Expression of endomembrane calcium pumps in colon and gastric cancer cells.

Induction of SERCA3 expression during differentiation.

Pascal Gélébart1, Tünde Kovács2, Jean-Philippe Brouland3, Roosje van Gorp1, Johannes Grossmann4, Nathalie Rivard5, Yves Panis6, Virginie Martin1, Raymonde Bredoux1, Jocelyne Enouf1 and Béla Papp1

1U. 348 INSERM, IFR-6, Hôpital Lariboisière, Paris, France, 2National Institute of Haematology and Immunology, Budapest, Hungary, 3Service d’Anatomie Pathologique, Hôpital Lariboisière, Paris, France, 4Department of Medicine I, University of Regensburg, Germany, 5Faculté de Medecine, Université de Sherbrooke, Québec, Canada and 6Service de Chirurgie Générale et Digestive, Hôpital Lariboisière, Paris, France.

Corresponding author: Béla Papp, U. 348 INSERM, Hôpital Lariboisière, 8, rue Guy Patin, 75010 Paris, France. E-mail: bela.papp@inserm.lrb.ap-hop-paris.fr fax: +33 1 49 95 85 79
Abbreviations:

SERCA: sarco/endoplasmic reticulum calcium ATPase, ER: endoplasmic reticulum, IP3: \( \text{myo-inositol-1,4,5-trisphosphate} \), SCFA: short chain fatty acids, CEA: carcinoembryonic antigen, DPP-IV: dipeptidyl peptidase 4, ZO-1: zonula occludens protein-1, DMSO: dimethyl-sulfoxide

Key words:

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We dedicate our work to the memory of all victims of the tragedy of September 11, 2001.
Summary

Calcium mobilization from the endoplasmic reticulum (ER) into the cytosol is a key component of several signaling networks controlling tumor cell growth, differentiation or apoptosis. Sarco/Endoplasmic Reticulum Calcium transport ATPases (SERCA-type calcium pumps), enzymes that accumulate calcium in the ER, play an important role in these phenomena. We report that SERCA3 expression is significantly reduced or lost in colon carcinomas when compared to normal colonic epithelial cells, which express this enzyme at a high level. To study the involvement of SERCA enzymes in differentiation, in this work differentiation of colon and gastric cancer cell lines was initiated, and the change in the expression of SERCA isoenzymes, as well as intracellular calcium levels were investigated. Treatment of the tumor cells with butyrate or other established differentiation inducing agents resulted in a marked and specific induction of the expression of SERCA3 whereas the expression of the ubiquitous SERCA2 enzymes did not change significantly or was reduced. A similar marked increase in SERCA3 expression was found during spontaneous differentiation of post-confluent Caco-2 cells, and this closely correlated with the induction of other known markers of differentiation. Analysis of the expression of the SERCA3 alternative splice isoforms revealed induction of all three known iso-SERCA3 variants (3a, 3b and 3c). Butyrate treatment of the KATO-III gastric cancer cells led to higher resting cytosolic calcium concentrations and, in accordance with the lower calcium affinity of SERCA3, to diminished ER calcium content. These data taken together indicate a defect in SERCA3 expression in colon cancers as compared to normal colonic epithelium, show that the calcium homeostasis of the endoplasmic reticulum may be remodeled during cellular differentiation and indicate
that SERCA3 constitutes an interesting new differentiation marker that may prove useful for the analysis of the phenotype of gastrointestinal adenocarcinomas.

**Introduction**

Cellular calcium concentration gradients and calcium ion fluxes are important components of several signaling networks controlling cell growth, differentiation or apoptosis (1-2). In a resting cell, the cytosolic free calcium concentration is approximately 50-100 nM, whereas the endoplasmic reticulum (ER) or the extracellular medium contains calcium in the high micromolar to low millimolar range. Binding of several growth factors, hormones, chemokines or bioactive peptides to their cell surface receptors leads to the formation of the second messenger inositol-1,4,5-trisphosphate (IP3), which induces calcium release from the endoplasmic reticulum into the cytosol through IP3 receptor calcium channels. The ensuing decrease of the calcium content of the ER lumen induces the opening of calcium channels in the plasma membrane, allowing calcium influx into the cytosol from the extracellular space. Calcium release from the ER and ensuing calcium influx lead to the augmentation of the cytosolic free calcium concentration. As many key components of intracellular signaling networks, such as various calmodulin-activated kinases (3), protein kinase C (4), calcineurin (5), calpains (6), as well as the PYK-2 tyrosine-kinase (7), the Ras guanine nucleotide exchange factor Ras-GRF (8), the apoptosis-associated kinase DAP-2 (9) or the apoptosis-linked calcium-binding protein ALG-2 (10) are directly activated by increased cytosolic calcium concentrations, cellular calcium fluxes constitute an important component of several signal transduction networks of the cell.

In addition to its role played in signaling in the cytosolic compartment, calcium stored within the ER lumen is required for the posttranslational modification and processing of
newly synthesized proteins transiting across the organelle (11). It is becoming increasingly clear that calcium stored in the ER is involved in homeostatic, synthetic as well as signaling functions also within the lumen of the organelle (12-14).

Refilling of calcium into the ER from the cytosol by active, ATP-driven ion transport is assured by Sarco-Endoplasmic Reticulum Calcium transport ATPases, also called SERCA enzymes (12). These enzymes, by pumping calcium into the ER against a steep concentration gradient, decrease cytosolic calcium levels after an episode of activation, and make calcium available in the ER lumen for intra-ER calcium dependent functions, as well as for being released into the cytosol during a next signaling event. Three SERCA genes are known, which by alternative splicing can give rise to several protein isoforms. SERCA1a and 1b are expressed in adult and neonatal skeletal muscle, respectively (15). SERCA2a is found in cardiac and smooth muscle, whereas SERCA2b has been found in all non-muscle cell types studied so far (16, 17). Expression of SERCA3 has been detected in a selected group of cell types, including cells of hematopoietic origin, where this enzyme is constitutively expressed (18-21), and the existence of three SERCA3 alternative splice isoforms has been reported (22-24). Although SERCA3 mRNA has been detected in various tissues, including normal intestinal epithelium (25, 26) the expression of SERCA-type enzymes has not been studied in colon and gastric cancer so far.

The calcium content of the ER lumen is a key determinant controlling apoptosis induced by physiologic stimuli (14, 27). The modulation of the calcium content of the ER by Bcl-2 is involved in the regulation of the apoptotic potential of the cell (14, 28), and the regulation of SERCA expression and function by Bcl-2 is thought to be involved in this process (29). In short term experimental settings the direct pharmacological inhibition of calcium pumping activity has been shown to lead to growth arrest, differentiation or caspase-12-dependent apoptosis, depending on the cell type (30-33), and highly specific SERCA
inhibitors such as thapsigargin or 2,5-di-tert-butyl-1,4-hydroquinone are known tumor promoters \textit{in vivo}, as well as \textit{in vitro} (34, 35) when applied chronically. Moreover, endogenously expressed truncated SERCA variants have recently been implicated in the modulation of the apoptotic potential of the cell by interfering with SERCA-dependent calcium transport (36). In addition, peptide hormone receptors that mobilize calcium from the endoplasmic reticulum have been shown to be involved in positive feedback mechanisms regulating colon cancer cell proliferation and behavior (37, 38). All these data taken together suggest that cellular phenotype, proliferation status, apoptotic potential and stage of differentiation are intricately connected to ER calcium homeostasis. However, the mechanisms involved in these processes are poorly understood. In order to better understand the role of SERCA enzymes in epithelial maturation, and to shed light on the involvement of the calcium homeostasis of the ER in epithelial malignancies, in this work we investigated the expression of SERCA enzymes in a series of human colon and gastric cancer cell lines, carcinoma tissue and primary cells, and studied the modulation of SERCA expression and function during cell differentiation.

\textbf{Materials and Methods.}

Cells and treatments.

The Caco-2, SW-48, SW-403, LS-174T, LoVo, SW-620, DLD-1, HT-29 (wild type) and COLO-205 colon cancer cell lines, as well as the KATO-III, NCI-SNU-1, NCI-SNU-16, NCI-N87 and RF-48 gastric cancer lines were purchased from, and were cultivated according to the instructions of ATCC (Manassas, VA), with the modification that RPMI-based media contained Glutamax-I (alanyl-glutamine) in addition to 2 mM glutamine. HT29-5M21 cells were cultured in the presence of 10 \textmu M methothrexate as previously described (39). The
culture of cells obtained from crypts of human fetal ileum (HIEC cells), as well as the isolation and culture of primary differentiated ileal epithelial cells (PCDE) have been described earlier (40, 41). Adult primary colonic epithelial cells were obtained as previously described (42).

Exponentially growing cells were trypsinized and seeded into 20 cm² cell culture dishes at a density of 2x10⁴ cells/cm². When cells reached 80% confluency by microscopic examination (day 2 or 3 post-plating, depending on the rate of growth), medium was renewed and drugs were added from concentrated stock solutions. Cells that grow in suspension or in a semiahesive manner (KATO-III, NCI-SNU-1, NCI-SNU-16, RF-48) were seeded at an initial density of 2x10⁵ cells/ml at the beginning of treatments. Sodium salts of short chain fatty acids and of their analogs were dissolved in phosphate buffered saline at a concentration of 0.3 M. When only the free acid forms were available commercially (Sigma-Aldrich, France), these were neutralized by dissolving in 0.3 M sodium bicarbonate at the same concentration and sterile filtered. Due to their hydrophobicity, 1,2,3-tributyrlylglycerol (tributyrin, Fluka, Germany) and pyvaloyloxy-methylbutyrate (Calbiochem, Darmstadt, Germany) were dispersed to the desired final concentrations as fine emulsions in complete medium by sonication immediately prior experiments. Suberoylanilide hydroxamic acid (SAHA) was purchased from Upstate Biotechnology (Lake Placid, NY), and apicidin from Calbiochem (Darmstadt, Germany). Herbimycin-A, HC-toxin and thapsigargin were from Sigma Aldrich France. Drugs were added to the cell cultures from concentrated stock solutions made in dimethyl sulfoxide (DMSO). The final concentration of DMSO vehicle did not exceed 0.1%, was included in control experiments and did not interfere with the assays. Untreated control cells were harvested in the exponential phase of non-confluent growth.

After treatments as indicated on figures, the cells were quickly washed with ice cold NaCl (150 mM) twice, precipitated with 5% trichloroacetic acid overnight at 4°C and
The protein pellet was then dissolved in sample buffer (20), and equal amounts of lysates (100 µg protein per well) were run on 8% SDS-polyacrylamide gels and electroblotted onto nitrocellulose (Hybond ECL, Amersham-Pharmacia, UK). Transfer of proteins onto nitrocellulose was controlled by Ponceau red staining. Immunodetection of SERCA proteins using the IID8 (SERCA2 specific) and the PLIM430 (SERCA3 specific) monoclonal antibodies was performed as described previously (20), with the modification that the sample lysis buffer contained also 1mM phenylmethylsulfonyl-fluoride, 0.1 mM aminoethyl-benzylsulfonyle fluoride, 10 µg/ml leupeptin and 10 µg/ml pepstatin-A, added freshly from 1000-fold concentrated stock solutions made in DMSO.

Rabbit polyclonal antibodies used in this work that specifically recognize the various SERCA3 isoenzymes (SERCA3a, 3b and 3c) have been characterized previously in detail (24). Immunostaining for the detection of carcinoembryonic antigen and of dipeptidyl peptidase IV was performed using the C6G9 (Sigma) and the HBB 3/775/42 (43) monoclonal antibodies, respectively, at a 1000-fold dilution of the ascites using the electrophoresis and immunostaining system as outlined above. ZO-1 protein was immunostained using an affinity-purified rabbit polyclonal antibody obtained from Zymed (CA) that recognizes both the α- and α+ isoforms, after electrophoresis of cellular proteins in 6.5% polyacrylamide gels. Luminescent signal obtained using the Amersham Enhanced Chemiluminescene system was quantified by scanning non-saturated luminograms (on Kodak Biomax ML films) with an Epson Perfection Photo 1240U scanner using the Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA) and quantitated using the Scion Image software (version 4.0.2, Scion Corporation, www.scioncorp.com). Due to the absence of detectable SERCA3 in untreated KATO-III or Caco-2 cells, SERCA expression in these cells is expressed on figures in percentages, with endpoint signal being taken arbitrarily as 100%.
Primary cells.

Primary colon cancer cells were obtained from fresh surgical specimens. Homogenous tumor tissue was carefully separated from adjacent structures, cut into sub-millimeter sized pieces with a scalpel and placed into 24 well plates in a medium consisting of a mixture of equal volumes of RPMI-1640 and Ham’s F12 nutrient medium supplemented with glutamax-I, glutamine, sodium pyruvate, nonessential amino acids, vitamins and reduced glutathione (all reagents obtained from Gibco-Life Technologies, France) plus 20% decomplemented fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin-B. After 3 days in culture, floating tissue debris were aspirated, and growth of adherent cells and morphology, as well as the absence of fibroblasts or microbial contamination was monitored microscopically. Only cultures devoid of fibroblast contamination were used. CEA synthesis was detected by immunoblotting of total cell lysates as indicated above. The cells were first treated with short chain fatty acids at day 6 post-plating in the wells in which they had been originally plated, and then after four months of continuous growth and regular subculturing in 20 cm² Petri dishes.

Immunohistochemistry.

Staining of 5 µm thick cryostat sections of freshly frozen normal and malignant colon and stomach tissue with the SERCA3 specific PLIM430 monoclonal antibody was performed as follows: slides were allowed to dry overnight at room temperature at the sections were then fixed in acetone for ten minutes at room temperature, were allowed to dry and were rehydrated in Tris-buffered saline (TBS, pH=7.4) containing 0.1% Tween-20 (TBS-Tween) for ten minutes. Inhibition of nonspecific protein binding was performed by incubation for 30 min. in TBS-Tween supplemented with 5% nonfat dry milk. The PLIM430 antibody, previously purified by protein-A affinity chromatography, was then applied upon the sections
in the above solution at 1 µg/ml concentration and incubated at room temperature for 90 minutes. The slides were then rinsed with distilled water three times and incubated in TBS-Tween-milk for ten minutes. After repeating this washing step once, the slides were rinsed three times with TBS-Tween, and incubated for ten min. in TBS containing 1/30 vol. normal horse serum. Slides were incubated with biotinylated anti-mouse secondary antibody (Vectastain ABC kit, Vector Laboratories, CA) for one hour, followed by incubation with avidin-biotin-peroxidase complex (Vectastain ABC kit) for 45 min. according to the protocol of the manufacturer. As chromogen 3,3'-diaminobenzidine was used and the slides were counterstained with hematoxylin. Omission of primary antibody and isotype matched irrelevant antibody were used as negative controls and this resulted in no staining.

Calcium fluorimetry.

Fluorescence from suspensions of Fura-2-loaded KATO-III cells was recorded at 37°C with a Shimadzu RF-1501 spectrofluorimeter (Shimadzu Europe, Duisburg, Germany) equipped with a stirring apparatus. For calculation of cytosolic calcium concentrations, the ratio of fluorescence at excitation wavelengths of 340 and 380 nm (emission at 510 nm) was calibrated according to Grynkievicz (44). 13x10^6 KATO-III cells were loaded with 1.7 µM Fura-2-AM (Sigma-Aldrich France) in 6 ml complete culture medium for 45 min at 37°C. The cells were then centrifuged for 8 min at 350xg and resuspended in 10 ml fresh complete medium. After 15 min at 37°C the cells were washed by centrifugation with 10 ml buffer containing 136 mM NaCl, 2.7 mM KCl, 10 mM HEPES pH=7.45 adjusted with NaOH, 2 mM MgCl2, 1 mg/ml D-glucose and 1 mM CaCl2 and resuspended in 14 ml of this buffer. Measurements were performed with cuvets containing 2 ml cell suspension. In order to measure SERCA-dependent intracellular calcium storage capacity, calcium mobilization from
the ER into the cytosol was induced with 1 \( \mu \)M thapsigargin in the presence of 2 mM extracellular EGTA, and peak cytosolic calcium concentration was recorded.

RT-PCR amplification of SERCA transcripts.

Total cellular RNA was isolated from cells using the Trizol reagent (GibcoBRL, UK) according to the instructions of the manufacturer. 500 ng total RNA was reverse transcribed using the MuLV reverse transcriptase (Perkin Elmer France). After inactivation of the reverse transcriptase, semiquantitative PCR was initiated by adding 0.625 units of AmpliTaq Gold DNA polymerase (Perkin Elmer) in a 25 \( \mu \)l reaction mixture. Touch-down PCR (45) was performed for ten cycles with an annealing temperature decrement from 65\( ^\circ \)C to 56\( ^\circ \)C in order to increase the specificity of priming during initial cycles of amplification. PCR was then conducted essentially as described (45) with slight modifications as follows: 18 cycles of PCR were conducted for SERCA2b, 22 cycles for SERCA3a, 23 cycles for 3b and 24 cycles for 3c, with each cycle consisting of successive periods of 1 min at 95\( ^\circ \)C, 1 min at 58\( ^\circ \)C and 1 min at 72\( ^\circ \)C with a final extension step of 10 minutes at 72\( ^\circ \)C. The SERCA isoform-specific oligonucleotide primers used were the following: SERCA2-5\' : 2861 TCA TCT TCC AGA TCA CAC CGC T2882, SERCA2b-3\' : 3129 TCA AGA CCA GAA CAT ATC GC 3110, SERCA3-5\' (same for the various isoforms): 2674 GAG TCA CGC TTC CCC ACC ACC 2694, SERCA3a-3\' : 2992 GGC TCA TTT CTT CGT GCA TGT GGT TC 2967, SERCA3b-3\' : 3080 GGC TCA TTT CTT CAA AGA GGC CAA C 3058, SERCA3c-3\' : 3093 GGC TCA TTT CTT CAA AGA GGC CAA C 3069. As internal control glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was amplified as in (45). The amplification products were separated in 1.5% agarose gels and visualized by ethidium bromide staining. The apparent molecular masses of the PCR products corresponded to those calculated based on the reported sequences, moreover the identity of the PCR products was also confirmed by direct sequencing (Genome Express, Grenoble,
France). Quantitative data were obtained using the Scion Image software (see above) on
digitalized images of ethidium bromide stained gels. Data in this work correspond to at least
three independent experiments and are presented as means +/- S.E.

Results

SERCA3 expression is lost in colon carcinomas.

In order to study SERCA3 expression in human tissue, we developed an
immunohistochemical staining protocol using the SERCA3-specific PLIM430 monoclonal
antibody. As illustrated in Fig. 1, Panel A SERCA3 was readily detected in normal colonic
crypt epithelium in enterocytic, as well as mucus secreting cells, as well as in stomach mucosa
(Panel B). In colonic crypts stronger SERCA3 staining could be seen in more mature cells
residing in the luminal region. However, SERCA3 could also be detected in deeply located,
less mature cells, suggesting that SERCA3 expression is induced early in colonic epithelial
differentiation. On the other hand, when SERCA3 expression was investigated in colon
cancer tissue, in 9 cases out of 12 examined, a complete lack of staining in the malignant cells
was observed (Panels C-G), and in the remaining cases a faint staining could be observed in
the apical region of the cells (Panel H). At the same time, adjacent normal epithelial tissue
stained strongly positive for SERCA3 in all specimens in a highly reproducible manner.
These data show that SERCA3 expression is dramatically decreased or completely lost in
colon adenocarcinomas, although present at high levels in normal epithelium.

In accordance with immunoblotting data presented in Figure 2, SERCA3 expression could
also be detected by immunocytochemistry in the COLO-205 cell line (Panel J), whereas the
KATO-III cell line was negative (Panel I). SERCA3 expression in COLO-205 cells was,
however, markedly weaker than that observed in normal colonic epithelial cells.
SERCA3 expression in colon and gastric cancer cell lines.

In order to study SERCA3 expression in various colon and gastric cancer cell lines, cells in the exponential phase of growth were harvested, and total cellular protein was probed with the PLIM430 (pan-SERCA3 specific) monoclonal antibody in a Western blot format as in (20), using a very sensitive chemiluminescent detection method. As an internal control, SERCA2 was detected using the IID8 monoclonal antibody as well. As shown in Figure 2, whereas SERCA2 was expressed in all cell types at comparable levels, the expression of SERCA3 was undetectable in DLD-1, Caco-2, KATO-III or NCI-SNU-1 cells. Although SERCA3 could be detected at various levels in HT29, SW-403, SW-48, LS-174T, COLO-205, NCI-N87, and NCI-SNU-16 cells, freshly isolated normal adult colonic epithelial cells expressed SERCA3 protein at a higher level than any of the cell lines (Figure 2). SERCA3 protein was also detected in primary differentiated embryonic ileal epithelial cells (PCDE cells) and in cells obtained from crypts of human fetal ileum (HIEC cells, not shown).

SERCA3 expression increases during drug-induced cell differentiation.

As blockage of differentiation is a general hallmark of malignancy, in order to investigate the implication of SERCA3 in colon cancer differentiation, cell lines were treated with sodium butyrate, a known physiological differentiation-inducing agent of colonic mucosa, and SERCA expression was investigated. As shown in Figure 3, Panel A, butyrate induced SERCA3 expression in various colon and gastric cancer cell lines in a concentration-dependent manner in the low millimolar range. At the same time, SERCA2 expression did not change, or decreased. Induction of SERCA3 by butyrate occurred during 2-5 days (Figure 3, Panel B) with maintained viability. Similar results were obtained in the LoVo, SW-620 and RF-48 cell lines, as well (not shown). In accordance with data in the literature (46-48),
butyrate treatment was accompanied by growth arrest, and longer treatments at higher concentrations led to apoptosis.

Based on their negligible basal expression of SERCA3 and strong induction upon butyrate treatment, KATO-III cells were selected for further quantitative study. As illustrated in Figure 4, induction of SERCA3 expression and down-regulation of SERCA2 expression were manifest at low millimolar butyrate concentrations (Panel A), starting at day 1 following treatment (Panel B). In these conditions butyrate treatment induced growth arrest (Panel D) with maintained cell viability (Panel C). Similar results were obtained on DLD-1 cells as well (not shown).

Analysis of the modulation of SERCA expression upon butyrate treatment of single cell clones, obtained by limiting dilution cloning, showed that the cell line behaved in a clonally homogeneous manner. In all KATO-III clones tested, butyrate treatment strongly and homogenously induced SERCA3 expression, whereas SERCA2 expression was at the same time decreased (Figure 5). Similar results were obtained with single cell clones of the DLD-1 colon cancer cell line as well (not shown).

As shown in Figure 6 Panel A, other short chain fatty acids such as propionate, valerate, isobutyrate, isovalerate, caproate and valproate also induced SERCA3 expression, as well as the butyrate generating prodrugs tributyrin (tributyryl-glycerol) and pivaloyloxymethyl butyrate (49-51). Other histone-deacetylase inhibitors such as HC-toxin, apicidin and SAHA also induced SERCA3 expression, although, in accordance with the literature (52-54) to a lesser extent than butyrate. On the other hand, and similarly to previous data, acetate, and other chemicals structurally related to butyrate, such as, crotonate, cyclopropane-carboxylate, pentenoate, pentinoate, trimethylacetate were only marginally active or had no effect, and heptafluorobutyrate, pivaloate, γ-aminobutyrate or lactate were inactive (not shown). Aryl-derivatives such as 3-phenylpropionate and 4-phenylbutyrate also
induced SERCA3 expression, whereas trans-cinnamate or phenoxyacetate were without effect (not shown). These observations are in agreement with previous data in the literature regarding the pharmacological profile of these molecules (55, 56), and with the recently established three-dimensional tube-like pocket structure of the active site of histone-deacetylase where inhibitors bind (57).

Interestingly, as shown in Figure 6 Panel B, although being as potent as butyrate in terms of SERCA3 induction, valerate treatment did not induce growth arrest in KATO-III cells in the conditions used. In addition, when the cells were cultured without drugs in medium in which serum was replaced by 0.5% bovine serum albumin leading to a significant inhibition of proliferation, no SERCA3 expression was seen. These observations suggest that growth inhibition per se is not indispensable for induction of SERCA3 expression by short chain fatty acids.

Short chain fatty acids induced SERCA3 expression in primary cultures of colon cancer cells as well. As shown in Figure 7, left Panel, SERCA3 expression of primary cells was increased approximately seven to nine-fold by a five days treatment with 3 mM butyrate or valerate, whereas the expression of SERCA2 at the same time diminished. Essentially the same effect could be observed in cells, which had been grown continuously for four months in vitro (right Panel) before treatments.

In addition to butyrate and its analogs, the effect of structurally and pharmacologically unrelated molecules, such as the tyrosine kinase inhibitor herbimycin-A and suramine, drugs that have been reported to induce the differentiation of COLO-205 and NCI-SNU-16 cells, respectively (58, 59), has been studied. As shown in Figure 8, Panels A and B, both drugs
induced SERCA3 expression in their target cells, indicating that this effect is not restricted only to short chain fatty acids or other histone-deacetylase inhibitors.

SERCA expression during differentiation of Caco-2 and HT29-5M21 cells.

Caco-2 cells, a human colon adenocarcinoma cell line, spontaneously undergo differentiation in post-confluent cultures. The initially rapidly growing cells become quiescent, and display structural, biochemical as well as functional characteristics corresponding to a mature enterocytic phenotype (60). After reaching confluency the cells stop to proliferate, elaborate tight junctions, microvilli, transcellular solute transport, display transepithelial electric resistance, and express many differentiation markers such as carcinoembryonic antigen (CEA), sucrase-isomaltase, dipeptidyl peptidase-IV, alkaline phosphatase and others. Due to their differentiation potential Caco-2 cells constitute a widely used model of enterocytic differentiation and function.

When SERCA expression of post-confluent Caco-2 was analyzed, a marked induction of SERCA3 expression was seen (Figure 9, Panel A). While the expression of SERCA3 was undetectable in exponentially growing non-confluent and early post-confluent cultures, SERCA3 expression was manifest from day 5-6 post-confluency, and reached a plateau at day 20, while SERCA2 expression was only slightly increased. The induction of SERCA3 expression followed a time course very similar to that of carcinoembryonic antigen, a widely used marker of differentiation of this cell line (61). In addition, during this process the expression of dipeptidyl peptidase IV, another marker of differentiation (62) was induced, and the isoform switch from α- to α+ of the tight junction protein ZO-1 was seen as described earlier (63, 64).

SERCA expression was also investigated in HT29-5M21 cells, a well characterized, methotrexate-resistant clone of HT29. Although non-differentiated in the exponential phase
of growth, in post-confluent culture these cells display a well differentiated, goblet cell-like, mucus secreting phenotype (39). As shown in Figure 9, Panel B, SERCA3 expression was induced during the differentiation of post-confluent HT29-5M21 cells.

SERCA3 isoenzymes.

Recent data in the literature, including ours, indicate that the SERCA3 gene can give rise by alternative splicing in the 3′ region of the primary transcript to three mRNA isoforms, SERCA3a, b and c, coding for proteins that carry unique peptide sequences in their C-terminal region (22-24). In order to investigate SERCA3 expression on the isoform level, we performed semi-quantitative RT-PCR experiments using oligonucleotide primers that allow the specific amplification of the various SERCA3 transcripts (45), and studied the expression of the corresponding protein isoforms using recently developed antipeptide antibodies (24) that recognize unique peptide sequences in SERCA3a, b and c, respectively.

As shown in Figure 10, Panel A, normal primary adult intestinal epithelial cells strongly expressed all three SERCA3 isoforms, and the expression of these isoenzymes was significantly induced early during the differentiation of post-confluent Caco-2 cells on the mRNA level (Panels A and B). The corresponding protein isoforms could also be detected with isoform-specific antibodies in differentiating Caco-2 cells (Panel C), as well as in butyrate treated KATO-III cells. However, in KATO-III SERCA3a expression was predominant (not shown). The kinetics of the induction of SERCA3 mRNA was somewhat dissimilar when compared to that of the corresponding protein in Caco-2 cells. Induction of SERCA3 mRNA could be detected by RT-PCR as early as day one post-confluency for all three isoforms (Figure 10, Panel A) and increased severalfold during early post-confluency (Figure 10, Panel B), after which mRNA levels decreased again (not shown). On the other hand, protein induction could be detected by Western blotting from day five post-confluency,
and protein expression levels followed thereafter a plateau-type time course. The markedly earlier detection of SERCA3 mRNA is probably due to the higher sensitivity of RT-PCR when compared to that of Western blotting. In addition, as post-confluent differentiation of Caco-2 cells is accompanied by growth arrest, the plateau-type kinetics of the accumulation of SERCA3 protein is compatible with transitory induction of corresponding mRNA, assuming that SERCA3 protein is more stable in these cells than the corresponding mRNA.

Calcium homeostasis of differentiating KATO-III cells.

In order to study cellular calcium homeostasis in functional terms during drug-induced cell differentiation, untreated, as well as butyrate treated KATO-III cells were loaded with Fura-2 and analyzed by calcium spectrofluorimetry. Resting cytosolic calcium levels and calcium mobilization from the ER into the cytosol upon complete inhibition of cellular SERCA activity using supramaximal concentrations of the specific SERCA inhibitor thapsigargin (32, 65) were quantified. As shown in Figure 11, the resting cytosolic calcium concentration of butyrate treated cells was significantly higher than that of controls (Panel A), whereas the amount of calcium released from the ER into the cytosol upon SERCA inhibition by thapsigargin (Panel B) was decreased in butyrate treated cells, indicating decreased calcium storage in the ER. These data show, that the calcium homeostasis of the KATO-III cell line undergoes a significant remodeling during drug-induced differentiation.

Discussion

As shown in this work, SERCA3 expression is strongly decreased or completely lost in colon carcinomas, while being abundantly expressed in normal colonic and gastric epithelial cells. Similarly, in several colon and gastric cancer cell lines this enzyme was
absent, and in others, in which SERCA3 expression could be detected by a highly sensitive chemiluminescent immunoblotting method, this was significantly inferior to that observed in purified primary normal colonic epithelial cells. At the same time, the ubiquitous SERCA2b isoform was present at similar levels in all studied cell types.

Independently from the initial level of SERCA3 expression, the differentiation of cell lines of enterocytic (such as Caco-2), as well as of mucus secreting phenotype (HT29-5M21) was accompanied by a marked increase of the expression of this enzyme. SERCA3 expression could be induced by several physiologically relevant short chain fatty acids such as butyrate, valerate, and to a lesser extent by propionate or caproate, by synthetic butyrate analogues such as phenylbutyrate, phenylpropionate or phenylacetate (57), or butyrate-releasing prodrugs such as pivaloyloxymethyl-butyrate (66) or tributyrin (50), molecules with clinical potential for differentiation induction therapy of malignancies (49, 50).

SERCA3 expression was also obtained with drugs such as the tyrosine kinase inhibitor herbimycin-A or by suramin, known to induce the differentiation of the cell lines studied (58, 59). In addition, a strong induction of SERCA3 expression including all three isoforms, was observed also during the spontaneous differentiation of post-confluent Caco-2 cells, with a time course comparable to that of other established markers of differentiation, such as carcinoembryonic antigen (61) or dipeptidyl peptidase IV expression (62), or the shift from the α- to the α+ isoform of the ZO-1 protein (63, 64).

To study the effect of short chain fatty acids on primary colon cancer cells we attempted to establish short term cultures, as well as continuously growing cell lines from tumor tissue from freshly obtained surgical specimens. In spite of the difficulties inherent to this technique due to microbial contamination of the specimens and poor plating efficiency and growth of the primary tumor cells in vitro, out of twelve attempts we successfully established primary cultures as well as a permanently growing, CEA-expressing cell line from
a moderately differentiated mucus secreting primary adenocarcinoma of the descending colon of a 61 years old man. Cells in short term culture behaved very similarly to other established cell lines in terms of SERCA3 induction by SCFA, and inductibility of SERCA3 expression was maintained even after four months of continuous growth and repeated subculturing. Although the detailed characterization of the obtained cell line requires more detailed studies, and despite potential heterogeneity of the primary cultures, this observation suggests, however, that short chain fatty acids can induce SERCA3 expression in primary colon cancer cells similarly to that seen in permanent cell lines, and therefore studies performed on established cell lines constitute a physiologically relevant approach in this regard.

Short chain fatty acids are produced by the fermentation of dietary fibers by the colonic flora, and the induction of the differentiation followed by apoptosis, of microscopic precancerous lesions by these molecules (and in particular by butyrate) is considered as being a main mechanism of the protective effect of a fiber-rich diet against colorectal cancer (67, 68). From these data it is tempting to speculate, that cellular calcium homeostasis may be modulated by short chain fatty acids in the colonic epithelium. In addition, our data suggest that this effect may also operate in the case of gastric cancer, as well.

Inhibition of histone-deacetylases is a key component of the mechanism of action of differentiation induction by butyrate (69). Highly specific histone-deacetylase inhibitors such as HC-toxin, apicidin or SAHA also induced SERCA expression in our hands, indicating that this mechanism may be involved in the modulation of SERCA3 expression by short chain fatty acids. Butyrate appeared, however, to be a more potent inducer of SERCA3 expression. This is in accordance with data in the literature obtained in various experimental systems (52-54), and may be due to differences in stability of the drugs in cell culture conditions, and to that in addition to histone-deacetylase inhibition, butyrate and other SCFAs also interact with other intracellular targets as well (70).
The functional implications of the modulation of SERCA expression during epithelial differentiation are complex. The pattern of a calcium signal is shaped by the concerted and coordinated action of mechanisms that increase cytosolic calcium levels (i.e., calcium channels) and that decrease it (calcium pumps), leading in many instances to oscillatory calcium signals. SERCA enzymes are actively resequestering calcium into the endoplasmic reticulum even during calcium mobilization from this organelle, and thus clearly contribute to the shaping of calcium transients (71, 72). The various geometrical characteristics of a calcium transient, and the frequency and amplitude of repetitive calcium oscillations convey key information to calcium-activated intracellular targets. The modulation by the cell of the spatiotemporal characteristics of calcium oscillations confers specificity and selectivity to calcium signals, because calcium activated target molecules such as calmodulin-dependent protein kinases, calcineurin or protein kinase C isoenzymes are optimally activated at distinct frequencies and amplitudes of calcium oscillations (73-75). This can lead to differential activation of transcription factors such as NF-AT, NF-κB and others, leading to the modulation of gene expression by calcium oscillations (76-78). The biochemical characteristics of SERCA2b and of SERCA3 enzymes are distinct (79). In particular, the calcium affinity of SERCA3 is lower ($K_{Ca} = 1.2 \mu M$) than that of SERCA2b ($K_{Ca} = 0.2 \mu M$). Quantitative changes of the relative abundance of various SERCA isoenzymes thus may alter resting cytosolic calcium levels, and may modify the shape of a calcium transient and of calcium oscillations (80), leading to an altered responsiveness of the cell to stimuli. For example if calcium elimination from the cytosol by SERCA enzymes is modified due to the replacement of SERCA2b by SERCA3, quantitatively identical IP3 generation may lead to modified calcium transients. Data presented in this work showing that resting cytosolic calcium levels are higher, and calcium mobilization is smaller in butyrate-treated KATO-III cells, where SERCA2b expression is decreased and SERCA3 expression is in parallel
increased, are compatible with these observations. Modulation of SERCA expression may thus contribute to the fine-tuning of calcium signaling, and may reset and modify activation thresholds and thus may contribute to redirect calcium signals towards distinct intracellular target proteins and signaling pathways.

Moreover, as the endoplasmic reticulum consists of heterogeneous sub-compartments within a single cell in terms of calcium content, relative abundance of IP3 receptors and SERCA enzymes (81-86), modulation of SERCA expression may also reflect the remodeling of structurally, as well as functionally distinct sub-compartments of the organelle. The present work indicates that colonic epithelial cells may constitute an interesting model system for the study of ER heterogeneity.

The inability to follow a complete normal differentiation program is a common hallmark of most malignant cells. Although the degree of malignancy and the state of differentiation of colon cancers may vary somewhat independently, cancerous cells almost always present defects in the expression of genes associated with a fully mature normal phenotype. The data presented in this work show for the first time, that SERCA3 expression is deficient in colon cancers and that differentiation of these cells leads to the induction of its expression. On the other hand, in normal epithelium, although more strongly expressed in more luminally located cells, SERCA3 expression could be detected already in less mature cells, residing in deeper regions of crypts, suggesting that SERCA3 expression may be a relatively early event during normal differentiation.

An in depth understanding of the complex structural and functional implications of the reorganization of the endoplasmic reticulum during differentiation will require the simultaneous analysis of the expression of many genes. In particular, due to the extreme complexity and plasticity of the different calcium regulatory systems that are interconnected and work in concert to handle cellular calcium fluxes, it is not possible at the present time to
associate growth control and cellular differentiation to one particular calcium pump species. An in depth understanding of the implications of the modulation of SERCA expression on cellular calcium homeostasis and differentiation will require further investigation on several cell types, using inducible expression vectors coding for SERCA isoenzymes, as well as isoform-specific gene knock out techniques. Our work shows, for the first time, however, that induction of SERCA3 expression is taking place during the differentiation of colon cancer cells, and data obtained on the KATO-III cell line suggest that cellular calcium homeostasis of the ER may also be reorganized in functional terms during differentiation. In addition, our data indicate that SERCA3 may serve as a useful new marker for the study of colon, as well as gastric cancer phenotype.
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References

1. Berridge, M. J., Lipp, P. and Bootman, M. D. (2000) Nature Reviews (Mol. cell biol.) 1, 11-21
2. Putney, J. W. and Riberio, C. M. (2000) Cell. Mol. Life Sci. 57, 1272-1286
3. Corcoran, E. E. and Means, A. R. (2001) J. Biol. Chem. 276, 2975-2978
4. Musashi, M., Ota, S. and Shiroshita, N. (2000) *Int. J. Hematol.* **72**, 12-19
5. Crabtree, G. R. (2001) *J. Biol. Chem.* **276**, 2313-2316
6. Huang, Y. and Wang, K. K. (2001) *Trends Mol. Med.* **7**, 355-362
7. Keely, S. J., Calandrilla, S. O. and Barrett, K. E. (2000) *J. Biol. Chem.* **275**, 12619-12625
8. Zippel, R., Balestrini, M., Lomazzi, M. and Sturani, E. (2000) *Exp. Cell Res.* **258**, 403-408
9. Kawai, T., Nomura, F., Hoshino, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Akira, S. (1999) *Oncogene* **18**, 3471-3480
10. Missotten, M., Nichols, A., Rieger, K. and Sadoul, R. (1999) *Cell Death Differ.* **6**, 124-129
11. Cooper, G. R., Brostrom, C. O. and Brostrom, M. A. (1997) *Biochem. J.* **325**, 601-608
12. Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) *Physio. Rev.* **74**, 595-636
13. Corbett, E. F. and Michalak, M. (2000) *TIBS* **25**, 307-311
14. Pinton, P., Ferrari, D., Rapizzi, E., Di Virgilio, F., Pozzan, T. and Rizzuto, R. (2001) *EMBO J.* **20**, 2690-2701
15. Brandl, C. J., deLeon, S., Martin, D.R. and MacLennan, D. H. (1987) *J. Biol. Chem.* **262**, 3768-3774
16. Lytton, J. and MacLennan, D. H. (1987) *J. Biol. Chem.* **263**, 15024-15031
17. Gunteski-Hamblin, A-M., Greeb, J. and Shull, G. E. (1988) *J. Biol. Chem.* **263**, 15032-1540
18. Papp, B., Enyedi, A., Pászty, K., Kovács, T., Sarkadi, B., Gárdos, G., Magnier, C., Wuytack, F. and Enouf, J. (1992) *Biochem. J.* **288**, 297-302
19. Launay, S., Bobe, R., Lacabaratz-Porret, C., Bredoux, R., Kovács, T., Enouf, J. and Papp, B. (1997) *J. Biol. Chem.* **272**, 10746-10750
20. Launay, S., Gianni, M., Kovacs, T., Bredoux, R., Bruel, A., Gélébart, P., Zassadowski, F., Chomienne, C., Enouf, J. and Papp, B. (1999) Blood 93, 4395-4405
21. Lacabaratz-Porret, C., Launay, S., Corvazier, E., Bredoux, R., Papp, B. and Enouf, J. (2000) Biochem. J. 350, 723-734
22. Poch, E., Leach, S., Snape, S., Cacic, T., MacLennan, D. H. and Lytton, J. (1998) Am. J. Physiol. 275, C1449-1458
23. Dode, L., De Greef, C., Mountian, I., Attard, M., Town, M. M., Casteels, R. and Wuytack, F. (1998) J. Biol. Chem. 273, 13982-13994
24. Kovacs, T., Felfoldi, F., Papp, B., Paszty, K., Bredoux, R., Enyedi, A. and Enouf, J. (2001) Biochem. J. 358, 559-568
25. Burk, S. E., Lytton, J., MacLennan D. H. and Shull, G. E. (1989) J. Biol. Chem. 264, 18561-18568
26. Kwan-Dun, W., Wen-Sen, L., Wey, J., Bungard, D. and Lytton, J. (1995) Am. J. Physiol. 269, (Cell Physiol. 38) C775-C784
27. McConkey, D. J. and Nutt, L. K. (2001) Methods in Cell Biol. 66, 229-246
28. Foyouzi-Youssefi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demaurex, N. and Krause, K. H. (2000) Proc. Natl. Acad. Sci. USA, 97, 5723-5728
29. Kuo, T. H., Kim, H. R., Zhu, L., Yu, Y., Lin, H. M. and Tsang, W. (1998) Oncogene 17, 1903-1910
30. Waldron, R. T., Short, A. D., Meadows, J. J., Ghosh, T. K. and Gill, D. (1994) J. Biol. Chem. 269, 11927-11933
31. Schaefer, A., Magocsi, M., Stocker, U., Kosza, F. and Marquardt, H. (1994) J. Biol. Chem. 269, 8786-8791
32. Treiman, M., Caspersen, C. and Christensen, S. B. (1998) Trends Pharmacol. Sci. 19, 131-135
33. Rao, R. V., Hermel, E., Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M. and Bredesen, D. E. (2001) *J. Biol. Chem.* **276**, 33867-33874

34. Hakii, H., Fujiki, H., Suganuma, M., Nakayasu, M., Tahira, T., Sugimura, T., Scheuer, J. P. And Christensen, S. B. (1986) *J. Cancer Res. Clin. Oncol.* **111**, 177-181

35. Sakai, A. And Teshima, R. (2001) *Cancer Lett.* **168**, 183-190

36. Chami M., Gozuacik, D., Lagorce, D., Brini, M., Felson, P., Peaucellier, G., Pinton, P., Lecoeur, H., Gougeon, M-L., le Maire, M., Rizzuto, R., Bréchot, C. and Paterlini-Bréchot, P. (2001) *J. Cell Biol.* **153**, 1301-1313

37. Hellmich, M. R., Rui, X-L., Hellmich, H. L., Fleming, R. Y. D, Evers, B. M. and Townsend, C. M. Jr. (2000) *J. Biol. Chem.* **275**, 32122-32128

38. Carroll R. E., Matkowskyj, K. A., Tretiakova, M. S., Battey, J. F. and Benya, R. V. (2000) *Cell Growth and Differentiation* **11**, 385-393

39. Lesuffleur, T., Violette, S., Vasile-Pandrea, I., Dussaulx, E., Barbat, A., Muleris, M. and Zweibaum, A. (1998) *Int. J. Cancer* **76**, 383-392

40. Perreault, N. and Beaulieu, J. F. (1996) *Exp. Cell Res.* **224**, 354-364

41. Deschênes, C., Vézina, A., Beaulieu, J. F. and Rivard, N. (2001) *Gastroenterology* **120**, 423-438

42. Grossmann, J., Walther, K., Artinger, M., Kiessling, S. and Schölmerich, J. (2001) *Cell Growth and Differentiation* **12**, 147-155

43. Hauri, H. P., Sterchi, E., E., Bienz, D., Fransen, J. A. and Marxer, A. (1985) *J. Cell Biol.* **101**, 838-851

44. Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440-3450.

45. Martin, V., Bredoux, R., Corvazier, E., Papp, B. and Enouf, J. (2000) *Hypertension* **35**, 91-101
46. Hague, A., Manning, A. M., Hanlon, K. A., Huschtscha, L. I., Hart, D. and Paraskeva, C. (1993) \textit{Int. J. Cancer} \textbf{55}, 498-505
47. McBain, J. A., Eastman, A., Novel, C. S. and Mueller, G. C. (1997) \textit{Biochem. Pharmacol.} \textbf{53}, 1357-1368
48. Litvak, D. A., Hwang, K. O., Evers, B. M. and Townsend, C. M. (2000) \textit{Anticancer Res.} \textbf{20}, 779-784
49. Rephaeli, A., Rabizadeh, E., Aviram, A., Shaklai, M., Ruse, M. and Nudelman, A. (1991) \textit{Int. J. Cancer} \textbf{49}, 66-72
50. Conley, B. A., Egorin, M. J., Tait, N., Rosen, D. M., Sausville, E. A., Dover, G., Fram, R. J. and Van Echo, D. A. (1998) \textit{Clin. Cancer Res.} \textbf{4}, 629-634
51. Maier, D., Reich, E., Martin, R., Bachem, M., Altug, V., Hautmann, R. E. and Gschwend, J. E. (2000) \textit{Int. J. Cancer} \textbf{88}, 245-251
52. McCaffrey, P. G., Newsome, D. A., Fibach, E., Yoshida, M. and Su, M. S-S. (1997) \textit{Blood} \textbf{90}, 2075-2083
53. Wu, J. T., Archer, S. Y., Hinnebusch, B., Meng, S. and Hodin, R. A. (2001) \textit{Am. J. Physiol.} \textbf{280}, G482-490
54. Siavoshian, S., Segain, J. P., Kornprobst, M., Bonnet, C., Cherbut, C., Galmiche, J. P. and Blottièrè, H. M. (2000) \textit{Gut} \textbf{46}, 507-514
55. Heerdt, B. G., Houston, M. A. and Augenlicht, L. H. (1994) \textit{Cancer Res.} \textbf{54}, 3288-3293
56. Shack, S., Miller, A., Liu, L., Prasanna, P., Thibault, A. and Samid, D. (1996) \textit{Clin. Cancer Res.} \textbf{2}, 865-872
57. Finnin, M. S., Donigian, J. R., Cohen, A., Rich, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. and Pavletich, N. P. (1999) \textit{Nature} \textbf{401}, 188-193
58. Choe, G., Kim, W. H., Park, J. G. and Kim, Y. I. (1997) *J. Korean Med. Sci.* **12**, 433-442
59. Wang, Y., Hung, C., Koh, D., Cheong, D. and Hooi, S. C. (2001) *Int. J. Oncol.* **18**, 617-622
60. Pinto, M., Robine-Leon, S., Appay, M-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assman, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) *Biol. Cell* **47**, 323-330
61. Hauck, W. and Stanners, C. P. (1991) *Cancer Res.* **51**, 3526-3533
62. Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Troto, P., Barba, A. and Trugnan, G. (1992) *J. Biol. Chem.* **267**, 4824-4833
63. Willott, E., Balda, M. S., Heintzelman, M., Jameson, B. and Anderson, J. M. (1992) *Am. J. Physiol.* **262**, C1119-1124
64. Anderson, J. M., Van Itallie, C. M., Peterson, M. D., Stevenson, B. R., Carew, E. A. and Mooseker, M. S. (1989) *J. Cell Biol.* **109**, 1047-1056
65. Inesi, G. and Sagara, Y. (1994) *J. Membr. Biol.* **141**, 1-6
66. Aviram, A., Zimrah, Y., Shaklai, M., Nudelman, A. and Rephaeli, A. (1994) *Int. J. Cancer* **56**, 906-909
67. Trock, B., Lanza, E. and Greenwald, P. (1990) *J. Natl. Cancer Inst.* **82**, 650-661
68. McIntyre, A., Gibson, P. R. and Young, G.P. (1993) *Gut* **34**, 386-391
69. Della Ragione, F. Criniti, V., Della Pietra, V., Borriello, A., Loiva, A., Indaco, S., Yamamoto, T. and Zappia, V. (2001) *FEBS Lett.* **499**, 199-204
70. Cuisset, L., Tichonicky, L., Jaffray, P. and Delpech, M. (1998) *J. Biol. Chem.* **272**, 24148-24153
71. Yu, R. and Hinkle, P. M. (2000) *J. Biol. Chem.* **275**, 23648-23653
72. Gilon, P., Arredouani, A., Gailly, P., Gromada, J. and Henquin, J-C. (1999) *J. Biol. Chem.* **274**, 20197-20205
73. Pralong, W-F., Spät, A. and Wollheim, C. B. (1994) *J. Biol. Chem.* **269**, 27310-27314
74. Oancea, E. and Meyer, T. (1998) *Cell* **95**, 307-318
75. De Koninck, P. and Schulman, H. (1998) *Science* **279**, 227-230
76. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. and Healy, J. I. (1997) *Nature* **386**, 855-858
77. Li, W-h., Llopis, J., Whitney, M., Zlokarnik, G. and Tsien, R. Y. (1998) *Nature* **392**, 936-941
78. Dolmetsch, R. E., Xu, K. and Lewis, R. S. (1998) *Nature* **392**, 933-936
79. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E. and MacLennan, D. H. (1992) *J. Biol. Chem.* **267**, 14483-14489
80. Camacho, P. and Lechleiter, J. D. (1993) *Science* **260**, 226-229
81. Golovina, V. A. and Blaustein, M. P. (1997) *Science* **275**, 1643-1648
82. Golovina, V. A. and Blaustein, M. P. (2000) *Glia* **31**, 15-28
83. Papp, B., Pászty, K., Kovács, T., Sarkadi, B., Gárdos, G., Enouf, J. and Enyedi, A. (1993) *Cell Calcium* **14**, 531-538
84. Engelender, S., Wolosker, H. and de Meis, L. (1995) *J. Biol. Chem.* **270**, 21050-21055
85. Cavallini, L., Coassin, M. and Alexandre, A. (1995) *Biochem J.* **310**, 449-452
86. Lee, M. G., Xu, X., Zeng, W., Diaz, J., Kuo, T. H., Wuytack, F., Raeymaekers, L. and Muallem, S. (1997) *J. Biol. Chem.* **272**, 15771-15776
87. Mariadason, J. M., Corner, G. A. and Augenlicht, L. H. (2000) *Cancer Res.* **60**, 4561-4572
Legends to Figures.

Figure 1.
SERCA3 expression in normal colonic and gastric mucosa and in colonic neoplasia.

Cryostat sections were labeled with the SERCA3-specific PLIM430 antibody using an avidin-biotin-peroxidase system and revealed as a brown coloration with diaminobenzidine on a blue hematoxylin counterstain.

Panel A: normal colonic epithelium, longitudinal section.

Panel B: gastric mucosa, longitudinal section.

Panel C: moderately differentiated adenocarcinoma (SERCA3\textsuperscript{neg}) surrounded by normal epithelial crypts (SERCA3\textsuperscript{pos}; cross sectioned). Arrowheads delineate the interface between normal (brown coloration; SERCA3\textsuperscript{pos}) and cancerous tissue (blue coloration; SERCA3\textsuperscript{neg}).

Panel D: Higher magnification view of a region of the specimen presented in Panel C, displaying a normal (SERCA3\textsuperscript{pos}) and an adenocarcinomatous gland (SERCA3\textsuperscript{neg}).

Panels E, F and G: Colon adenocarcinoma specimens (SERCA3\textsuperscript{neg}) and adjacent normal epithelium (SERCA3\textsuperscript{pos}). In Panel F arrowheads delineate the interface between normal (brown coloration; SERCA3\textsuperscript{pos}) and cancerous tissue (blue coloration; SERCA3\textsuperscript{neg}).

Panel H: Colon adenocarcinoma displaying weak apical SERCA3 staining with adjacent normal epithelium. Inset: Higher magnification view of malignant cells with weak apical staining for SERCA3.

Panels I and J: Immunocytochemical staining for SERCA3 in KATO-III (SERCA3\textsuperscript{neg}) and COLO-205 (SERCA3\textsuperscript{pos}) cells, respectively. A moderate SERCA3 staining can be observed in COLO-205 cells.
Figure 2.

Expression of SERCA-type calcium pumps in colon- and gastric cancer cell lines.

Equal amounts of total cellular protein from various exponentially growing colon- (Panel A) and gastric (Panel B) cancer cell lines, as well as from normal adult colonic epithelial cells (Panel C) were immunostained for SERCA2 and SERCA3 expression with the IID8 and PLIM430 antibodies, respectively.

Whereas SERCA2 expression is relatively homogenous, that of SERCA3 varies among cell lines. In all cell lines, however, SERCA3 expression is inferior to that of normal colonic epithelial cells.

Figure 3.

Induction of SERCA3 expression by butyrate in various colon- and gastric cancer cell lines.

Panel A: Cells were cultured in the presence of various concentrations of sodium butyrate for 4 days and the expression of SERCA2 and SERCA3 enzymes was determined by Western blotting. Butyrate treatment induced the expression of SERCA3 in a concentration dependent manner in the low millimolar range. Induction was manifest in cells with undetectable initial SERCA3 levels (KATO-III, DLD-1), as well as in cells, which express this enzyme already in the untreated state (LS-174T, SW-48, SW-403, NCI-N87).

Panel B: Time course of SERCA3 induction by butyrate.

Cells were cultured in the presence of 3 mM butyrate and SERCA expression was measured daily. Induction of SERCA3 expression is detectable as early as day 1, and reaches a plateau after 4-5 days of treatment in most cell lines.

Figure 4.

Quantitative evaluation of expression of SERCA proteins in butyrate treated KATO-III cells.
Panel A: Concentration dependency of SERCA expression. The cells were treated with various concentrations of butyrate for 4 days and SERCA expression was determined. Full circles: SERCA3, empty circles: SERCA2.

Panel B: Time course of SERCA expression. KATO-III cells were treated with 3 mM butyrate and SERCA expression was determined daily for 4 days. Full circles: SERCA3, empty circles: SERCA2.

Panel C: Viability of butyrate treated KATO-III cells. The cells were treated with 3 mM butyrate for 4 days and viability was determined by Trypan blue exclusion daily. Full circles: butyrate treated cells, empty circles: untreated control cells.

Panel D: Inhibition of proliferation of KATO-III cells by butyrate. The cells were treated with 3 mM butyrate for 4 days and cell density was measured daily by a hemocytometer. Full circles: butyrate treated cells, empty circles: untreated cells.

Figure 5.

Induction of SERCA3 expression in single cell clones of KATO-III.

The expression of SERCA2 and SERCA3 enzymes of single cell clones obtained from the KATO-III cell line was compared prior and after treatment with 3 mM butyrate for 4 days. Induction of SERCA3 expression was obtained in all KATO-III clones (12 clones tested).

Figure 6.

Panel A: Induction of SERCA3 expression by butyrate analogs.

KATO-III cells were treated with various naturally occurring short chain fatty acids (acetate, propionate, butyrate, valerate and caproate, all at 3 mM), with butyrate-releasing prodrugs (tributyrin, 1 mM, pivaloyloxy-methylbutyrate, 0.5 mM), with synthetic analogs containing double and triple bonds (crotonate, vinylacetate, pentenoic- and pentinoic acid), a branching
chain (isobutyrate, isovalerate, trimethylacetate, valproate), a cycloalkyl group (cyclopropane-carboxylic acid) or an aryl group (phenylacetate, phenylpropionate, phenylbutyrate) at 3 mM concentration for 5 days, and with the highly specific histone-deacetylase inhibitors HC-toxin (0.1 µM), SAHA acid (1 µM) or apicidin (1 µM). Short chain fatty acids, their prodrugs, as well as aryl-substituted analogs and various histone-deacetylase inhibitors induced SERCA3 expression.

**Panel B:** Growth inhibition is not required for SERCA3 induction.

KATO-III cells were incubated with 3 mM butyrate, 3mM valerate, or cultured without drugs in serum free medium containing 0.5% bovine serum albumin (BSA). Cell densities (lower panel), as well as SERCA3 expression (upper panel) were detected at day 3. Valerate treatment induced SERCA3 expression without growth arrest, and growth inhibition induced by serum withdrawal was without effect.

**Figure 7.**

Induction of SERCA3 expression in freshly isolated primary colon cancer cells.

Cells at an early stage following plating (day 6, left) and after four months of continuous growth and subculturing (right) were treated with 3 mM butyrate or valerate for 5 days. In both cases a strong induction of SERCA3 expression was obtained by both SCFA.

**Figure 8.**

Induction of SERCA3 expression by various differentiation inducing agents.

**Panel A:** COLO-205 cells were treated with 0.52 µM herbimycin-A and the time course of the expression of SERCA2 and SERCA3 proteins was determined.

**Panel B:** NCI-SNU-16 cells were treated with various concentrations of suramin for 7 days and SERCA2, and SERCA3 expression was measured.
Treatment of the cells with these differentiation inducing agents led to the induction of the expression of SERCA3 protein.

**Figure 9.**
Differentiation of Caco-2 and HT29-5M21 cells.

**Panel A:** Caco-2 cells were allowed to reach confluency and cultured further for 26 days. Cells were harvested at different time points and the expression of SERCA2 and SERCA3 proteins was measured. The expression of various established markers of enterocytic differentiation was determined in parallel. Whereas SERCA2 was constitutively expressed in the cells, and its expression slightly increased during the differentiation of post-confluent cells, SERCA3 was undetectable prior confluency, and was strongly induced during post-confluent cell differentiation. The time course of SERCA3 expression closely paralleled that of the induction of the expression of carcinoembryonic antigen or dipeptidyl peptidase IV, as well as that of the isoform switch from $\alpha$- to $\alpha$+ of the tight junction associated protein ZO-1.

**Panel B:** Induction of SERCA3 expression in HT29-5M21 cells. During growth of the cells in post-confluent conditions that allow differentiation of cells towards a mucus secreting phenotype, induction of SERCA3 expression was observed.

**Figure 10.**
Expression of SERCA3 isoenzymes in differentiating Caco-2 and normal adult colonic epithelial cells.

**Panel A:** Induction of the synthesis of SERCA3a, 3b and 3c mRNA in Caco-2 cells after confluency as detected by SERCA3 isoform-specific RT-PCR. As control G3PDH, as well as
SERCA2b was amplified in the same RNA preparations. The various SERCA3 isoforms were abundantly expressed in normal adult human colonic epithelial cells.

**Panel B:** Estimation of SERCA isoform mRNA levels in differentiating Caco-2 cells by semiquantitative RT-PCR using isoform-specific primers. As internal controls SERCA2b and G3PDH were used. Differentiation of Caco-2 cells leads to the induction of the expression of all three SERCA3 isoforms.

**Panel C:** Induction of the expression of SERCA3a, 3b and 3c protein in post-confluent Caco-2 cells detected by Western blotting using isoform-specific antibodies. Induction of SERCA3a, b and c protein expression could be detected during differentiation.

**Figure 11.**

Calcium homeostasis of KATO-III cells differentiated by butyrate.

KATO-III cells were treated for 5 days with 3 mM butyrate and cytosolic calcium concentration [Ca^{2+}]_i was compared to untreated controls in resting cells (Panel A), and following treatment with a supramaximal dose of the SERCA inhibitor thapsigargin (1 µM, Panel B). Resting cytosolic calcium concentration was significantly higher in butyrate treated cells than in controls, and the amount of calcium stored in intracellular pools that could be released into the cytosol by thapsigargin was significantly decreased in butyrate treated cells.
Figure 2

| A | B | C |
|---|---|---|
| HT-29 | KATO-III | Normal colonic epithelial cells |
| SW-403 | NCI-SNU-1 | |
| SW-48 | NCI-N87 | |
| LS-174T | NCI-SNU-16 | |
| COLO 205 | | |
| Caco-2 | | |
| DLD-1 | | |

SERCA2

Colon carcinoma

Gastric carcinoma
Figure 3b

|               | KATO-III          | DLD-1           |
|---------------|-------------------|-----------------|
| **Time (days)** | ![Diagram](image) | ![Diagram](image) |
| **SERCA2**    | ![Diagram](image) | ![Diagram](image) |
| **SERCA3**    | ![Diagram](image) | ![Diagram](image) |

|               | LS-174T           | SW-48           |
|---------------|-------------------|-----------------|
| **Time (days)** | ![Diagram](image) | ![Diagram](image) |
| **SERCA2**    | ![Diagram](image) | ![Diagram](image) |
| **SERCA3**    | ![Diagram](image) | ![Diagram](image) |

|               | SW-403            | HT-29           |
|---------------|-------------------|-----------------|
| **Time (days)** | ![Diagram](image) | ![Diagram](image) |
| **SERCA2**    | ![Diagram](image) | ![Diagram](image) |
| **SERCA3**    | ![Diagram](image) | ![Diagram](image) |
Figure 4

A

SERCA expression (%)

Butyrate (mM)

0 1 2 3 4

0 20 40 60 80 100

B

SERCA expression (%)

Time (Days)

0 1 2 3 4

0 20 40 60 80 100

C

Viability (%)

Time (Days)

0 1 2 3 4

0 20 40 60 80 100

D

Cell density (10^6/mL)

Time (days)

0 1 2 3 4

0 2 3 4 5 6 7 8 9

Figure 4
Figure 5

KATO-III

| Butyrate (3 mM) | - | + | - | + | - | + | - | + | - | + | - | + | - | + |
|-----------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SERCA2          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| SERCA3          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
Figure 7

|                | Primary cells | Cell line |
|----------------|---------------|-----------|
| CEA            | ![Image]      | ![Image]  |
| SERCA2         | ![Image]      | ![Image]  |
| SERCA3         | ![Image]      | ![Image]  |

Butyrate (3mM) - - + - +
Valerate (3mM) - - + - +

SERCA3 fold increase

Butyrate (3mM) - + - +
Valerate (3mM) - - + +
**Figure 8**

| A | Herbinycin-A (0.52μM) | Time (days) | COLO-205 |
|---|-----------------------|-------------|-----------|
|   | SERCA2                | 0 1 2 3 4 5 6 |           |
|   | SERCA3                |             |           |

| B | Suramine (μM) | 0 35 70 105 140 175 | NCI-SNU-16 |
|---|--------------|---------------------|------------|
|   | SERCA2       |                     |            |
|   | SERCA3       |                     |            |
Figure 10 A

| Days post-confluency | Caco-2 | Normal colonic epithelial cell |
|----------------------|--------|-------------------------------|
|                      | 0      | 1                             | 2 | 3 | 5 | 270 bp  |
| SERCA2b              |        |                               |   |   |   |         |
| SERCA3a              |        |                               |   |   |   | 319 bp  |
| SERCA3b              |        |                               |   |   |   | 407 bp  |
| SERCA3c              |        |                               |   |   |   | 420 bp  |
| G3PDH                |        |                               |   |   |   | 767 bp  |
|          | Days post- confluency |
|----------|-----------------------|
|          | 4  | 5  | 6  | 10 | 14 | 16  |
| SERCA3a  |    |    |    |    |    |     |
| SERCA3b  |    |    |    |    |    |     |
| SERCA3c  |    |    |    |    |    |     |

Figure 10 C
Figure 11

A  Basal cytosolic $[\text{Ca}^{2+}]$

$[\text{Ca}^{2+}]$ (nM)

|        | Control | Butyrate |
|--------|---------|----------|
| 0.0    |         |          |
| 50.0   |         |          |
| 100.0  |         |          |

B  Thapsigargin-induced calcium release

Increase in $[\text{Ca}^{2+}]$ (nM)

|        | Control | Butyrate |
|--------|---------|----------|
| 0.0    |         |          |
| 50.0   |         |          |
| 100.0  |         |          |
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Grossmann, Nathalie Rivard, Yves Panis, Virginie Martin, Raymonde Bredoux, Jocelyne 
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