the SB transposase, we used an enhanced version of the enzyme having an approximately hundred-fold higher activity than the originally reconstructed transposase [59, 60]. For transfection of exponentially growing A549 cells, the FuGENE® 6 (Roche Applied Science, Rotkreuz, Switzerland (http://www.roche-applied-science.com) reagent was used according to the instructions of the manufacturer. Cells were co-transfected with transposon and transposase plasmids in a 10:1 ratio to avoid overproduction inhibition of the transposase [56, 57]. Following transfections, cells were cultured for 3 days and then resuspended by trypsinization and sorted for GFP fluorescence using the Aria High Speed Cell Sorter of Beckton-Dickinson (San José, CA, http://www.bdbiosciences.com). The sorted cell population (containing approximately 60% GFP positive cells) was further cultured in bulk for 4 days, trypsinized and single cell cloned by limiting dilution in 96 well plates. Individual GCaMP2 expressing clones were identified by GFP fluorescence using an inverted fluorescent microscope and expanded.

SB-CAG-GCaMP2-carrying A549 clones were grown to 20-30% confluency in 8 chamber Lab-Tek™ glass bottom chamber slides and treated with 5 mM phenylbutyrate for 5 days. At the end of treatments, the cells were washed with calcium-free Hanks' solution. Cells were then observed with an Olympus IX-81/FV500 laser scanning confocal microscope using

an Olympus PLAPO 60× (1.4) oil immersion objective. For GCaMP2 imaging cells were excited with the 488 nm laser line and emission was collected between 505 and 535 nm. Under these conditions emitted fluorescence is proportional to the cytosolic free calcium concentration [53].

Calcium signal measurements were carried out in Hanks' balanced salt solution supplemented with 20 mM Hepes (pH=7.4) and 0.9 mM $MgCl_2$. This medium was supplemented with 100 μM $CaCl_2$ and 100 μM EGTA for the measurement of thapsigargin-induced calcium release from the ER. Under these conditions free calcium concentration in the medium is approximately 4 μM, and no calcium influx is observed. Capacitative calcium influx was thereafter induced by the addition of 2 mM free $CaCl_2$ to the medium. Time lapse sequences of cellular fluorescence were recorded and images were analyzed with the FluoView Tiempo (v4.3, Olympus, http://www.olympusmicro.com) time course software. In order to compensate for variability of fluorophore expression levels in individual cells, fluorescence values are expressed as F/F_0. A total of 36 untreated and 48 treated cells were recorded individually in 4 parallel experiments.

Statistical analysis

Data are presented as the mean+/-SEM and correspond to at least three independent experiments. Statistical analysis was done using Student's paired t-test. The number of experiments is indicated in brackets in Figures.

Acknowledgements

We express special thanks to Prof. Neville Crawford (Department of Biochemistry, the Hunterian Institute, London, UK) for giving us the PLIM430 hybridoma, and to Dr Tamás Orbàn (Membrane Research Group, Hungarian Academy of Sciences, Hungary) for his

contribution regarding the use of the transposon system used in this work. The excellent technical help of Mr. Patrice Castagnet (Service d'Anatomie et Cytologie Pathologiques, Hôpital Lariboisière, Paris, France) is gratefully acknowledged. This work was supported by Inserm, the Association pour la Recherche sur le Cancer, France and by the Hungarian Academy of Sciences grants CK80283 and K101064 (EA). This work is dedicated to the memory of Casper and Rose.

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Legends to Figures

Figure 1 - SERCA expression in lung adenocarcinoma cell lines and lung tumor tissue

Panel A: Expression of SERCA2 and SERCA3 proteins in untreated lung carcinoma cell lines, detected with the IID8 (SERCA2) and the PLIM430 (SERCA3) monoclonal antibodies by Western blotting. **Panel B:** Immunohistochemical detection of SERCA3 protein in normal lung parenchyma (Photograph 1), bronchial epithelium (Photograph 2), and grade 1 (Photographs 3 and 4), grade 2 (Photograph 6), and grade 3 (Photograph 5) lung adenocarcinomas. Black arrows: normal bronchial epithelial cells, white arrowheads: normal lymphocytes, black arrowhead: alveolar macrophage (original magnification: 40x).

Figure 2 - Selective induction of SERCA3 protein expression in various lung adenocarcinoma cell lines

The cells were treated with 5 mM phenylbutyrate (PB) for 5 days, and identical amounts of total cell protein lysates were analyzed using the IID8 (SERCA2-specific) and PLIM430 (SERCA3-specific) antibodies by Western blotting. SERCA protein expression in phenylbutyrate-treated cells (**+**) is compared to untreated controls (**-**).

Figure 3 - Dose-response relationship of SERCA3 induction

A549 cells were treated with various concentrations of butyrate (**Panel A**) and phenylbutyrate (**Panel B**), and SERCA expression was measured by semi-quantitative Western blotting. An approximately six-fold induction of SERCA3 expression (black columns) is obtained in the low millimolar concentration range for both molecules, whereas the expression of SERCA2 (empty columns) decreases slightly (butyrate) or is not modified significantly (phenylbutyrate; n=3).

Figure 4 - Expression of lung cancer cell differentiation markers by short chain fatty acids

Several lung adenocarcinoma cell lines (A549, NCI-H358, NCI-H1650, Cha-Go K1 and HCC-827) were treated with various short chain fatty acids (phenylbutyrate, 3 mM; valerate or butyrate, 5 mM) for 5 days, and SERCA expression, as well as the expression of gelsolin (82 kDa) and $p21^{CIP1/WAF1}$ (21 kDa) was observed by Western blotting. Similarly to SERCA3, the expression of gelsolin and $p21^{CIP1/WAF1}$ is increased in short chain fatty acid-treated cells in all investigated drug/cell line configurations.

Figure 5 - Induction of SERCA3 expression during the spontaneous differentiation of post-confluent Calu-3 cells - Superinduction by short chain fatty acids

Calu-3 lung adenocarcinoma cells were grown in pre-confluent conditions, and in post-confluency, in the absence or the presence of various short chain fatty acids (5 mM) for 5 days, and SERCA expression was determined by Western blotting. **Panels A** and **B:** Photomicrographs of pre- and post-confluent cultures. Formation of "domes" in post-confluent cultures (arrowheads) is indicative of trans-epithelial solute transport, a feature of a tight, differentiated epithelial monolayer. **Panels C** and **D:** Cells at day 1 of post-confluency were allowed to undergo differentiation for 5 days in the absence or the presence of 5 mM butyrate, valerate or phenylbutyrate. SERCA2 and SERCA3 expression was then detected by Western blotting and quantified. The selective induction of SERCA3 expression by post-confluent growth is further enhanced by short chain fatty acids. White bars: pre-confluent untreated cells, grey bars: post-confluent untreated cells, black bars: post-confluent, phenylbutyrate-treated cells (n=3).

Figure 6 - Calcium release from SERCA-dependent intracellular calcium pools in A549 cells

A549 cells carrying the GCaMP2 fluorescent calcium probe (BWV543-4 cells) were treated or not with 5 mM phenylbutyrate for 5 days, and cytosolic calcium fluorescence was recorded by confocal fluorescent microscopy after complete inhibition of SERCA activity with 10 μM thapsigargin in the absence of extracellular calcium. After completion of the thapsigargin-induced calcium release signal, extracellular medium was replenished with 2 mM Ca^{2+}, and capacitative calcium influx was observed. Phenylbutyrate treatment leads to a significant decrease of calcium release from intracellular pools upon complete SERCA inhibition, whereas capacitative calcium influx remains similar. A total number of 36 treated, and 48 untreated individual cells were recorded in 4 parallel experiments. **Right Panel:** Similarly to parental A549 cells, SERCA3 expression was induced by phenylbutyrate in A549 cells carrying the GCaMP2-carrying calcium probe.

Legends to Supplemental Figures

Supplemental Figure 1 - Time course of SERCA2 and SERCA3 expression in short chain fatty acid-treated A549 cells

Cells were treated with 3 mM butyrate (**Panel A**) or 3 mM phenylbutyrate (**Panel B**) during several days. SERCA3 expression in treated cells (■) increases and reaches a plateau of approximately four-fold and six-fold in butyrate, and phenylbutyrate treated cells, respectively, whereas SERCA2 expression in treated cells (●), as well as SERCA3 (□) or SERCA2 (◯) in untreated cells remains unchanged.

Supplemental Figure 2 - Induction of SERCA3 expression in A549 cells by various short chain fatty analogs

Cells were treated with various short chain fatty acid analogs (5 mM) of increasing alkyl chain length (acetate, propionate, butyrate, valerate and caproate), with branched chain analogs (valproate, isobutyrate and 4-methylvalerate), and with *omega*-aryl-substituted analogs (phenylacetate, phenylpropionate, phenylbutyrate and phenylvalerate for 5 days. Maximal induction of SERCA3 expression is observed with valerate and phenylbutyrate.

Figure 1

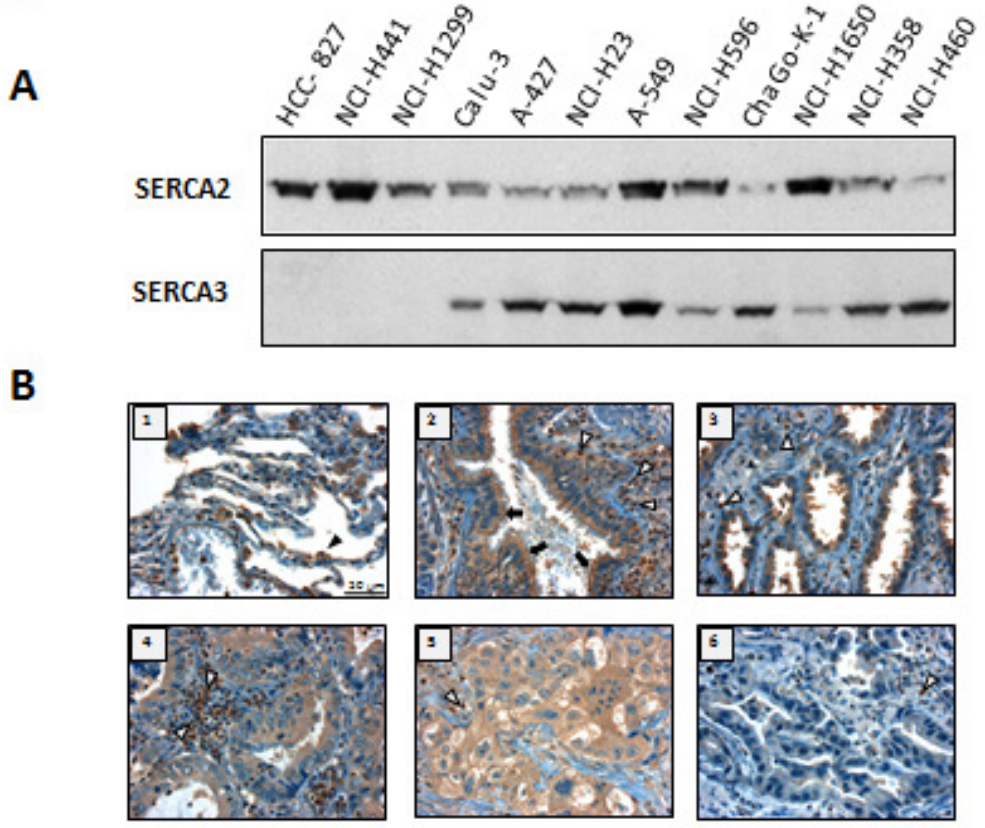

A
HCC- 827
NCI-H441
NCI-H1299
Calu-3
A-427
NCI-H23
A-549
NCI-H596
ChaGo-K-1
NCI-H1650
NCI-H358
NCI-H460
SERCA2
SERCA3
B
1
2
3
4
5
6

Figure 2

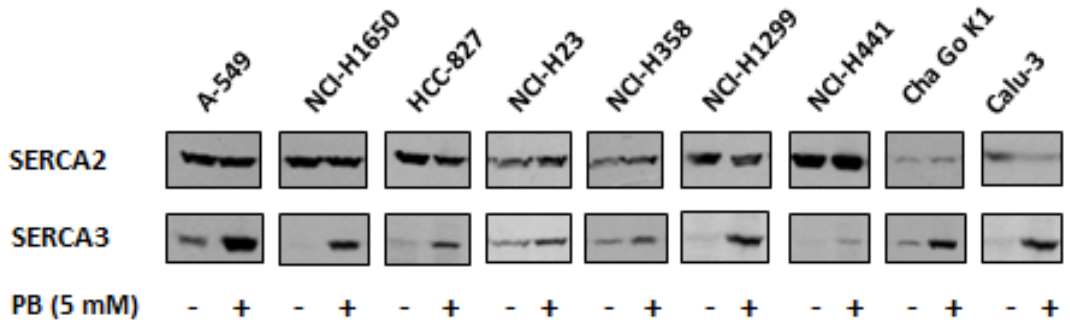

A-549
NCI-H1650
HCC-827
NCI-H23
NCI-H358
NCI-H1299
NCI-H441
Cha Go K1
Calu-3
SERCA2
SERCA3
PB (5 mM)
- + - + - + - + - + - + - + - + - +

Figure 3

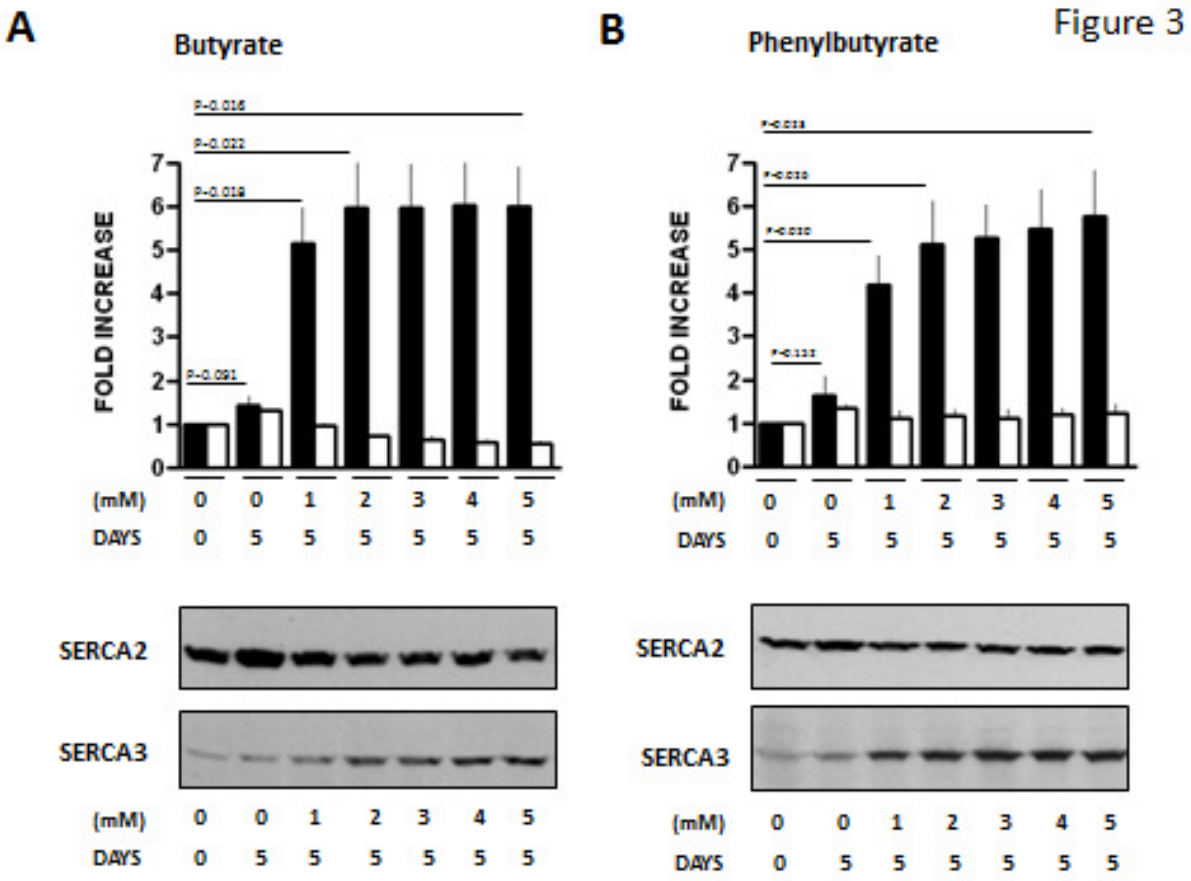

A
Butyrate
B
Phenylbutyrate
FOLD INCREASE
P=0.016
P=0.022
P=0.018
P=0.091
(mM) 0 0 1 2 3 4 5
DAYS 0 5 5 5 5 5 5
SERCA2
SERCA3
(mM) 0 0 1 2 3 4 5
DAYS 0 5 5 5 5 5 5

Figure 4

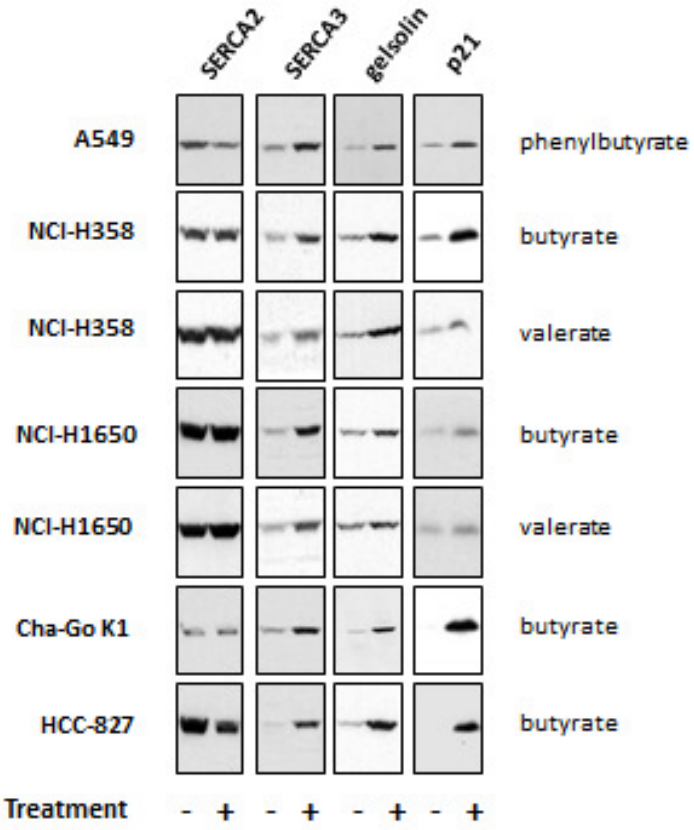

SERCA2
SERCA3
gelsolin
p21
A549
phenylbutyrate
NCI-H358
butyrate
NCI-H358
valerate
NCI-H1650
butyrate
NCI-H1650
valerate
Cha-Go K1
butyrate
HCC-827
butyrate
Treatment
- + - + - + - +

Figure 5

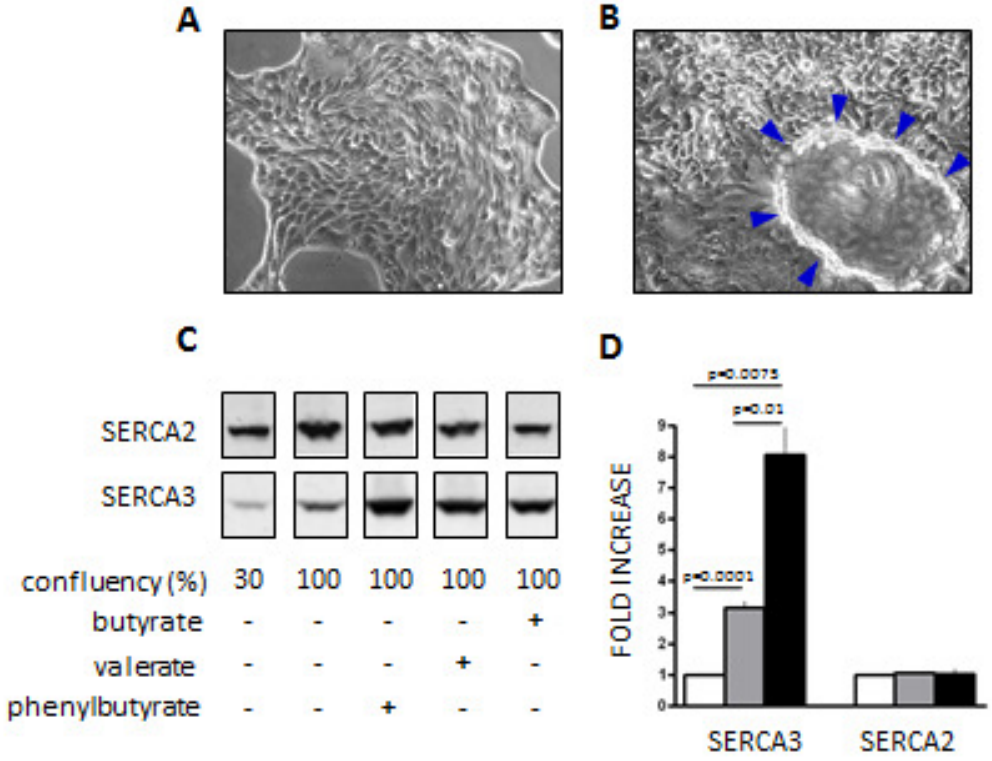
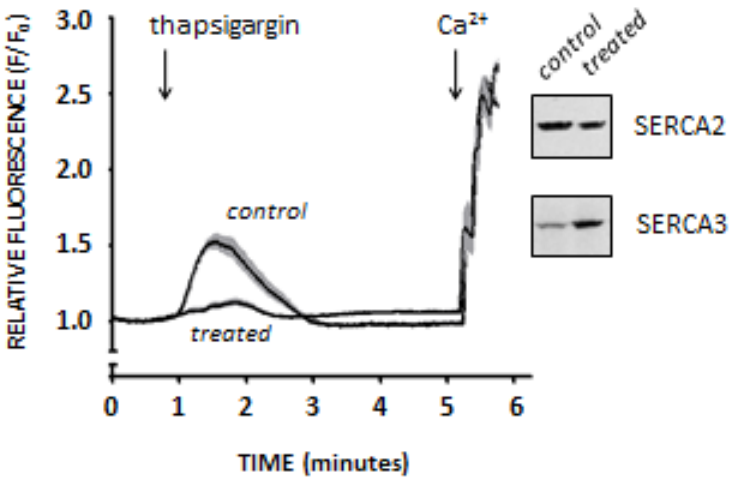
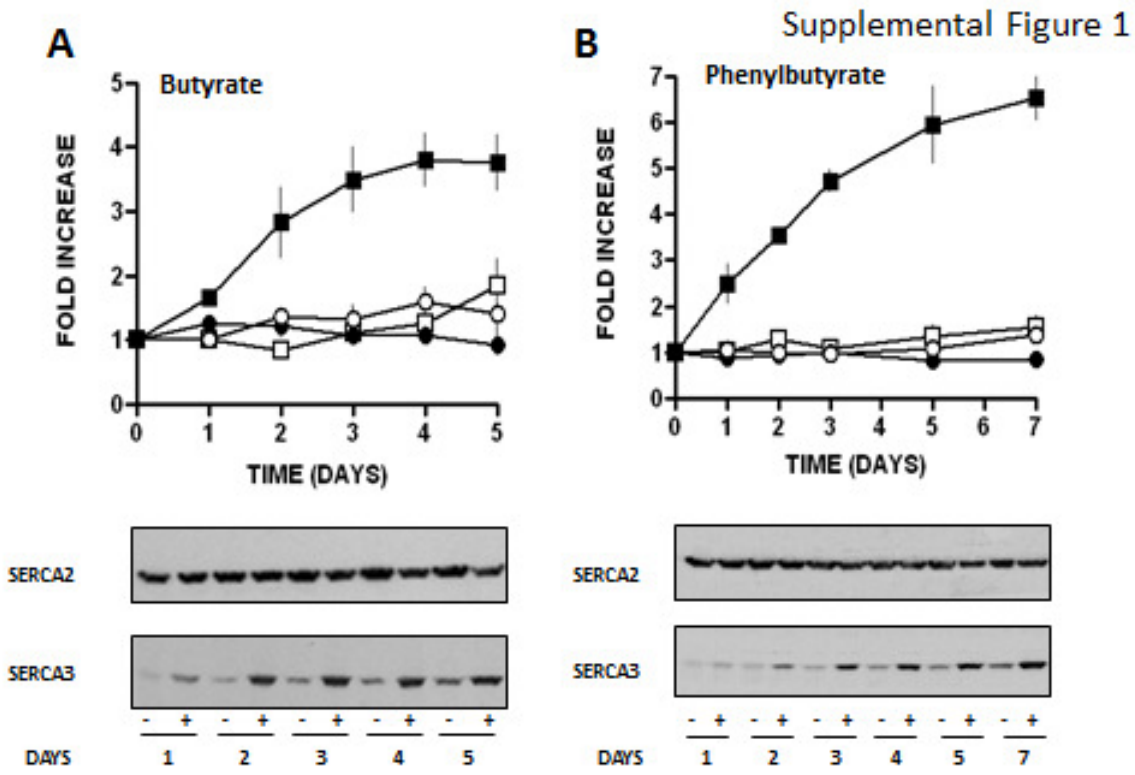
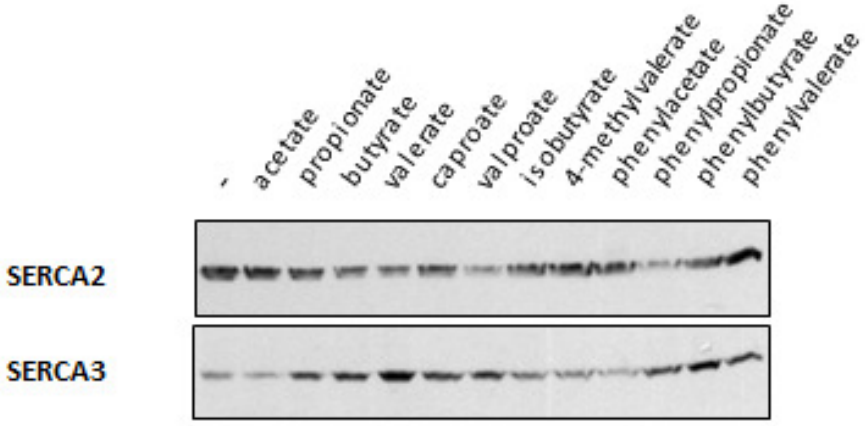

A
B
C
SERCA2
SERCA3
confluency (%) 30 100 100 100 100
butyrate - - - - +
valerate - - - + -
phenylbutyrate - - + - -
D
p=0.0075
p=0.01
p=0.0001
FOLD INCREASE
SERCA3
SERCA2

Figure 6


RELATIVE FLUORESCENCE (F/F0)
3.0
2.5
2.0
1.5
1.0
thapsigargin
Ca2+
control
treated
control
treated
SERCA2
SERCA3
0
1
2
3
4
5
6
TIME (minutes)

Supplemental Figure 1



Supplemental Figure 2