Original Paper

The Putative Role of the Non-Gastric H^+/K^+ -ATPase ATP12A (ATP1AL1) as Anti-Apoptotic Ion Transporter: Effect of the H^+/K^+ ATPase Inhibitor SCH28080 on Butyrate-Stimulated Myelomonocytic HL-60 Cells

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Key Words

H^+/K^+ -ATPase • ATP12A • ATP1AL1 • HL-60 cells • Butyrate • Apoptosis • Cell volume

Abstract

Background/Aims: The ATP12A gene codes for a non-gastric H^+/K^+ ATPase, which is expressed in a wide variety of tissues. The aim of this study was to test for the molecular and functional expression of the non-gastric H^+/K^+ ATPase ATP12A/ATP1AL1 in unstimulated and butyrate-stimulated (1 and 10 mM) human myelomonocytic HL-60 cells, to unravel its potential role as putative apoptosis-counteracting ion transporter as well as to test for the effect of the H^+/K^+ ATPase inhibitor SCH28080 in apoptosis. **Methods:** Real-time reverse-transcription PCR (qRT-PCR) was used for amplification and cloning of ATP12A transcripts and to assess transcriptional regulation. BCECF microfluorimetry was used to assess changes of intracellular pH (pH_i) after acute intracellular acid load (NH_4Cl prepulsing). Mean cell volumes (MCV) and MCV-recovery after osmotic cell shrinkage (Regulatory Volume Increase, RVI) were assessed by Coulter counting. Flow-cytometry was used to measure MCV (Coulter principle), to assess apoptosis (phosphatidylserine exposure to the outer leaflet of the cell membrane, caspase activity, 7AAD staining) and differentiation (CD86 expression). **Results:** We found by RT-PCR, intracellular pH measurements, MCV measurements and flow cytometry that ATP12A is expressed in human myelomonocytic HL-60 cells. Treatment of HL-60 cells with 1 mM butyrate leads to monocyte-

directed differentiation whereas higher concentrations (10 mM) induce apoptosis as assessed by flow-cytometric determination of CD86 expression, caspase activity, phosphatidylserine exposure on the outer leaflet of the cell membrane and MCV measurements. Transcriptional up-regulation of ATP12A and CD86 is evident in 1 mM butyrate-treated HL-60 cells. The H⁺/K⁺ ATPase inhibitor SCH28080 (100 μM) diminishes K⁺-dependent pH_i recovery after intracellular acid load and blocks RVI after osmotic cell shrinkage. After seeding, HL-60 cells increase their MCV within the first 24 h in culture, and subsequently decrease it over the course of the next 48 h. This effect can be observed in the overall- and non-apoptotic fraction of both untreated and 1 mM butyrate-treated HL-60 cells, but not in 1 mM butyrate-stimulated phosphatidylserine-positive cells. These cells do not shrink from 24 h to 72 h and have finally a higher MCV than untreated cells unless they are exposed to SCH28080. 10 mM butyrate induces apoptosis within 24 h. **Conclusion:** In summary we show that in HL-60 cells ATP12A is a functionally active H⁺/K⁺ ATPase that may counteract events during early apoptosis like intracellular acidosis, loss of intracellular K⁺ ions and apoptotic volume decrease. Its expression and/or susceptibility to the H⁺/K⁺ ATPase inhibitor SCH28080 becomes most evident in cells exposing phosphatidylserine on the outer leaflet of the cell membrane and therefore during early apoptosis.

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Introduction

In previous studies it has been shown that leucocyte functions are sensitive to inhibitors of the gastric H⁺/K⁺ ATPase like omeprazole, lansoprazole or pantoprazole and/or the K⁺-competitive inhibitor SCH28080. These proton pump inhibitors (PPIs) impair neutrophil adherence to endothelial cells, chemotaxis, transendothelial migration, degranulation, superoxide generation, translocation of cytochrome b-245, Ca²⁺ influx and p38 MAP kinase activation in human polymorphonuclear leucocytes (PMNs). PPIs inhibit cytotoxic activity in human natural killer cells [1], decrease the secretion of extracellular translationally controlled tumor protein (TCTP) in monocytic U937 cells [2] and have anti-inflammatory effects in monocytic THP-1 as well as in human microglial cells [3]. Moreover, in mouse microglia the existence of a PPI sensitive H⁺/K⁺ ATPase was shown [4]. PPIs have also been shown to reduce the capacity of osteoclasts to resorb bone *in vitro* and *in vivo* [5, 6]. In addition they induce apoptosis in Jurkat cells [7] and human PMNs [8] and potentiate hypericin-induced phototoxicity of HL-60 cells [9]. All these results imply the functional expression of an H⁺/K⁺ ATPase in distinct subsets of leucocytes. Indeed we could disclose the expression of the gastric H⁺/K⁺ ATPase and unravel a K⁺-dependent H⁺ extrusion mechanism sensitive to the PPIs omeprazole and SCH28080 in human PMNs and showed that the putative gastric H⁺/K⁺ ATPase regulates migration in these cells [10], an effect that can be reversed by addition of the H⁺/K⁺ ionophore nigericin [11].

H⁺/K⁺ ATPases comprise the gastric isoform serving to produce gastric acid and the non-gastric isoform, which is also referred to as the renal- and colonic H⁺/K⁺ ATPase. These pumps are members of the P2-type family of cation-transporting ATPases, which also includes the ubiquitous Na⁺/K⁺ ATPase [12-14]. The H⁺/K⁺ ATPase isoforms share functional and structural similarities. They form heterodimers composed of an ion-translocating transmembrane catalytic alpha-subunit and a glycosylated beta-subunit, and pump K⁺ (and/or NH₄⁺) into the cell in exchange for H⁺ (and/or Na⁺). While the gastric H⁺/K⁺ ATPase is insensitive to inhibition by ouabain, the catalytic alpha polypeptide (HKalpha2) encoded by the gene ATP12A (ATP1AL1) is a non-gastric H⁺/K⁺ ATPase which is sensitive to inhibition by both SCH28080 and ouabain [15-19].

Reverse-transcriptase (RT)-PCR screening of mouse, rat, rabbit, human, and dog tissues revealed significant ATP12A expression levels in the skin, kidney and distal colon of all species and strong expression in coagulating and preputial glands. Lower expression levels were detected in the brain, placenta, lung, heart, forestomach and uterus. In rabbit brain the expression was found to be specific to the choroid plexus and cortex [14, 20-25].

Functional expression of the non-gastric H⁺/K⁺ ATPase alpha-subunit (HKalpha2) requires dimerization with a glycosylated beta-subunit. No unique non-gastric beta-subunit has yet been identified, but it has been shown that both the Na⁺/K⁺ ATPase and the gastric H⁺/K⁺ ATPase beta-subunits can assemble with HKalpha2 *in vivo* or when co-expressed in heterologous expression systems to form functional pumps [15, 26-28]. In transfected MDCK cells the plasma membrane expression of human ATP1A1 is regulated by the PKC pathway and activation of PKC leads to internalization of the H⁺/K⁺ ATPase subunits [29]. The known functions of ATP12A include the regulation of acid-base and K⁺ homeostasis in the kidney and colon, where it is expressed in the apical membranes of renal cortical collecting ducts and colon brush border [30-32] and the acidification of prostate secretions in the anterior lobe of the organ, where it is localized in the apical membrane of the epithelium [21, 22]. In the ducts of the exocrine pancreas the non-gastric H⁺/K⁺ ATPase appears to be involved in transepithelial bicarbonate secretion [33]. In newborn rats HKalpha2 is specifically up-regulated but becomes undetectable in adult rats. Hence it appears to play an important role in early growth and development [26, 30, 34]. In kidney cells heterologously expressing ATP12A and the gastric H⁺/K⁺ ATPase beta-subunit, ATP12A is sorted to the apical membrane of MDCK cells, but to the lateral membrane in LLC-PK1 cells [28, 35]. It has been shown that in some colorectal adenocarcinomas ATP12A is up-regulated and recently we could demonstrate that in benign hyperplasia and cancerous tissue of the prostate ATP12A shows modified sorting in the luminal cells of the glandular epithelium while the expression of the gene was unaltered in these conditions [20, 22, 36]. Given the wide tissue distribution of ATP12A and deranged expression and/or cellular distribution in tumor cells and tissues further specific functions of the pump can be assumed.

The aim of the present study was to test for the existence as well as the identification and characterization of the functional and molecular properties of an H⁺/K⁺ ATPase in myelomonocytic HL-60 cells. HL-60 cells are predominantly promyelocytes, which can be differentiated towards granulocytes, macrophages/monocytes or basophils by different agents such as retinoic acid, DMSO or the short chain fatty acid butyrate [37-45]. Beside induction of differentiation butyrate has also been shown to exert a plentitude of other effects including inhibition of cell proliferation or induction of apoptosis [46-52]. Importantly, butyrate is an inhibitor of histone deacetylases [53, 54]. Here we show that HL-60 cells possess a SCH28080- and ouabain-sensitive, K⁺-dependent H⁺ extruding mechanism consistent with the expression of a functional H⁺/K⁺ ATPase, and demonstrate that these cells express the non-gastric H⁺/K⁺ ATPase ATP12A (ATP1A1). In addition we show up-regulation of ATP12A expression upon treatment with low (1 mM) and induction of apoptosis with high (10 mM) concentrations of butyrate. The data demonstrate that in HL-60 cells ATP12A is a molecular entity of the H⁺/K⁺ ATPase, which is transcriptionally up-regulated by induction of the cells with 1 mM butyrate. ATP12A appears to regulate cell volume during apoptosis. The susceptibility of HL-60 cells to the H⁺/K⁺ ATPase inhibitor SCH28080 seems to be restricted to cells that expose phosphatidylserine on the outer leaflet of the cell membrane and therefore this protein might serve as an anti-apoptotic ion transport mechanism presumably during the early phase of apoptosis.

Materials and Methods

Salts, Chemicals, Drugs and Solutions

All salts, chemicals, and drugs (*p.a.* grade) were purchased from Sigma-Aldrich (Germany) unless otherwise stated. Cell culture reagents were from Invitrogen (Life Technologies, Thermo Fisher Scientific, Germany). Optical filters were from AHF Analysentechnik (Tübingen, Germany). Ouabain was purchased from Sigma-Aldrich and SCH28080 (2-methyl-8-(phenylmethoxy)imidazopyridine-3-acetonitrile) was obtained from Schering-Plough Research Institute (Kenilworth, New Jersey, USA) and Sigma-Aldrich. For measurements of intracellular pH (pH_i) and mean cell volume (MCV) the solutions were composed as indicated in Table 1 unless otherwise stated.

Table 1. Solutions used for measurements of intracellular pH and regulatory volume increase (RVI) measurements. Concentrations are given in mM. pH_i and RVI measurements were performed at room temperature and 37°C, respectively

	Control	NH ₄ Cl	0Na ⁺ 0K ⁺	0Na ⁺	C6	C7	C8
NaCl	130	90			5	5	5
NH ₄ Cl		40					
KCl	5	5		5	130	130	130
BaCl ₂			2	2			
MgCl ₂	1	1	1	1	2	2	2
CaCl ₂	1	1	1	1	2	2	2
Choline Chloride			135	130			
Glucose	5	5	5	5	5	5	5
KH ₂ PO ₄	2	2		2			
TRIS	10	10	10	10			
HEPES						10	10
PIPES					10		
Nigericin					0.01	0.01	0.01
pH adjusted by HCl or KOH	7.4 or 8.0	7.4	7.4 or 8.0	7.4 or 8.0			
pH adjusted by HCl or KOH					6.0	7.0	8.0

Cell culture and incubation

The promyelocytic leukaemia cell line HL-60 was obtained from the American Type Culture Collection (ATCC CCL 240; Rockville, MD USA) and maintained in suspension culture. Culturing was performed in 50 ml tissue culture flasks (Nunc, Thermo Scientific) in RPMI 1640 medium that was supplemented with 1× MEM sodium pyruvate, 1×MEM NEAA, 10 % FCS, 2 mM L-glutamine and 1× antibiotic/antimycotic (penicillin 10,000 U, streptomycin 10,000 U, amphotericin B 25 µg/ml (GIBCO). About 10⁶ cells were seeded per culture flask in 10 ml medium, cultured at 37°C, 5 % CO₂ in a humidified incubator, and passaged (ratio ~1:4) every 2 to 3 days.

Reverse transcriptase (RT) PCR

Total RNA was extracted from HL-60 cells using the RNA Bee reagent (AMS Biotechnology) according to the protocol of the supplier. 1 µg of total RNA was reverse transcribed using oligo(dT)₁₈ primers and the 'First Strand cDNA Synthesis Kit' (Thermo Scientific) utilizing M-MuLV reverse transcriptase. The cDNA was then subjected to PCR (35 cycles, annealing temperature 62°C) using specific primers for the human isoform of ATP12A (GenBank accession numbers U02076 (ATP1AL1), NM_001185085 (ATP12A transcript variant 1), NM_001676.5 (ATP12A transcript variant 2). Primer sequences were 5' GGGGCACACTTGTTCATCTTCTGA 3' and 5' GCAAAACATCAGTGAGCATCCTG 3' for the forward and reverse primer, respectively, yielding a 128-bp amplicon. 18S ribosomal RNA (GenBank accession number X03205.1) served as a reference gene and was amplified using the following primers: 5'-TACCACATCCAAGGAAGGCAGCA-3' (forward) and 5'-TGGAATTACCGCGGCTGCTGGCA-3' (reverse) giving a 180-bp fragment. PCR products were separated on a 2 % agarose gel and visualized by ethidium bromide staining. The 'GeneRuler 100 bp Plus DNA Ladder' (Thermo Scientific) was used as a size marker.

Amplification and Cloning of ATP12A Transcripts in HL-60 cells

Total RNA was isolated from promyelocytic leukaemia HL-60 cells (ATCC CCL 240) with Trizol Reagent according to the manufacturer's protocol (Invitrogen). First-strand cDNA synthesis was performed on 2 µg total RNA using 1 U Moloney murine leukemia virus reverse transcriptase, 3.5 U RNase inhibitor, 1 mM dNTPs and 5 µM random hexamer primers in 1× transcriptase buffer (all from MBI Fermentas) for 1 h at 37 °C. First strand cDNA was amplified by PCR using specific primers (Table 2) for the non-gastric H⁺/K⁺-ATPase alpha subunit ATP12A/ATP1AL1 (accession numbers NM_001676.5/U02076.1; [55]) and Herculanase DNA polymerase (Stratagene, Agilent Technologies) according to standard protocols. Amplification products were separated by agarose gel electrophoresis (GTG Seakem, Cambrex), purified using the QIAquick Gel Purification Kit (Qiagen) and cloned into the pGEM-T vector (Promega) for sequencing. Sequencing of cDNA clones was carried out using IRD800 labeled SP6 or T7 primers, the SequiTherm EXCEL II DNA sequencing kit (Epicentre) and an automatic sequencer (Gene ReadIR 4200, LI-COR Biosciences).

Measurement of intracellular pH (pH_i)

pH_i measurements were performed using BCECF/AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethylester (Molecular Probes) as described earlier [56]. In brief, HL-60 cells were grown on poly-lysine coated glass coverslips and loaded in the dark with 10 μ M BCECF-AM at 37° C for 15–20 minutes and thereafter mounted on an inverted microscope (Olympus IX70) connected to a monochromator (TILL Photonics Polychrome IV) and a CCD camera (TILL Photonics IMAGO) controlled by the TILLvision 4.01 imaging software. Experiments were performed at room temperature. Cells were superfused with solutions given in Table 1 and first allowed to adapt to control solution for ~20 minutes. BCECF was alternately excited through a 40× oil immersion objective by light of 440 nm and 505 nm wavelengths, respectively, and the emitted fluorescence at 560 nm was monitored from regions of interest within the cells. Raw intensity data were corrected for background- and auto-fluorescence. NH_4Cl prepulsing experiments were performed to test for the cells' ability to recover from an acute acid load. Intracellular acidification was obtained by superfusion of cells with NH_4Cl solution (for 1 or 3 minutes). For long-term pH_i measurements (pH_i recovery time 14.5 minutes, image acquisition interval 30 seconds; Fig. 3A) the change of pH_i is expressed as relative change of the F505/440 ratio with respect to the mean control value before addition of NH_4Cl and as absolute change in pH_i (ΔpH_i). pH_i recovery was investigated in the absence of both Na^+ and K^+ ($0Na^+ 0K^+$) for 2.5 or 5 minutes and under $0Na^+$ in the absence and presence of the H^+/K^+ ATPase inhibitor SCH28080 (100 μ M). In addition short-term pH_i recovery measurements were performed (pH_i recovery time 5 minutes, image acquisition interval 10 seconds; Fig. 3B). In these experiments the change of pH_i after acidification is expressed as relative change of the F505/440 ratio (in %/min) immediately after acidification and 5 minutes after re-addition of control solution (control), in the nominal absence of Na^+ ($0Na^+$), absence of both Na^+ and K^+ ($0Na^+ 0K^+$), in the absence of Na^+ and presence of 100 μ M SCH28080 ($0Na^+ SCH28080$) and in the absence of Na^+ and presence 100 μ M ouabain ($0Na^+ ouabain$). 2 mM $BaCl_2$ was added to block K^+ channels to prevent eventual recirculation of K^+ ions *via* an H^+/K^+ ATPase. Where indicated the F505/F440 ratio was converted to absolute pH_i by the high- K^+ /nigericin technique (calibration solutions C6, C7 and C8, Table 1) [56, 57].

Cell volume measurements

Mean cell volume (MCV) was measured by cell sizing using a Schärfe cell analyzer (CASY1 TT, Schärfe, Reutlingen, Germany) as described earlier [58]. MCV was calculated from the medians of volume distribution curves. During measurement cells were kept at 34–37°C. Absolute MCVs were obtained using latex beads (13.7 μ m diameter, Coulter Electronics, UK) as standard. Before the MCV measurements, the cells were suspended in isotonic control solution (Table 1) and allowed to adapt for about 5 minutes. Cell shrinkage was induced by addition of 50 mM NaCl. Where indicated, the NHE1 inhibitor HOE 694 (10 μ M), the NKCC inhibitor bumetanide (10 μ M) and/or the H^+/K^+ ATPase inhibitor SCH28080 (100 μ M) were added. Regulatory volume increase (RVI) is expressed as % gain of the MCV after maximum shrinkage.

Apoptosis, differentiation and cell volume measurements by flow cytometry

For the assessment of phosphatidylserine exposure and MCV measurements cells were subcultured two days prior to incubation for 0, 24, 48 and 72 hours with 1 or 10 mM sodium butyrate alone (BUT1 and BUT10, respectively), 100 μ M SCH28080 alone (UT-DMSO-SCH), both 1 or 10 mM sodium butyrate plus 100 μ M SCH28080 (BUT1-DMSO-SCH and BUT10-DMSO-SCH, respectively), 0.25 % DMSO alone (UT-DMSO; solvent control for 100 μ M SCH28080), or 0.25 % DMSO plus 1 mM butyrate (BUT1-DMSO). Untreated (UT) cells served as controls (UT-CO). 0.5 ml of cell suspension per sample (approximately 5×10^5 cells) was centrifuged for 5 minutes at 500×g. The cell pellets were re-suspended in a buffer containing (in mM): 140 NaCl, 5.6 KCl, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES-FA, 5 D-glucose, 1 % bovine serum albumin (BSA), pH 7.4 adjusted with NaOH and subsequently incubated with a primary mouse monoclonal anti-phosphatidylserine

Fig. 1. RT-PCR of ATP12 and 18S-rRNA (housekeeping gene). M, 100-bp marker; NTC, non-template control.

Fig. 2. Schematic representation of the cloning and sequencing strategy for the human ATP12A cDNA from HL-60 cells. The light gray rectangle represents the coding region (CDS) and the dark grey rectangles the untranslated regions (UTR) of ATP12A (GenBank accession number NM_001676.5). The black bars represent the cDNA fragments obtained by PCR using the primers (arrows) specified in Table 1.

(PS) antibody (clone 1H6; Upstate) at a 1:50 dilution and a secondary fluoresceinisothiocyanate (FITC)-conjugated anti-mouse IgG (1:50). Incubations were performed for 30–45 minutes at room temperature in the dark and each incubation step was followed by a washing step with 300 μ l buffer. Finally cells were re-suspended in 400 μ l buffer and analyzed by flow cytometry within 1 hour. Caspase activity was quantified using the CaspACE™ FITC-VAD-FMK *in situ* marker (Promega), a FITC conjugate of the membrane permeable pan-caspase inhibitor VAD-FMK. UT-CO, UT-DMSO, BUT1, BUT10, UT-DMSO-SCH, BUT1-DMSO-SCH and BUT1-DMSO cell samples incubated for 0, 24, 48 and 72 hours were harvested by centrifugation (5 minutes at 500 $\times$ g), washed twice with PBS, incubated with caspase inhibitor reagent for 30 min at 37 °C, washed again twice and re-suspended to 1 $\times$ 10⁶ cells/ml for the measurements. For CD86 staining 500 μ l HL-60 cell suspension was centrifuged for 5 min at 500 $\times$ g. The cell pellet was washed once with PBS. Thereafter cells were re-suspended in PBS containing a phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD86 IgG1 antibody (CD86-PE; BD Biosciences) at a dilution of 1:40 and incubated for 30–45 min in the dark on ice. After a washing step the cell pellet was re-suspended in 400 μ l PBS and immediately used for measurements. For ATP12A staining 0.5–1 $\times$ 10⁶ cells were incubated with a primary rabbit anti-human ATP12A polyclonal antibody (ATP12A (N-12), Santa Cruz Biotechnology) and stained with a secondary FITC-conjugated goat anti-rabbit antibody (eBioscience). 40,000 cells per sample were analyzed by flow cytometry. Flow cytometry was performed on a Cell Lab Quanta™ SC flow cytometer (Beckman Coulter). Cell diameter/volume given as mean cell volume (MCV in fl) was directly measured employing the Coulter principle for volume assessment. The electronic volume channel was calibrated using 10 μ m Flow-Check fluorospheres (Beckman-Coulter) by positioning this size bead in channel 200 on the volume scale. Fluorescence emission of FITC on FL-1 passed through a 525 nm band pass filter was measured upon excitation with a 488 nm argon laser. Unstained samples were used for setting the electronic volume (EV) gain and the FL-1 PMT-voltage. Debris (particles with a diameter <7 μ m) and cell aggregates (>14 μ m) were excluded from analysis. 20,000–40,000 single cells with a diameter $\geq$ 7 μ m and $\leq$ 14 μ m were analyzed per sample.

Real-time reverse-transcription PCR (qRT-PCR)

The effect of butyrate stimulation of HL-60 cells on ATP12A mRNA expression was determined in two independent experimental series using qRT-PCR. In the first series HL-60 cells were either stimulated with 1 mM butyrate for 24 hours or left untreated. Total RNA was extracted with RNA Bee reagent (AMS Biotechnology) based on phenol/chloroform extraction and isopropanol precipitation. 1 μ g of total RNA was reverse-transcribed using random hexamer primers. Primer pairs P14/P16 and P18/P19 (Table 3) specific for ATP12A and the reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) respectively, were used for the PCR reaction containing SYBR® green as intercalating dye, yielding a 135-bp (ATP12A) and 112-bp fragment (GAPDH). In the second set of experiments HL-60 cells were either stimulated with 1 mM butyrate, treated with 0.25 % DMSO or left untreated. After 24 hours total RNA was isolated using the NucleoSpin® RNA II preparation kit (Macherey-Nagel). 1 μ g of total RNA was used for the reverse transcriptase (RT) reaction with RevertAid Premium Reverse Transcriptase, RiboLock RNase Inhibitor and oligo(dT)₁₈ primers (Thermo Scientific). Real-time PCR quantification was done by means

Table 2. Primers used for RT-PCR and cloning of the non-gastric H⁺/K⁺ ATPase alpha subunit ATP12A (accession numbers: NM_001185085 (transcript variant 1) and NM_001676.5/U02076.1 (transcript variant 2), respectively. Positions refer to the CDS of accession number NM_001676.5

Forward Primers	Sequence	Position
P1	TATCTCCACCGCCAACACCTCAGC	-128 to -105
P2	CACCAGCCCAGCATGCACCAG	-12 to 9
P3	CGGAACCTAAGGACATCGTGAAGAAC	42 to 65
P4	TGTCTTCTCAGGGGTGTCGG	662 to 681
P5	GAAAAAGAAACCGCAAAGTAGC	1457 to 1478
P6	TTGCTGGACGACAACCTTCGC	2293 to 2312
P7	AACCTGCGGGTAGAATGGGAGAAG	2698 to 2721
Reverse Primers	Sequence	Position
P8	AAAATGACCACCAGACCAAGCACACAGCCC	470-441
P9	TCATGGGTAAACTCAGAGGAGCGG	746-723
P10	TGGGGGTCATCCATCTCGTG	1541-1522
P11	TGGTCAGGGAATAAGCAATAGTC	2389-2367
P12	CTTCCGCACCTCATCATAACC	3066-3045
P13	TGCTGCTGAGAGACATAGGAAGGG	3152-3129

Table 3. Primers used for quantitative Real-time reverse-transcription PCR Sequence and position of primers and probe correspond to the CDS of GenBank accession numbers NM_001676.5 (ATP12A) and NM_002046.5 (GAPDH)

ATP12A		
Forward primers	Sequence	Position
P14	GGGTGTCGGGTGGATAACTCATC	673-695
P15	GAGCACTTTGTTACATTGTGG	910-931
Reverse primers	Sequence	Position
P16	GACAGTGCTTCCAGACACGTTG	807-785
P17	ATGGAGTCCAGGACTTGATACTTC	1007-984
Probe A	FAM-AGTGGCTGTCTCCATCGGCATCCT-TAMRA	936-959
GAPDH		
Forward primer	Sequence	Position
P18	GTGAAGGTCCGAGTCAACG	10-28
Reverse primer	Sequence	Position
P19	TGAGGTCAATGAAGGGGTC	121-103

of a 5' nuclease assay using the ATP12A specific primer pair P15/P17 (Table 3) and probe A (Microsynth AG, Balgach, Switzerland; Table 3). Hypoxanthine-guanine phosphoribosyltransferase (HPRT1) was used as reference gene, which was detected by the HPRT1 VIC TaqMan® Gene Expression Assay Hs02800695_m1 (Applied Biosystems). A Rotor-Gene 6000 real-time PCR cyclor (Qiagen) was used for both series of experiments. Samples were run in triplicates or quadruplicates. The specificity of the PCR reactions were tested by agarose gel loading and melt curve analysis in case of the first experimental series using SYBR® green. Data are presented as Ct value ratios ($Ct_{ATP12A}/Ct_{reference}$), where a decrease in the ratio indicates an increased ATP12A expression given stable reference gene expressions. Data were further analyzed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method [59] assuming approximately equal amplification efficiencies for ATP12A and the housekeeping genes.

Statistics

Data were carefully checked for outliers and for normal distribution. Where indicated, Students t-test and ordinary one-way or two-way ANOVA followed by Tukey's multiple comparisons test was applied. For data given in Tables 4 and 5 mixed models were applied with group as fixed, time as repeated factor and a corresponding interaction effect. Covariance and correlations between repeated measurements were taken into account. For doing so, compound symmetry as the repeated pattern was used (Covariance Pattern Model). 95 % confidence intervals for means and difference of means were computed and post-hoc tests were used to further analyze data. Unless otherwise indicated, data analyses were done using STATISTICA 10 data analysis software system (StatSoft, Inc.; 2011; www.statsoft.com); NCSS 8 (NCSS, LLC. Kaysville, Utah, USA; www.ncss.com); MATHEMATICA 7 (Wolfram Research, Inc.; www.wolfram.com) and GraphPad Prism version 6.03 (GraphPad Software, Inc. La Jolla, CA, USA; www.graphpad.com). A p-value less than 0.05 indicates a statistical significant difference.

Fig. 3. Prepulsing measurements of the intracellular pH (pH_i). A: Long-term pH_i recovery within 14.5 minutes after intracellular acidification in the absence of both Na⁺ (0Na⁺) and K⁺ (0K⁺), in the sole absence of Na⁺ as well as in the absence of Na⁺ and presence of 100 μM SCH28080. Extracellular pH (pH_o) was 8.0 and 2 mM BaCl₂ was added to prevent eventual recirculation of K⁺ ions via an H⁺/K⁺ ATPase. The starting pH_i was 7.02 ± 0.01 ($n=46$). C, control. B: Short-term pH_i recovery within 5 minutes after intracellular acidification under control conditions (130Na⁺/5K⁺), under Na⁺-free conditions (0Na⁺/5K⁺), as well as under 0Na⁺/0K⁺ in absence and presence of inhibitors (100 μM SCH28080 or 100 μM ouabain). Means ± SEM; * $p < 0.05$.

Results

K⁺-dependent H⁺ extrusion

Under culture conditions with an extracellular pH (pH_o) of 7.4, pH_i was 7.22 ± 0.062 ($n=7$) in untreated HL-60 cells. Addition of 10 mM sodium butyrate to the cell culture medium for 12 h leads to a drop in pH_i to 7.15 ± 0.14 ($n=13$). To test for the presence of a functional H⁺/K⁺-ATPase in HL-60 cells, their ability to recover from an acute intracellular acid load was tested. Cells were acidified by NH₄Cl prepulsing and acute withdrawal of Na⁺, and the pH_i recovery was monitored. To facilitate a potential secretion of H⁺ ions, measurements of pH_i were performed after acidification for 12 h with 10 mM butyrate at an extracellular pH of 8.0 and in the presence of 2 mM Ba²⁺ to prevent eventual recirculation of K⁺ ions via an H⁺/K⁺ ATPase. Under this condition the starting pH_i was 7.02 ± 0.01 ($n=46$). As shown in Fig. 3A, no pH_i recovery occurred in the absence of both Na⁺ and K⁺. After re-addition of K⁺ a slow significant Na⁺-independent recovery could be observed within 14.5 minutes which could be completely blocked in the presence of 100 μM SCH28080. To test for a possible Na⁺/K⁺ ATPase-independent inhibitory effect of ouabain, short-term pH_i recovery within 5 minutes after intracellular acidification was measured (Fig. 3B). The respective pH_i recovery rates were 8.4 ± 5.5 %/min ($n=11$) under control conditions, 3.3 ± 2.8 %/min in the absence of Na⁺ ($n=27$; 0Na⁺/5K⁺), -2.7 ± 0.9 %/min in the absence of both Na⁺ and K⁺ ($n=30$; 0Na⁺/0K⁺; $p < 0.05$ compared to 0Na⁺), -11.4 ± 4.1 % in the absence of Na⁺ and the presence of 100 μM SCH28080 ($n=26$; 0Na⁺/5K⁺/SCH28080; $p < 0.05$ compared to 0Na⁺) and -6.3 ± 1.5 % in the absence of Na⁺ and the presence of 100 μM ouabain ($n=18$; 0Na⁺/5K⁺/ouabain; $p < 0.05$ compared to 0Na⁺). Taken together these results disclose the existence of an ouabain-sensitive K⁺-dependent H⁺-extrusion mechanism that can be inhibited by SCH28080, which is therefore most likely an H⁺/K⁺-ATPase.

Regulatory Volume Increase (RVI)

Since the cellular import of K⁺ ions by an H⁺/K⁺ ATPase activity is expected to drive the entry of osmotically obliged water, its action may to some extent serve for regulatory volume increase (RVI) after cell shrinkage. Therefore HL-60 cells were treated with a hypertonic solution in the presence or absence of the Na⁺/H⁺ (NHE1) exchange inhibitor HOE 642,

Fig. 4. Regulatory volume increase of HL 60 cells after cell shrinkage in the absence (control, circles, n=8) and presence of either the NHE1 inhibitor HOE 694 and the NKCC inhibitor bumetanide (squares, n=5), or of HOE 694, bumetanide and the H^+/K^+ ATPase inhibitor SCH28080 (triangles, n=15). Means $\pm$ SEM. pH_o was 8.0. Data were analyzed by 2-way ANOVA followed by Tukey's multiple comparisons test and second order polynomial (quadratic) fitted (GraphPad Prism 6). ** $p<0.001$.

Fig. 5. Transcriptional up-regulation of ATP12A in HL-60 cells by treatment with 1 mM butyrate for 24 h. Data are expressed as dCt values (see Methods) using HPRT1 which served as reference gene (Table 3, probe A). The decrease of dCt corresponds to a ~35-fold increase in ATP12A mRNA. * $p<0.05$ compared to DMSO. Students t-test. Means $\pm$ SEM.

the $Na^+/K^+/2Cl^-$ (NKCC) cotransport inhibitor bumetanide and the H^+/K^+ ATPase inhibitor SCH28080. The experiments were performed at 34–37°C and at an extracellular pH (pH_o) of 8.0 to facilitate cellular extrusion of H^+ ions. Under these conditions (untreated control) HL-60 cells had a mean cell volume (MCV) of 773 ± 8 fl (n=8). In the presence of both HOE 642 (10 μ M) and bumetanide (10 μ M) MCV was 746 ± 6 fl (n=15; $p<0.05$ compared to untreated control) and in the presence of HOE 694, bumetanide and 100 μ M SCH28080 MCV was 680 ± 15 fl (n=6; $p<0.0001$ compared to HOE694+bumetanide), respectively. After addition of 50 mM NaCl to the extracellular solution, HL-60 cells shrunk to 89 ± 1 % of the initial MCV and this cell shrinkage was followed by a partial RVI (Fig. 4). Under control conditions HL-60 cells regained 4.9 ± 0.5 % (n=8) of the lost MCV within 5.5 minutes. Addition of HOE 642 and bumetanide blunted RVI (3.3 ± 0.3 %, n=15). In the additional presence of the H^+/K^+ -ATPase inhibitor SCH28080, a significant inhibition of RVI was observed (2.3 ± 0.6 %, n=5, $p<0.05$ compared to control).

Verification of ATP12A expression in HL-60 cells

To determine if ATP12A is expressed in HL-60 cells, total RNA was purified, cDNA transcribed and subsequently amplified by PCR using ATP12A specific primers (Fig. 1, 2 and Table 2). Several PCR fragments of expected lengths that overlapped the entire open reading frame of the ATP12A mRNA including 120 bp of the predicted 5'-untranslated region could be obtained. To verify the specificity of the PCR, amplified PCR products were cloned and sequenced. The alignment of these sequences with the published mRNA sequence of ATP12A/ATP1A1 (accession numbers NM_001676.5/U02076.1 [55]) identified ATP12A as the amplified product.

Transcriptional regulation of ATP12A in HL-60 cells

Real time-qPCR using primers and probes for ATP12A (Table 3) was performed to assess the transcriptional regulation of ATP12A. As is evident from Fig. 5, treatment of HL-60 cells with the solvent DMSO does not change the transcription of ATP12A, whereas treatment of

Fig. 6. ATP12A expression and determination of cell volume was performed by flow cytometry in unstimulated HL-60 cells and cells stimulated with 1 mM butyrate for 24 h. A: Histograms of FITC-fluorescence measured at 520 nm (upper panels), side-scatter versus cell diameter dot plots (middle panels) and diameter histograms (lower panels) of untreated (left panels) and butyrate-stimulated (right panels) HL-60 cells. ATP12A positive (ATP12A+) and ATP12A negative (ATP12A-) cells are shown in green and grey, respectively. The whole cell population without differentiation between ATP12A+ and ATP12A- cells is depicted in black. B: Upper panel: percentage of ATP12A+ (green bars) and ATP12A- cell populations in untreated and 1 mM butyrate-stimulated cells. Lower panel: Cell diameters (μm) of the entire cell population (black bars), ATP12A- cells (grey bars) and ATP12A+ cells (green bars) of untreated (bars without pattern) and butyrate-stimulated (hatched bars) HL-60 cells, respectively. Means $\pm$ SEM. 40,000 cells were analyzed per sample.

Fig. 7. Flow cytometric assessment of CD86 expression. HL-60 cells were stained with a PE-conjugated anti-CD86 antibody as described in Materials and Methods. HL-60 cells were either left untreated or induced with 1 mM butyrate for a period of 0–72 h. UT, untreated cells; BUT, 1 mM butyrate-treated cells. Means $\pm$ SEM; * and # $p < 0.05$. * Significantly different from the corresponding value at 0 h, # significantly different from untreated (UT) at the same time point.

the cells for 24 h with 1 mM butyrate (BUT1) led to an up-regulation of ATP12A expression, as indicated by a significantly lower dCt value which corresponds to a ~35-fold increase in ATP12A mRNA.

Assessment of ATP12A protein expression and mean cell volume (MCV) of HL-60 cells by flow cytometry

ATP12A protein expression and determination of cell volume was performed by flow cytometry in unstimulated and 1 mM butyrate-stimulated HL-60 cells using a primary rabbit anti-human ATP12A polyclonal antibody and a secondary FITC-conjugated goat anti-rabbit antibody. As shown in Fig. 6A, only a small fraction (5.1 ± 0.7 %, $n=3$) of HL-60 cells display a positive staining for ATP12A. Upon treatment with 1 mM butyrate for 24 h, however, the ATP12A positive cell fraction significantly increases to 28.0 ± 5.8 % ($n=3$, $p=0.047$). While in untreated cells no difference in cell diameter could be detected between ATP12A- and ATP12A+ cells (14.69 ± 0.07 versus 14.67 ± 0.09 μm ; $n=3$, $p=0.77$), treatment of HL-60 cells with 1 mM butyrate leads to a significantly larger cell diameter and hence cell volume in ATP12A+ cells as compared to ATP12A- cells (15.27 ± 0.17 versus 14.03 ± 0.20 μm ; $n=3$, $p=0.008$; Fig. 6A,B).

Expression of CD86

CD86 expression was determined by flow cytometry. Stimulation of HL-60 cells with butyrate (1 mM) elicits the expression of CD86 within 48–72 h (Fig. 7). By 48 h and 72 h of 1 mM butyrate treatment, 43.4 ± 4.9 % ($n=4$) and 34.1 ± 2.0 % ($n=3$) of cells, respectively, were positive for CD86 staining, indicating a butyrate-induced differentiation of HL-60 cells towards the monocytic lineage.

Assessment of apoptosis and mean cell volume (MCV)

Apoptosis was assessed by determining the fraction and the mean cell volume (MCV) of cells that show phosphatidylserine (PS) exposure at the outer leaflet of the cell membrane (PS⁺ cells), loss of cell membrane integrity (7AAD⁺ cells) and activation of key caspases (casp⁺ cells). These fractions were determined for HL-60 cell populations with a cell diameter of 7–14 μm . Particles with a diameter $>7 \mu\text{m}$ were classified as cells and those with a diameter $<7 \mu\text{m}$ as cell-debris. PS[−]/7AAD[−] cells were classified as non-apoptotic (living) cells. PS⁺ cells and casp⁺ cells were classified as apoptotic cells. PS⁺/7AAD⁺ cells were classified as neither apoptotic nor non-apoptotic. This fraction and the cell-debris fraction were not separately considered for the analysis. Since the solvent DMSO (0.25 %) might eventually *per se* exert effects on differentiation and/or apoptosis its effect was tested in all fractions investigated.

Apoptosis and mean cell volume (MCV) were measured after 0, 24, 48, and 72 h of culture in the absence of any drugs (untreated control, UT-CO) or in the continuous presence of 1 mM (BUT1) or 10 mM (BUT10) butyrate, respectively. UT-CO and BUT1 were tested for an effect of the solvent DMSO (0.25 %; UT-DMSO, BUT1-DMSO) and the H⁺/K⁺ ATPase inhibitor SCH28080 (100 μM ; UT-DMSO-SCH, BUT1-DMSO-SCH and BUT10-DMSO-SCH).

Percent gated cells. A descriptive statistics is given in Table 4. No significant differences were observed between the experimental groups UT-CO, UT-DMSO, UT-DMSO-SCH, BUT1 and BUT1-DMSO at any time point and fraction tested (all-cells, non-apoptotic, PS⁺, Casp⁺; some data for Casp⁺ cells are lacking for some time points). This demonstrates that DMSO and/or 100 μM SCH28080 *per se* exert no effects on the investigated cell fractions. The results also show that – in contrast to time point 0 (0 h) – after 72 h in the presence of SCH28080 the numbers of all-cells and non-apoptotic cells treated with 1 mM butyrate (BUT1-DMSO-SCH) were significantly lower compared to their respective untreated counterparts (UT-DMSO-SCH; both $p < 0.0001$). Consequently these values were significantly higher in the PS⁺ ($p=0.005$) and Casp⁺ ($p < 0.0001$) fraction. In the PS⁺ fraction this effect was already detectable after 48 h ($p=0.0266$). The groups UT-CO, UT-DMSO, UT-DMSO-SCH, BUT1 and BUT1-DMSO significantly increased from 0–72 h in the all-cell fraction. In the non-apoptotic fraction the BUT1-DMSO-SCH values remained unchanged until 48 h, but significantly decreased below the 0 h value ($p=0.0025$) 72 h after initiation of treatment. Similarly, the Casp⁺ cell fraction in the BUT1-DMSO-SCH group significantly increased over time. After 72 h a comparison between the UT-DMSO-SCH and BUT1-DMSO groups *versus* the BUT1-DMSO-SCH group revealed significant differences ($p=0.0001$ for both). These results indicate that 1 mM butyrate *per se* does not induce apoptosis but renders the cells sensitive for the induction

Table 4. Flow cytometric assessment of apoptosis in untreated (UT) cells in the absence of drugs (control, UT-CO), in the presence of the solvent DMSO (UT-DMSO) and in the presence of SCH28080 (UT-DMSO-SCH) as well as in cells stimulated with 1 mM (BUT1) or 10 mM (BUT10) butyrate in the absence of drugs (BUT1, BUT10), in the presence of the solvent DMSO (BUT1-DMSO) and in the presence of SCH28080 (BUT1-DMSO-SCH, BUT10-DMSO-SCH). Measurements were made at time points 0 h, 24 h, 48 h and 72 h of treatment of the cells. Data are presented as means $\pm$ SEM. Relevant statistical differences are mentioned in the text

	Group	Fraction (%)											
		all cells			non-apoptotic			PS+			Casp+		
		Mean	±SEM	n	Mean	±SEM	n	Mean	±SEM	n	Mean	±SEM	n
0 h	UT-CO	85.4	1.8	7	91.41	0.75	7	7.5	0.6	7	10.66	1.02	4
	UT-DMSO	85.2	1.9	4	90.56	0.63	4	8.1	0.4	4	10.83	2.64	4
	UT-DMSO-SCH	85.4	1.1	7	91.21	0.69	7	7.7	0.5	7	9.33	1.25	4
	BUT1	86.0	2.0	4	90.03	0.99	4	8.3	0.6	4			
	BUT1-DMSO	86.0	1.9	4	90.95	0.91	4	7.8	0.5	4	13.88	2.34	4
	BUT1-DMSO-SCH	84.5	2.0	4	90.65	0.62	4	8.1	0.4	4	10.08	1.76	4
	BUT10	86.4	2.5	3	92.95	1.14	3	6.2	1.0	3			
	BUT10-DMSO-SCH	86.5	2.3	3	93.99	0.74	3	5.2	0.6	3			
24 h	UT-CO	89.9	0.7	7	93.74	0.73	7	5.3	0.5	7	9.29	1.16	8
	UT-DMSO	90.5	1.1	4	94.41	1.00	4	4.7	0.7	4	9.47	1.85	4
	UT-DMSO-SCH	89.8	0.8	7	93.36	0.76	7	5.4	0.8	7	7.81	0.77	7
	BUT1	90.4	0.5	4	92.06	0.57	4	6.2	0.2	4	9.96	2.55	4
	BUT1-DMSO	90.1	0.6	4	92.15	0.70	4	6.2	0.5	4	10.54	1.23	4
	BUT1-DMSO-SCH	89.3	0.7	4	89.28	1.04	4	7.6	0.9	4	11.33	1.45	4
	BUT10	80.6	2.6	3	79.24	5.19	3	18.0	5.1	3	33.23	6.08	3
	BUT10-DMSO-SCH	75.0	3.1	3	83.28	2.22	3	12.7	2.3	3			
48 h	UT-CO	90.2	0.8	7	94.56	0.63	7	4.3	0.5	7	14.15	1.96	8
	UT-DMSO	91.4	0.8	4	93.68	0.50	4	5.0	0.5	4	10.96	0.66	4
	UT-DMSO-SCH	92.1	0.6	7	94.47	0.98	7	4.3	0.8	7	9.11	1.14	4
	BUT1	92.6	0.7	4	91.72	0.98	4	6.7	0.9	4	12.58	2.40	4
	BUT1-DMSO	93.3	0.8	4	92.52	0.71	4	5.8	0.6	4	11.51	0.88	4
	BUT1-DMSO-SCH	90.1	0.9	4	87.86	1.88	4	8.4	1.4	4	15.75	1.01	4
	BUT10	79.3	1.0	3	59.70	8.46	3	33.0	8.4	3	72.37	5.26	4
	BUT10-DMSO-SCH	79.3	0.8	3	61.04	6.78	3	24.0	5.8	3			
72 h	UT-CO	90.4	1.0	7	89.60	1.49	7	8.2	1.0	7	11.61	1.98	4
	UT-DMSO	92.1	0.6	4	87.64	0.97	4	8.9	0.3	4	20.08	5.13	4
	UT-DMSO-SCH	92.7	0.6	7	90.69	1.63	7	6.0	0.7	7	12.04	2.40	7
	BUT1	92.0	0.4	4	90.95	0.87	4	6.7	0.9	4			
	BUT1-DMSO	91.6	0.5	4	91.92	0.21	4	5.8	0.4	4	12.69	1.83	4
	BUT1-DMSO-SCH	83.1	0.7	4	73.48	1.14	4	12.7	2.1	4	40.85	9.72	4
	BUT10	77.2	2.6	3	45.29	7.05	3	38.1	5.3	3			
	BUT10-DMSO-SCH	74.7	2.4	3	37.66	6.68	3	45.7	11.0	3			

of apoptosis by the H⁺/K⁺ ATPase inhibitor SCH28080. The values of the groups BUT10 and BUT10-DMSO-SCH in non-apoptotic fractions markedly decreased from 24–72 h. After 24, 48 and 72 h the BUT10 value decreased by ~14 % (p=0.0001), ~33 % (p<0.0001) and ~48 % (p<0.0001), respectively. However, comparing BUT10 and BUT10-DMSO-SCH, SCH28080 had no apparent aggravating or accelerating effect in this cell fraction. This was mirrored by an increase of cells in the PS⁺ fraction. This indicates that 10 mM butyrate induced apoptosis within 24 h, which was, however, not accelerated by SCH28080.

Since the solvent DMSO *per se* had no effect on the fractions of living or apoptotic cells in any of the conditions studied, the data for UT-CO/UT-DMSO and BUT1/BUT1-DMSO respectively, were pooled and further analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. The results are shown in Fig. 8. (A) The percentage of living cells remained unchanged over time in untreated cells both in the absence and presence of SCH28080. (B) The cell number in 1 mM butyrate-treated samples remained constant over time, whereas in the presence of SCH28080 they significantly decreased after 72 h compared to both the value at 0 h and to the percentage of gated cells in absence of the inhibitor. (C) The percentage of PS⁺ cells in untreated samples significantly decreased from 0 to 48 h in the absence and presence of SCH28080. No significant differences could be measured after 72 h. (D) In contrast, the fraction of 1 mM butyrate-treated PS⁺ cells remained constant up to 72 h in the absence of SCH28080. In the presence of the inhibitor the cell fraction was significantly increased after 72 h compared to the both the value at 0 h and to the percentage of gated cells in absence of the inhibitor. (E) In untreated casp⁺ cells there was no change irrespective of the absence or presence of SCH28080. (F) 1 mM butyrate-treated casp⁺ cells behaved similar to PS⁺ cells and responded to SCH28080 with a dramatic increase after 72 h compared to the both the value at 0 h and the percentage measured in the absence of the inhibitor.

Absolute mean cell volumes (MCV). The MCVs were assessed simultaneously and under the same conditions as described above. The results are given in Table 5. At time point 0 (0 h) the absolute MCV of the all-cell fraction and the non-apoptotic cells fraction of all groups ranged from 626 $\pm$ 15 to 680 $\pm$ 25 fl (n=3–4) and from 630 $\pm$ 14 to 688 $\pm$ 25 fl (n=3–4),

Fig. 8. Flow cytometric assessment of the fraction of untreated (A, C, E) or 1 mM butyrate-treated (B, D, F) cells (in %) that are either negative for both phosphatidylserine (PS) exposure at the outer leaflet of the cell membrane and 7AAD staining (non-apoptotic cells, living cells, A, B), positive for phosphatidylserine (PS⁺; C, D) or positive for caspase activation (Casp⁺ cells; E, F). Measurements were made at time points 0 h, 24 h, 48 h and 72 h after starting treatment of the cells. Means±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 denote significant difference from the corresponding value in at 0 h; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 denote significant difference from the corresponding value in the absence of SCH28080.

respectively. Comparisons of the MCVs revealed significant differences between the groups BUT10 and BUT10-DMSO-SCH, which had a slightly but significantly smaller MCV compared to groups BUT1, BUT1-DMSO and BUT1-DMSO-SCH. Within 24 h the MCV of all groups and cell fractions increased significantly. From 24–72 h the MCV decreased in all groups of the fractions all-cells and non-apoptotic cells. Each 72 h value of the all-cells and non-apoptotic cells fraction was significantly lower compared to the corresponding 0 h value ($p<0.0005$). The MCVs of apoptotic cells (PS⁺ or Casp⁺ cells) were significantly smaller compared to the fractions all-cells and non-apoptotic cells and ranged from 575 ± 12 to 606 ± 13 fl ($n=3-4$) in PS⁺ cells and from 547 ± 46 to 578 ± 51 fl ($n=4$) in Casp⁺ cells, respectively. In PS⁺ cells the absolute MCV increased from 0–24 h in all groups and subsequently remained unchanged in BUT-1 and BUT1-DMSO cells, but significantly decreased in all other groups including BUT1-DMSO-SCH cells ($p<0.0006$). In Casp⁺ cells MCV increased within 24 h in the UT-CO,

Table 5. Flow cytometric assessment of mean cell volume (MCV) of untreated (UT) cells in the absence of drugs (control, UT-CO), in the presence of the solvent DMSO (UT-DMSO) and in the presence of SCH28080 (UT-DMSO-SCH) as well as in cells stimulated with 1 mM (BUT1) or 10 mM (BUT10) butyrate in the absence of drugs (BUT1, BUT10), in the presence of the solvent DMSO (BUT1-DMSO) and in the presence of SCH28080 (BUT1-DMSO-SCH, BUT10-DMSO-SCH). Measurements were made at time points 0 h, 24 h, 48 h and 72 h of treatment of the cells. Data are given as means $\pm$ SEM. Relevant statistic differences are mentioned in the text

	Group	Fraction											
		all cells			non-apoptotic			PS+			Casp+		
		MCV (fl)	±SEM	n	MCV (fl)	±SEM	n	MCV (fl)	±SEM	n	MCV (fl)	±SEM	n
0 h	UT-CO	651	16	7	658	17	7	586	15	7	561	60	4
	UT-DMSO	669	25	4	676	26	4	606	13	4	547	46	4
	UT-DMSO-SCH	646	15	7	652	16	7	585	11	7	593	57	4
	BUT1	677	28	4	685	28	4	602	26	4			
	BUT1-DMSO	679	25	4	686	25	4	592	21	4	565	65	4
	BUT1-DMSO-SCH	680	25	4	688	25	4	600	21	4	578	51	4
	BUT10	626	15	3	630	14	3	582	19	3			
	BUT10-DMSO-SCH	627	13	3	630	13	3	575	12	3			
24 h	UT-CO	780	7	7	786	8	7	673	5	7	637	31	8
	UT-DMSO	790	6	4	795	7	4	693	7	4	637	38	4
	UT-DMSO-SCH	774	12	7	780	12	7	677	7	7	606	35	4
	BUT1	727	8	4	733	8	4	653	10	4	602	31	4
	BUT1-DMSO	714	11	4	719	11	4	661	14	4	589	28	4
	BUT1-DMSO-SCH	740	7	4	750	8	4	654	5	4	583	26	4
	BUT10	771	9	3	812	1	3	599	11	3	650	38	4
	BUT10-DMSO-SCH	772	14	3	800	17	3	607	31	3			
48 h	UT-CO	665	15	7	666	15	7	637	11	7	543	22	8
	UT-DMSO	685	18	4	686	18	4	660	12	4	508	23	4
	UT-DMSO-SCH	669	16	7	671	17	7	643	11	7	491	11	4
	BUT1	625	3	4	623	3	4	646	2	4	581	28	4
	BUT1-DMSO	607	5	4	604	5	4	654	3	4	505	34	4
	BUT1-DMSO-SCH	612	9	4	614	10	4	616	3	4	449	13	4
	BUT10	599	5	3	620	3	3	548	7	3	590	12	4
	BUT10-DMSO-SCH	625	5	3	647	9	3	548	15	3			
72 h	UT-CO	582	13	7	583	14	7	583	11	7	535	44	4
	UT-DMSO	579	10	4	578	10	4	590	3	4	482	22	4
	UT-DMSO-SCH	565	10	7	565	10	7	568	12	7	496	20	4
	BUT1	608	8	4	603	8	4	674	12	4			
	BUT1-DMSO	571	12	4	568	12	4	629	15	4	457	35	4
	BUT1-DMSO-SCH	544	11	4	548	9	4	584	12	4	381	23	4
	BUT10	521	11	3	492	18	3	517	6	3			
	BUT10-DMSO-SCH	534	13	3	511	22	3	533	8	3			

UT-DMSO, and UT-DMSO-SCH groups but remained unchanged in the other groups. Except for UT-CO the MCV of all groups significantly decreased within 72 h below the corresponding 0 h and 24 h value.

Since (with the only exception of BUT1 and BUT1-DMSO at 72 h, see below) the solvent DMSO alone had no significant effect, the MCV data for UT-CO/UT-DMSO and BUT1/BUT1-DMSO, respectively, were pooled for further analysis by one-way ANOVA followed by Tukey's multiple comparisons test. The results are shown in Fig. 9. (A) In untreated cells both non-apoptotic (living, black bars) and early-apoptotic (PS⁺, white bars) HL-60 cells underwent shrinkage from 24–72 h after seeding. The MCV of non-apoptotic cells was not different from that of PS⁺ cells after 72 h. Untreated cells were insensitive to the H⁺/K⁺ ATPase inhibitor SCH28080 (gray and dotted bars). (B) Treatment with 1 mM butyrate revealed remarkable differences between non-apoptotic and PS⁺ cells. In contrast to non-apoptotic cells (black bars) PS⁺ cells (white bars) did not shrink from 24–72 h. After 72 h of treatment with butyrate, PS⁺ cells showed a significantly higher MCV (651 $\pm$ 12 fl, n=8) compared to non-apoptotic cells (586 $\pm$ 9 fl, n=8; p<0.003), compared to PS⁺ cells at 0 h (597 $\pm$ 16 fl; p<0.04) and compared to untreated PS⁺ cells (587 $\pm$ 7, n=11; p=0.0001; unpaired student's t-test; compare white bars upper left and right panels at 72 h). This effect could not be observed in non-apoptotic butyrate-treated cells. Furthermore, in contrast to untreated PS⁺ cells, the MCV of butyrate-treated PS⁺ cells did show sensitivity to SCH28080 (B, dotted bars), which led to shrinkage of cells from 651 $\pm$ 12 fl (n=8) to 584 $\pm$ 12 fl (n=4; data pooling did not corrode this comparison, i.e. p=0.02 and 0.03 for unpooled and pooled data, respectively). (C) and (D)

Fig. 9. Flow cytometric assessment of the mean cell volumes (MCV in fL) of untreated (UT; A, C) or 1 mM butyrate (BUT1; B, D)-treated HL-60 cells. Measurements were taken at time points 0 h, 24 h, 48 h and 72 h after starting treatments. Living (non-apoptotic) cells (black bars); PS+ cells (white bars); non-apoptotic, SCH28080-treated cells (grey bars); PS+, SCH28080-treated cells (dotted bars). Means $\pm$ SEM. $^{##}p < 0.003$; $^{*}p < 0.05$; $^{§}p < 0.04$ denote significant differences as indicated by the lines; $^{****}p < 0.001$ significantly different from the corresponding value of untreated cells (A) at 72 h; ns, not significant.

Neither in untreated nor in 1 mM butyrate-treated cells significant effects could be observed in late apoptotic caspase positive cells.

Discussion

In the present study we show by RT-PCR (Fig. 1 and 5), cloning of the coding sequence (Fig. 2), and protein staining (Fig. 6) that human myelomonocytic HL-60 cells express the non-gastric H^{+}/K^{+} ATPase ATP12A. Furthermore the transcriptional (Fig. 5) and translational (Fig. 6) up-regulation of ATP12A by 1 mM butyrate, the existence of a SCH28080- and ouabain-sensitive K^{+} -dependent H^{+} extrusion mechanism (Fig. 3) and the ability of SCH28080 to elicit cell shrinkage (Table 5 and Fig. 9) as well as to inhibit regulatory volume increase after hyperosmotic cell shrinkage (Fig. 4) point to the existence of a functional H^{+}/K^{+} ATPase in these cells.

Using electron microprobe analysis it could be demonstrated that unstimulated HL-60 cells possess a Rb^{+} -uptake mechanism which is not mediated by the Na^{+}/K^{+} ATPase and which is sensitive to both ouabain and SCH28080. In these experiments the actual

measured inhibition of ouabain-sensitive cellular Rb^+ uptake within 1 h was 4 times higher than calculated assuming a $3\text{Na}^+/2\text{K}^+$ exchange stoichiometry. Hence it was concluded that ouabain not only blocks Rb^+ uptake *via* the Na^+/K^+ ATPase but also by inhibition of another SCH28080-sensitive transporter, presumably an H^+/K^+ ATPase (A. Dörge, I. Mühlhahn, et al., unpublished measurements [60]). In agreement with this observation we show here, that ouabain is able to inhibit pH_i recovery after cellular acidification in a Na^+/K^+ ATPase-independent manner (Fig. 3B).

The basal transcription and functional activity of ATP12A in HL-60 cell seems to be fairly low, as indicated by the low abundance of ATP12A mRNA/cDNA in the overall cell fraction, the small percentage of ATP12A positive cells as assessed by flow cytometry (Fig. 6), the slow pH_i recovery rates after cellular acid load (Fig. 3) as well as the shallow SCH28080-sensitive RVI process after hyperosmotic cell shrinkage (Fig. 4). However, while butyrate at a concentration of 10 mM induces apoptosis within 24 h (Table 4), 1 mM butyrate leads to a significant up-regulation of both ATP12A mRNA/cDNA (Fig. 5) as well as ATP12A positive cells (Fig. 6). Importantly, the effects of SCH28080 on the number of non-apoptotic (living) cells (Fig. 8B), apoptotic PS^+ cells (Fig. 8D) and caspase $^+$ cells (Fig. 8F), respectively, are only evident after 72 h if the cells are stimulated by 1 mM butyrate (Fig. 8 B, D and F). Moreover, while the number of untreated PS^+ cells decreases significantly within 48 h and the cells exhibit no SCH28080 sensitivity (Fig. 8C), this is not the case in 1mM butyrate-treated PS^+ cells which remain constant over time and exhibit SCH28080 sensitivity after 72 h (Fig. 8D).

Interestingly, the up-regulation of ATP12A is paralleled by enhanced expression of the pan-monocytic marker CD86 (Fig. 7 and [44, 52]), indicating that butyrate-induced ATP12A expression occurs along with the differentiation of HL-60 cells towards the monocyte lineage. Genes responsive to sodium butyrate contain a so-called butyrate response element in their promoter sequence. As mentioned above, butyrate is an inhibitor of histone deacetylases [53, 54] and the promoters of both CD86 and ATP12A (HKalpha2) harbor several SP1 binding sites, which are under control of histone deacetylases. In mIMCD3 cells it was demonstrated that the transcription factor Sp1 up-regulates ATP12A (HKalpha2) gene expression [44, 61, 62]. Therefore, the parallel up-regulation of ATP12A and CD86 protein expression in HL-60 cells by sodium butyrate may be mediated by an up-regulation of ATP12A and CD86 gene expression *via* the transcription factor Sp1 [62, 63]. Since HL-60 cells that differentiate towards monocytes can be regarded to be at increased risk of cell death in fulfilling their function, i.e. phagocytosis, the parallel expression of ATP12A might counteract key events during early apoptosis, like apoptotic volume decrease (AVD), intracellular acidification and decrease in intercellular K^+ concentration (see below).

Cell volume changes and changes of the intracellular ion composition are essential steps and earliest hallmarks of programmed cell death. During the cationic gradient reversal the cells lose K^+ and gain Na^+ ions mainly due to impaired function of the Na^+/K^+ ATPase [64]. Cell shrinkage or AVD are mainly elicited by activation of cell volume-regulated potassium- and anion channels. The changes result in the cellular exit of K^+ , Cl^- and HCO_3^- ions and consequently osmotically obliged water [65-68]. The intracellular loss of these ions, the resulting intracellular acidosis, as well as the cell condensation are obligatory events in activating downstream steps for the execution of apoptosis. Counteracting these processes may therefore delay or even prevent apoptosis (for a recent crosstalk debate see [69-71]). The activation of an H^+/K^+ ATPase can be expected to blunt intracellular acidosis, and to counteract the loss of K^+ ions and AVD. However, it has to be noted that in epithelial Madin-Darby canine kidney (C7-MDCK) cells and vascular endothelial cells from porcine aorta (PAEC) mild intracellular acidification prevented cell death induced by ouabain and other cardiotoxic steroids. The cell death-inducing action of these compounds was triggered *via* interaction with the Na^+/K^+ ATPase but was independent of the inhibition of ion fluxes and the ratio of intracellular Na^+ to K^+ and is most likely accompanied by the *de novo* synthesis of pH_i -sensitive genes involved in the suppression of the cell death machinery [72].

As H^+/K^+ ATPases are primary active transporters that have a demand for sufficient ATP, such a mechanism is expected to be only effective during the early phase of apoptosis

in which ATP supply is sufficient [73-75]. This phase is characterized by exposure of phosphatidylserine (PS) at the outer leaflet of the cell membrane. Indeed, in this study we demonstrate that the loss of cell volume in the time window between 24 and 72 h after seeding of the cells can be prevented only in early apoptotic PS⁺ cells if the expression of the non-gastric H⁺/K⁺ ATPase ATP12A is stimulated by treatment of the cells with 1 mM butyrate (Table 5 and Fig. 9). During this period only PS⁺ cells resist to shrink and show a significantly higher MCV than PS⁻ cells 72 h after initiation of treatment and compared to PS⁺ cells at 0 h. Accordingly, the effect of the H⁺/K⁺ ATPase inhibitor SCH28080 is not evident in untreated cells, but becomes detectable only in PS⁺ cells stimulated with 1 mM butyrate. PS⁺ cells treated with SCH28080 show similar MCVs as butyrate-treated non-apoptotic cells. This indicates that the ability of SCH28080 to induce cell shrinkage may depend on exposure of phosphatidylserine at the outer leaflet of the cell membrane[§].

In conclusion we demonstrate that HL-60 cells express the non-gastric H⁺/K⁺ ATPase ATP12A/ATP1A1, which might act as a SCH28080- and ouabain-sensitive K⁺-dependent H⁺ extrusion mechanism to increase cell volume and counteract cellular loss of K⁺ ions as well as intracellular acidosis. ATP12A⁺ cells exhibit a slightly higher cell volume than ATP12A⁻ cells. ATP12A is up-regulated upon differentiation of the cells with 1 mM butyrate. The functional activity of the H⁺/K⁺ ATPase and/or its susceptibility for the inhibitor SCH28080 becomes mainly evident or may even be restricted to phosphatidylserine positive cells and hence to early apoptosis. Since induction of apoptosis is frequently applied in the therapy of malignancies, the finding that inhibition of ATP12A during early apoptosis pushes programmed cell death in leukemic cells may be of significant clinical relevance.

Acknowledgements

The skilled technical and administrative assistances of Anna Baumgartner, Davide Mercurillo, Clemens Hufnagl, Marlena Beyreis and Leman Emin are gratefully acknowledged. We are deeply grateful to Anton Dörge[†] and Ines Mühlhahn for Electron Microprobe Analysis and Franz Xaver Beck for critical and helpful discussion. This project was supported by grants R-09/04/009-JAK, R-11/02/024-JAK and K-11/01-JAK to MJ.

Disclosure Statement

The authors declare to have no conflicts of interests. [§] Some of the MCV data presented in Fig. 9 and the related text were previously contributed to the crosstalk debate cited in reference [71].

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