Monovalent ions control proliferation of Ehrlich Lettre ascites cells

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Klausen TK, Preisler S, Pedersen SF, Hoffmann EK. Monovalent ions control proliferation of Ehrlich Lettre ascites cells. Am J Physiol Cell Physiol 299: C714–C725, 2010. First published June 30, 2010; doi:10.1152/ajpcell.00445.2009.—Channels and transporters of monovalent ions are increasingly suggested as putative anticarcinogenic targets. However, the mechanisms involved in modulation of proliferation by monovalent ions are poorly understood. Here, we investigated the role of K⁺, Na⁺, and Cl⁻ ions for the proliferation of Ehrlich Lettre ascites (ELA) cells. We measured the intracellular concentration of each ion in G₀, G₁, and S phases of the cell cycle following synchronization by serum starvation and release. We show that intracellular concentrations and content of Na⁺ and Cl⁻ were reduced in the G₀–G₁ phase transition, followed by an increased content of both ions in S phase concomitant with water uptake. The effect of substituting extracellular monovalent ions was investigated by bromodeoxyuridine incorporation and showed marked reduction after Na⁺ and Cl⁻ substitution. In spectrofluorometric measurements with the pH-sensitive dye BCECF, substitution of Na⁺ was observed to upregulate the activity of the Na⁺/H⁺ exchanger NHE1 as well as of Na⁺-independent acid extrusion mechanisms, facilitating intracellular pH (pHₗ) recovery after acid loading and increasing pHₑ. Results using the potential sensitive dye DiBac₄(3) showed a reduced Cl⁻ conductance in S compared with G₁ followed by transmembrane potential (Em) hyperpolarization in S. Cl⁻ substitution by impermeable anions strongly inhibited proliferation and increased free, intracellular Ca²⁺ ([Ca²⁺]), whereas a more permeable anion had little effect. Western blots showed reduced chloride intracellular channel CLIC1 and chloride channel CIC-2 expression in the plasma membrane in S compared with G₁. Our results suggest that Na⁺ regulates ELA cell proliferation by regulating intracellular pH while Cl⁻ may regulate proliferation by fine-tuning of Em in S phase and altered Ca²⁺ signaling.

Cell cycle; chloride channel; volume regulatory anion channel; Na⁺/H⁺ exchanger 1

Cell cycle progression depends on an increase in cell volume before cell division and is delayed in osmotically shrunken cells (see Refs. 23, 34, and 41). An increase in cell volume during the G₁-S phase transition has been directly demonstrated in human monokaryon cells (50), and we previously showed in Ehrlich Lettre ascites (ELA) tumor cells that the maximal swelling-induced Cl⁻ current is decreased in G₁ and increased in early S phase, compared with that in G₀ (30). Cell volume can change during the cell cycle by two distinct processes: either through accumulation of osmolytes through increased production or metabolism of larger molecules or through the movement of osmotically active substances across the plasma membrane. Specifically, the movement of the monovalent ions Na⁺, K⁺, H⁺, and Cl⁻ is important in the context of cell volume regulation. However, in addition to their role in regulating cell volume, changes in the cellular content of free, monovalent ions regulate cellular pH (pHₑ), transmembrane potential (Em), and the activity of a wide range of proteins with important functions in cell proliferation including cyclin B1, cyclin-dependent kinase 2 (cdk2) (52), mitogen-activated protein kinases (46, 47), ion transporters, and enzymes involved in cellular metabolism (15).

In agreement with these central functions of monovalent ions, channels and transporters controlling their cellular concentrations have repeatedly been shown to be important for cellular proliferation, and Na⁺, K⁺, and Cl⁻ channels have all been shown to be dysregulated in various cancers (33, 34, 58). However, the specific roles of monovalent ions in cell cycle progression are poorly understood and ion movements during the cell cycle have obtained little focus. Especially the role of Cl⁻ is elusive. In glia cells, Cl⁻ loss during M phase is essential for cytoplasmic condensation assisting chromatin condensation (20–22). However, this mechanism is dependent on the atypically high intracellular Cl⁻ concentration ([Cl⁻]) in glia cells, and in other cells Cl⁻ channel blockers will typically arrest cells in the G₀/G₁ phase (10, 25, 37, 61). Complicating the interpretation of such data, blocking monovalent ion channels is likely to affect multiple mechanisms involved in cell cycle progression. For instance, K⁺ channel blockers also tend to inhibit progression to the S phase, leading to accumulation in G₁ (see Refs. 33 and 66). This is generally taken to reflect that K⁺ channels set the driving force for [Ca²⁺] oscillations, but K⁺ channel inhibitors will, for instance, also affect pHₑ (see Refs. 7, 33, and 34). Similarly, Na⁺ is central in regulating pHₑ via the ubiquitous Na⁺/H⁺ exchanger isoform 1 (NHE1) (see Ref. 45) but is also very important for regulating [Cl⁻], via Na⁺-K⁺-2Cl⁻ cotransporter during cell proliferation (see Ref. 42).

Here, we investigated the roles of the main monovalent cellular ions in the proliferation of ELA cells during G₀–G₁ and G₁-S phase transition. We show that the cellular content of Na⁺, Cl⁻, and water content, yet not of K⁺, is altered during cell cycle progression, and that substitution of Na⁺ with N-methyl-D-glucamine (NMDG⁺) or Cl⁻ with impermeable anions, as well as inhibition of Cl⁻ channels or NHE1, inhibits cell proliferation. Moreover, despite the previously demonstrated increase in volume regulatory anion channel (VRAC) currents in S phase, total Cl⁻ conductance is reduced and Em is hyperpolarized in S compared with G₁. Finally, Cl⁻ replacement with impermeable, yet not with permeable, anions, depolarizes Em and increases [Ca²⁺]. We suggest that in ELA cells, Cl⁻ may regulate proliferation through at least two mechanisms: fine-tuning of Em in the G₁-S transition, possibly through chloride intracellular channel CLIC1 and chloride channel CIC-2 internalization, facilitating dynamic Ca²⁺ signaling; and VRAC-mediated protection of cell volume regulation during S phase and further cell cycle progression.

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MATERIALS AND METHODS

Unless otherwise stated, all materials were purchased from Sigma-Aldrich (St. Louis, MO) or Baker (Deventer, The Netherlands). Draq5 was from Biostatus. The CLIC1 antibody was a kind gift from Samuel N. Breit (St. Vincent Hospital and University, Sidney, Australia), and CIC-3 antibodies a kind gift from William J. Hatton (University of Nevada, Reno). CIC-1 and -2 antibodies were obtained from Alpha Diagnostics (San Antonio, TX) and Alamar Labs (Jerusalem, Israel), respectively. The mitosis-specific Alexa Fluor 488-conjugated antibody (Ab) phospho-histone H3 (Ser10) was from Cell Signaling Technology (Danvers, MA), the anti-cyclin D antibody was from Upstate (Lake Placid, NY), and the NHE1 antibody was from Chemicon. DIDS, niflumic acid, tamofoxen, EIPA (Sigma Aldrich), and NS3728 (Neurosearch, Ballerup, Denmark) stock solutions were prepared in DMSO. Antagonists were further diluted in DMSO to maintain constant DMSO concentration (1:1,000) in working solutions.

Cell culture. Ehrlich Lettre ascites cells were maintained in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C-5% CO2. Passages 11–28 were used for experiments.

Flow cytometry. Cells were synchronized in G0 by serum starvation for 72 h and sampled with 1-h intervals following release in serum-containing medium. Cells were trypsinized and harvested by centrifugation (600 g, 5 min, 4°C). After 1 × wash in PBS, cells were slowly resuspended in absolute ethanol during continuous mixing and fixed for 30 min on ice. Cells were collected by centrifugation (600 g, 5 min, 4°C), resuspended in ice-cold PBS, transferred to Partec 30-μm filters, and filtered during centrifugation. Supernatant was removed and cells were incubated in Triton buffer (1% BSA-0.25% Triton-X 100 in PBS) for 15 min. Cells were collected by centrifugation (600 g, 2 min, 4°C) and resuspended in blocking buffer (1% BSA in PBS). Alexa Fluor 488-conjugated phospho-histone H3-Ser10 Ab (1:20) was added for specific staining of mitotic cells and incubated 1 h. Cells were washed 3× in blocking buffer and resuspended in PBS containing 30 μM Draq5 for DNA staining. Flow cytometry was performed on a DAKO CyAn flow cytometer and analyzed using DAKO Summit 4.3 software.

Cell surface protein purification. Cell surface proteins were purified by biotinylation and avidin pull-down using a Cell Surface Protein Purification kit from Pierce (Rockford, IL) according to the manufacturer’s instructions. Briefly, cells were grown in 10-cm petri dishes and synchronized as above. Cells were quickly washed with 2 × 8 ml ice-cold PBS and incubated with 10 ml sulfo-NHS-SS-biotin (2.5 mg) in PBS for 30 min at 4°C. The reaction was quenched, and the monolayer was collected using a rubber policeman, and pooling four petri dishes for each sample. Cells were washed and collected by centrifugation (500 g, 4°C, 3 min). The pellet was redissolved in 500 μl lysis buffer containing Halt Protease Inhibitor cocktail (Pierce) and homogenized by 5 × 2 s sonication every 10 min for 30 min and centrifuged by precipitation (10,000 g, 4°C, 2 min). Total lysate (50 μl) was collected for protein measurement and Western blot analysis while 425 μl lysate was incubated 60 min in rotating immobilized NeutrAvidin gel column at room temperature. The column was washed 3 × by centrifugation (1,000 g, 25°C, 1 min) and incubated for 60 min at room temperature with 400 μl NuPage LDS sample buffer (Invitrogen) containing 53 mM DTT. Finally, biotinylated surface proteins were eluted by centrifugation (1,000 g, 25°C, 2 min).

SDS-PAGE and Western blot analysis. SDS-PAGE was carried out under denaturing and reducing conditions using 10% Bis-Tris gels with NuPage MOPS-SDS running buffer and a Novex XCell (E19001) system (Novex, San Diego, CA). The protein concentration of cleared total cell lysates was estimated using a BCA protein kit (Bio-Rad, Hercules, CA) and BSA as the protein standard. The amount of protein loaded per well was 30 μg for total lysates and an amount corresponding to 155 μg total lysate protein for purified surface proteins. Separated proteins were electrophoresed to Protran nitrocellulose membranes (Whatman, Dassel, Germany), membranes were stained with 1% Ponceau S Red solution (Sigma-Aldrich), blocked [5% nonfat dry milk in 1× TBST (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20)] for 75 min, incubated for 2 h with primary antibody in blocking buffer, washed extensively in TBST, and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody in blocking buffer, all at room temperature. Membranes were washed extensively in TBST and developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Band intensity was evaluated by densitometric scanning using the program Un-Scan-IT (Silk Scientific, Orem, UT).

Measurement of ion and water content was performed essentially as described previously (18). In short, cells were grown and synchronized in petri dishes as above. To estimate the intracellular water space, cells were incubated 1 h before experiments with 0.72 nM of the nonmetabolizable hexose, 3-O(14C-methyl)-D-glucose (GMO) (27.6 nCi/ml) which equilibrates across the membrane through facilitated transport (32). Experiments were initiated by rapid wash in 3 × 4 ml ice-cold 0.1 M Mg(NO3)2 with 55 mM D-mannitol (295 mosM) containing 1 mM phloretin to inhibit GMO transport during washing. Cells were lysed by adding 2.5 ml ethanol (EtOH) which was allowed to evaporate. The water-soluble pool was extracted by rehydration for 1 h in 1 ml H2O. Finally, the protein fraction was recaptured by NaOH containing 0.5% sodium deoxycholate.

The content of K+ and Na+ in the water-soluble pool was measured by flame spectrophotometry using a FLM3 flame photometer (Radiometer, Copenhagen, Denmark). CI− content in the water-soluble pool was measured using a CMT10 chloride titrator (Radiometer).

Ninhydrin-positive reactants were measured by spectrophotometry. Ninhydrin reactant (15 μl; Sigma-Aldrich) was added to 30 μl sample, and the mixture was boiled at 98°C for 10 min and cooled to room temperature. EtOH (75 μl) was added and the reaction was evaluated by measuring absorbance at 570 nm using a Fluostar-Optima microplate reader (BMG Labtech, Offenburg, Germany).

Cell proliferation. Cell proliferation was estimated by measuring bromodeoxyuridine (BrdU) incorporation using a Chemiluminescent Cell Proliferation assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, cells were seeded in black 96-well dishes with 6,000 cells/well. Twenty-four hours after seeding, the medium was changed to the relevant substi-
tution or inhibitor-containing medium and the cells were incubated for another 24 h. Ion-substituted RPMI-1640 media were custom-made according to recipe of Sigma-Aldrich using best-quality chemicals [in mM: 0.423 Ca(NO₃)₂, 0.406 MgSO₄, 5.4 KCl, 23.8 NaHCO₃, 5.6 Na₂HPO₄, 102.7 NaCl, 11.1 glucose, and glutathione], RPMI-1640 amino acid, and RPMI-1640 vitamin solutions (Sigma-Aldrich) as well as 2.05 l-glutamine (GIBCO). Ionic substitution was performed equimolarly. Two hours before measurement, 10 µl 10 µM BrdU in culture medium was added. After incubation, the cells were fixed for 30 min and incubated with monoclonal peroxidase-conjugated BrdU antibody for 90 min. After thorough washing, 100 µl peroxidase substrate solution was added and chemiluminescence was measured (within 10 min of substrate addition) on a Fluostar-Optima microplate reader.

Table 1. Flow cytometric analysis of cell cycle distribution

<table>
<thead>
<tr>
<th></th>
<th>G₀/G₁</th>
<th>S</th>
<th>G₂/M</th>
<th>M</th>
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<tbody>
<tr>
<td>Interphase</td>
<td>69.5 ± 5.3</td>
<td>18.4 ± 1.4</td>
<td>11.1 ± 3.4</td>
<td>0.6 ± 0.3</td>
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<tr>
<td>0 h</td>
<td>88.8 ± 0.3</td>
<td>7.2 ± 1.25</td>
<td>4.0 ± 1.5</td>
<td>—</td>
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<tr>
<td>8 h</td>
<td>88.5 ± 2.2</td>
<td>7.6 ± 1.53</td>
<td>3.9 ± 2.16</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>16 h</td>
<td>57.1 ± 7.9</td>
<td>36.2 ± 8.3</td>
<td>6.7 ± 2.2</td>
<td>—</td>
</tr>
<tr>
<td>24 h</td>
<td>60.6 ± 4.9</td>
<td>19.4 ± 2.6</td>
<td>20.0 ± 6.82</td>
<td>1.55 ± 0.9</td>
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Values are means ± SE. Cells were serum starved for 72 h and incubated with serum as indicated. Subsequently, cells were harvested and labeled as described in MATERIALS AND METHODS and analyzed for DNA content by flow cytometry. M phase cells were recognized by labeling with phospho-histone H3 (Ser10) Alexa-Fluor 488-conjugated antibody. Results are summarized from 3 independent experiments.

Fig. 2. Ion concentrations during the cell cycle. Cells were synchronized by 72 h serum starvation and sampled 0 h (G₀), 8 h (G₁), and 16 h (S) after reentry into the cell cycle by reexposure to serum (see Fig. 1). Insets: paired results for total protein per sample (A) or cellular content of the ion in question (B–D). Cells were sampled and analyzed as described in MATERIALS AND METHODS. A: intracellular water content per microgram protein as a function of cell cycle stage. B: cellular K⁺ concentration ([K⁺]ᵢ) as a function of the cell cycle phase. Intracellular K⁺ concentration ([K⁺]ᵢ) was measured from the ionic fraction by flame spectrometry as described in MATERIALS AND METHODS. C: cellular Na⁺ concentration ([Na⁺]ᵢ) measured by flame spectrometry. D: cellular Cl⁻ concentration ([Cl⁻]ᵢ) as estimated by coulombic measurement from the ionic fraction. *Significant difference relative to G₀ values (P < 0.05); n = 8–10 for all figures.
**Ionic Movements in the Cell Cycle**

Transmembrane potential measurement. $E_m$ was estimated using the potential-sensitive dye DiBaC$_4$(3) (16). Cells were seeded on no. 1 coverslips and synchronized as described above. Coverslips were mounted in an open perfusion chamber on an inverted microscope (Nikon Eclipse Ti) and continuously perfused with RPMI-1640 salt solution [in mM: 0.423 Ca(NO$_3$)$_2$, 0.406 MgSO$_4$, 5.4 KCl, 102.7 NaCl, 5.6 Na$_2$HPO$_4$, 11 glucose, and 25 HEPES, pH 7.4]. Excitation wavelength was 470 nm and emission was measured at 520–560 nm in regions of interest (ROI) surrounding individual cells using EasyRatioPro software (PTI, Seefeld, Germany). After a steady background signal was achieved, 0.5 μM DiBaC$_4$(3) was added. Signals were allowed to stabilize for 40–50 min before solution change. Data for all cells were corrected for ROI area (pixel$^2$) and background fluorescence. Calibration of fluorescence signals to $E_m$ was carried out by calculating membrane potentials from a modified Goldman-Hodgkin-Katz (GHK) equation at different extracellular Na$^+$ concentrations in the presence of gramicidin as previously described (13). Briefly, cells were perfused with RPMI-1640 salt solution (see above) with NaCl substituted with varying concentrations of choline-Cl and gramicidin. Since choline does not permeate gramicidin channels, $E_m$ could be calculated by a modulated GHK equation: $E_m = 60 \log([\text{Na}^+]/[\text{K}^+])_0 + ([\text{Na}^+]/[\text{K}^+])_i$. The relationship between $E_m$ and fluorescence intensity was fitted linearly, and $E_m$ was calculated from this correlation.

Calcium imaging. Calcium imaging was performed using the Ca$^{2+}$-sensitive dye Fura-2. Cells were seeded on glass coverslips 48 h before experiments. Twenty-four hours before experiments, growth medium was changed to purpose-made RPMI-1640 medium with NaCl/Na-glucuronate/Na-MSA, 5.6 Na$_2$HPO$_4$, 11 glucose, and 25 HEPES, pH 7.4. Excitation wavelength was 445 nm and emission was measured at 520–560 nm in regions of interest (ROI) surrounding individual cells using EasyRatioPro software (PTI, Seefeld, Germany). After a steady background signal was achieved, 0.5 μM DiBaC$_4$(3) was added. Signals were allowed to stabilize for 40–50 min before solution change. Data for all cells were corrected for ROI area (pixel$^2$) and background fluorescence. Calibration of fluorescence signals to $E_m$ was carried out by calculating membrane potentials from a modified Goldman-Hodgkin-Katz (GHK) equation at different extracellular Na$^+$ concentrations in the presence of gramicidin as previously described (13). Briefly, cells were perfused with RPMI-1640 salt solution (see above) with NaCl substituted with varying concentrations of choline-Cl and gramicidin. Since choline does not permeate gramicidin channels, $E_m$ could be calculated by a modulated GHK equation: $E_m = 60 \log([\text{Na}^+]/[\text{K}^+])_0 + ([\text{Na}^+]/[\text{K}^+])_i$. The relationship between $E_m$ and fluorescence intensity was fitted linearly, and $E_m$ was calculated from this correlation.

Intracellular pH measurements. Intracellular pH (pH$_i$) was estimated using the cell-permeable fluorescent dye BCECF, essentially as previously described (48). Cells were seeded on HCl- and ethanol-cleaned coverslips and loaded with BCECF-AM for 30 min at 37°C in RPMI 1640 salt solution. Cells were placed at an angle of 50° respective to the Xenon lamp light source in a thermostatted and perfused cuvette of a PTI Ratiomaster spectrophotometer. Fluorescence was measured at 525 nm after excitation at 445 and 495 nm. All data were corrected for background signals obtained from unloaded cells before calculation of the 445/495 nm ratio. BCECF ratios were converted to pH$_i$ values using a seven-point nigericin/high-K$^+$ calibration as previously described (48).

**RESULTS**

Cellular NaCl and water content changes during cell cycle progression. The first question asked was whether the cellular concentrations of, and hence the transmembrane driving force for, Cl$^-$, Na$^+$, and K$^+$ changed in ELA cells during cell cycle progression. For this purpose, cells were synchronized by serum starvation, and cell cycle phase distribution was monitored by cyclin D labeling (Fig. 1) and flow cytometry of cells labeled for phospho-histone 3 and DNA (Table 1). Cyclin D is a marker for interphase cells and is not expressed in G0 (39, 43), phospho-histone 3 labels M-phase cells (1, 2, 6, 19), and Draq5 monitors total DNA content. After 72 h of serum starvation, the cellular cyclin D level was markedly reduced (Fig. 1A), and the fraction of cells with G0/G1 phase DNA content was increased to almost 90% (Table 1). Following

![Fig. 3. Na$^+$ and Cl$^-$ substitution inhibits proliferation. Following 24 h substitution of K$^+$ (A) or Na$^+$ (B) by N-methyl-D-glucamine$^+$ (NMDG$^+$), cellular proliferation was estimated by bromodeoxyuridine (BrdU) incorporation. Cl$^-$ (C) was substituted by either methane-sulfonic acid (MSA), glucuronate, or glucuronate. BrdU incorporation relative to the control is depicted as a function of percentage substitution. *Significant difference from 0% substitution ($P < 0.05$); n = 4–9 in all figures.](http://ajpcell.physiology.org/)
readdition of serum, cyclin D1 expression normalized within the first 6–8 h, and S phase was predominant after 15–16 h after release (Table 1), in congruence with what we previously observed in ELA cells (30).

Whereas total protein content did not differ between different samples (Fig. 2A, inset), the amount of water per protein content was increased 16 h after release compared with G0 phase cells (Fig. 2A), implying an increase in the amount of intracellular osmotic substances. As will be discussed below, the water uptake can be explained by an uptake of NaCl.

Neither the concentration nor the cellular content of K+ was significantly changed during cell cycle progression (Fig. 2B). In contrast, Na+ and Cl− concentrations were significantly reduced by 15.0 and 11.3 mM, respectively, in the transition between G0 and G1 (8 h after release from G0) (Fig. 2, C and D), i.e., a decrease in intracellular osmolarity of ~26 mosM. As the water content did not change in this period (Fig. 2A), intracellular osmotic pressure must be kept constant via other means. However, we did not find significant changes in the content of either protein (Fig. 2A inset), K+ (Fig. 2B inset) or ninhydrin-positive reactants (n = 4, 35.7 ± 4.9 mM, 35.9 ± 3.6 mM, and 31.1 ± 3.3 mM at time 0, 8, and 16 h after release, respectively).

The loss of NaCl in the initial 8 h after cell cycle release was more than compensated for as the population was shifting toward S phase (16 h after release). Thus, a significant increase in Na+ (276 ± 27 to 330 ± 26 nmol/mg protein) and Cl− (284 ± 25 to 345 ± 28 nmol/mg protein) content and a significant increase in water content of 11.8 ± 4.5% was observed between the populations harvested 8 and 16 h after addition of serum (n = 8 paired experiments, P < 0.05 for all three parameters). Because of the concomitant change in water content, Na+ and Cl− concentrations did not differ between G1 and S.

**Substitution of Na+ and Cl−, yet not of K+, inhibits cell proliferation.** With concentrations of 5.4 mM K+, 113.9 mM Na+, and 108.1 mM Cl−, monovalent ions constitute the most abundant molecules in RPMI 1640 medium. Substituting extracellular K+ with the impermeable cation NMDG+ did not affect the rate of proliferation in ELA cells (Fig. 3A). In contrast, substituting Na+ with NMDG+ had a strong dose-dependent inhibitory effect on proliferation which was significant after 40% substitution and reached about one-third of the control rate after 90% substitution (Fig. 3B). [K+]i decreased from 28.3 ± 3.0 to 15.4 ± 2.4 and 16.2 ± 1.5 mM, respectively, following 36% and 90% Na+ substitution. [K+]i increased after 90% Na+ substitution, from 120.8 ± 8.1 mM to 174.6 ± 20.9 mM but was unchanged after 36% substitution (113.6 ± 9.5 mM). In line, [Cl−]i increased at 90% substitution (42.1 ± 3.9 mM and 61.4 ± 5.6 mM) but was unchanged after 36% substitution (42.5 ± 5.6 mM). Water content, however, was not significantly changed (9.8 ± 0.6 nl/μg protein, 9.3 ± 0.6 nl/μg protein, and 8.4 ± 0.9 nl/μg protein). Cl− was substituted using three different anions: MSA which exhibits intermediate permeability via Cl− channels (9, 68) and does not affect cell volume, ionic strength and pH (44) and the two more impermeable anions gluconate and glucuronate (Fig. 3C). The maximum inhibitory effect of Cl− substitution (45.5 ± 5%) was observed in response to the impermeable anions, whereas substitution with MSA only resulted in minor inhibition.

**Na+ substitution alkalizes pH** and upregulates NHE1 activity. The clear difference between the effects of Na+ and Cl− substitution on cell proliferation pointed to the existence

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**Fig. 4.** EIPA sensitivity of Ehrlich Lettre ascites (ELA) cell proliferation. BrdU incorporation assays were performed as in Fig. 3 with increasing concentrations of EIPA in the growth medium. *P < 0.05; n = 10.

**Fig. 5.** Na+/H+ exchanger isoform 1 (NHE1) expression. A: representative study of the NHE1 protein expression pattern. Cells were synchronized by serum starvation and harvested 0, 8, and 16 h after release from serum starvation. Total lysate and biotinylated protein fractions were prepared as described in MATERIALS AND METHODS. B: densitometric quantification of NHE1 Western blots as shown in A. Data are summarized results from 4–5 independent experiments.
of independent functions of the two ions in the cell cycle (see also DISCUSSION).

Given the importance of the Na\(^+\)/H\(^+\) exchanger, NHE1, for proliferation in numerous cell types (see Ref. 45) we determined the effect of the NHE1 inhibitor EIPA on ELA cell proliferation. As seen in Fig. 4, EIPA inhibited BrdU incorporation with an IC\(_{50}\) value of 14.5 \(\mu\)M. We therefore next investigated whether NHE1 was differentially expressed—in total and at the plasma membrane—during cell cycle progression. NHE1 runs as two bands in Western blots: an immature unglycosylated and a mature glycosylated form (35). As expected, the mature, glycosylated form was predominant in the cytosolic Ca\(^{2+}\) as a representative of 4–5 independent experiments. *Significant difference relative to control (\(P < 0.05\)).

\(\text{Cl}^-\) permeability decreases between cell cycle G\(_1\) and S phases. BrdU assays suggested a role for anion permeability rather than a direct role of Cl\(^-\) ions for cell proliferation in regulating cell cycle progression (see above and Fig. 3C). As measuring the small isotonic Cl\(^-\) currents constitutes a major difficulty in nystatin perforated patch-clamp studies due to difficulties in bringing nonpermeable cations to the cytoplasm, we assessed the Cl\(^-\) permeability indirectly via \(E_m\) measurements using the membrane-permeable anionic fluorescent dye DiBaC\(_4\)(3). Cl\(^-\) permeability was estimated from the shift in \(E_m\) when substituting extracellular Cl\(^-\) with the impermeable gluconate (Fig. 8A). These experiments showed a significant reduction in the Cl\(^-\)-sensitive potential of ELA cells at 16 h of independent functions of the two ions in the cell cycle (see also DISCUSSION).

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after the release compared with cells that have been released for 8 h, indicating a significant reduction in the Cl\textsuperscript{−}/H\textsubscript{11}O\textsubscript{2+} permeability in the S phase (Fig. 8C). Accordingly, substituting with the more permeable anion MSA had a significantly smaller effect (Fig. 8B).

\( E_{m} \) is hyperpolarized at 16 h (S phase) compared with at 8 h (G\textsubscript{0}–G\textsubscript{1} transition) after release from starvation. \( E_{m} \) was hyperpolarized (−69.6 ± 0.7 mV) 16 h after release from serum starvation (i.e., approaching S phase) when compared with cells 8 h after release, i.e., in G\textsubscript{0}–G\textsubscript{1} transition (−62.9 ± 1.2 mV) (Fig. 8D). This was expected based on the \( E_{Cl}\) of −20 to −30 mV and the observed reduction of Cl\textsuperscript{−} permeability as the cells shifted toward S phase. Cells in the G\textsubscript{0}–G\textsubscript{1} transition (8 h post release) also tended to be depolarized, albeit nonsignificantly, relative to G\textsubscript{0} cells. This cannot be explained by altered Cl\textsuperscript{−} conductance since no significant difference in \( E_{m} \) was seen upon Cl\textsuperscript{−} substitution (Fig. 8D).

**Cl\textsuperscript{−} substitution increases \([Ca^{2+}]_i\).** To investigate whether Cl\textsuperscript{−} channels regulate \([Ca^{2+}]_i\), we measured the effect of Cl\textsuperscript{−} substitution on \([Ca^{2+}]_i\). Maximal Cl\textsuperscript{−} substitution (95%) by either glucuronate or MSA resulted in increased \([Ca^{2+}]_i\) (Fig. 9). However, when substituting only 50% of extracellular Ca\textsuperscript{2+}, only glucuronate significantly increased \([Ca^{2+}]_i\). MSA did not significantly change \([Ca^{2+}]_i\) (Fig. 9).

Despite the role of Cl\textsuperscript{−} sensitive transporters in pH regulation, pH\textsubscript{i} was not affected by Cl\textsuperscript{−} substitution by either 50% or 95% glucuronate (\( P = 0.37 \) and 0.38, respectively; \( n = 3 \)).

**Cl\textsuperscript{−} channel antagonists inhibit cellular proliferation.** To further address the role of Cl\textsuperscript{−} permeability in ELA cell proliferation, we next tested the effect of pharmacologically inhibiting various Cl\textsuperscript{−} channels. The broad spectrum Cl\textsuperscript{−} channel inhibitor DIDS did not affect proliferation (Fig. 10A) and the ELA cell CaCC inhibitor niflumic acid only had minor effects at very high concentrations (Fig. 10B). NS3728, which blocks VRAC and CaCC channels in ELA cells (30), inhibited BrdU incorporation with an IC\textsubscript{50} value of 41 ± 2 \( \mu \)M (\( n_{Hill} = 4 \), Fig. 10C). Tamoxifen, which blocks VRAC in ELA cells potently inhibited proliferation with an IC\textsubscript{50} value of 5.8 ± 0.8 \( \mu \)M (\( n_{Hill} = 1.3 \), Fig. 10D). Notably, both compounds inhibited progression of the cell cycle almost 100%, in contrast to the more modest effect of Cl\textsuperscript{−} substitution (see also DISCUSSION).
CI− channels are differentially translocated to the plasma membrane during cell cycle. To identify molecular candidates for the Cl− channels involved in cell cycle regulation, we investigated the expression of known Cl− channels in ELA cells. In the CIC family of channels and transporters, CIC-2 yet neither CIC-3 nor CIC-4 appeared to be present in ELA cells (Fig. 11A). The recently cloned Cl− channels CLIC1 and TMEM16A were both present in ELA cells. To investigate whether these channels were differentially expressed during the cell cycle, plasma membrane proteins were isolated by biontination. From these experiments a clear regulation of Cl− channels during the cell cycle was observed. Both CLIC1 and CIC-2 were downregulated in the biontinated fraction of cells 16 h after release (S phase) compared with cells 8 h after release (G0–G1). A similar pattern was not observed in the total lysates, where neither CLIC1 nor CIC-2 expression was significantly changed. TMEM16A expression did not change in either the biont-avidin purified lysates nor in total lysates.

DISCUSSION

Ion channels and transporters are increasingly being investigated as molecular targets for anticancer drugs (4, 12, 36, 63); however, knowledge of the mechanisms through which ions regulate cell cycle progression is still rudimentary. In this study, we report changes in monovalent ion content and concentration during cell cycle progression in ELA cells and describe their possible effects on the cell cycle.

ELA cells showed a marked decrease in NaCl concentration during G0–G1 transition (starvation vs. 8 h in serum). It seems likely that this loss reflected the combined effect of water uptake and NaCl loss, although on their own, neither NaCl content nor water content changed significantly through this transition. A significant increase in NaCl content was observed between cells entering the cell cycle (8 h) and cells entering S phase (16 h). NaCl uptake was followed by water; hence, there was no difference in NaCl concentration and the cells swelled osmotically. A minor volume increase between G1 and S phase has previously been described in CNE-2Z nasopharyngeal carcinoma cells, where a decreased capacity for volume regulation in S phase compared with G1 was also observed (65). The role of osmotic volume changes in regulation of cell proliferation is still very rudimentarily understood, but cell swelling has been shown to promote, and cell shrinkage to inhibit, proliferation in a number of cell types (3, 14). The functional importance of these ion and water movements could be speculated to involve cell shape as changing cell morphology involves volume regulatory channels and transporters e.g., during migration (56, 57, 59). Because the nuclear volume is strictly regulated in relation to cell morphology, in a process that involves rearrangement of the cytoskeleton and that is particularly important in the G1–S phase transition (54), such morphological changes could very well be initiated by volume changes, which strongly affect the cytoskeleton arrangement in many cell types including the ELA related cell line, Ehrlich ascites tumor cells (38, 49).

Substituting either Na+ or Cl− with impermeable ions had significant effects on cell proliferation, demonstrating that these ions are important for normal cell cycle progression despite the lack of significant concentration differences for these ions between G1 and S phases. However, there was a marked difference in the potency of Na+ and Cl− substitution. Na+ substitution having a much stronger effect than Cl− substitution. This could imply that the two ions regulate the cell cycle via different mechanisms, or at least that Na+ has roles in cell cycle regulation beyond its role in cell volume regulation. Our data indicated that one such role may involve the Na+/H+ exchanger NHE1. Inhibition of NHE1 dose dependently inhibited ELA cell proliferation, in agreement with findings in other cell types (40, 67). Also in accordance with our previous findings (57), NHE1 expression was higher in G0 than in cells released for 8 h (G1). This may indicate that NHE1 is kept in a “readied, but quiescent” state in G0, allowing for a rapid increase in NHE1 activity once the cells are released from G0 (57). In CNS pericytes, NHE1 controlled pH oscillations which facilitated proliferation by stimulating Ca2+ oscillations (40). It has also been shown that NHE1 activity regulates G2/M progression by eliciting an increase in pH, which in turn regulates cyclin B1 expression and cdk2 activity (52). Hence, we hypothesized that Na+ depletion might inhibit proliferation by reducing pH, since, under these conditions, the cells cannot extrude acid equivalents via either NHE1 or Na+/H+-dependent HCO3− cotransport, the two major acid extruders in most cell types. Counter to our expectation, ELA cells grown in 0 Na+ medium for 24 h showed a significant increase in steady-state pH, measured in the same medium. Since, as noted above, cells in 0 Na+ medium cannot increase their pH via either NHE1 or Na+/H+-dependent HCO3− cotransport, this must reflect the upregulation of Na+/H+-independent acid extrusion mechanisms in these cells, possibly H+/ATPases. The capacity of the Na+-depleted cells for pH4 recovery in Na+ containing solutions after an acid load was also strongly increased relative to that in control cells. This could reflect either upregulation of NHE1 expression and/or the greater transmembrane Na+ gradient in depleted cells, a question not addressed here. In any event, however, it seems clear that at least at time 24 h, the effect of Na+ depletion does not reflect...
a decrease in pH. It seems likely that pH may have been reduced initially upon Na/H exchange removal, possibly leading to a proliferation delay, followed by an increase of pH as compensatory mechanisms are upregulated. Another possibility is that the altered transmembrane Na/H gradient will cause dysregulation of Ca transport via NCX, and indeed we observed an increase in Ca. Hence, it is very possible that Na/H is affecting the cell cycle by pH as well as Ca regulation. However, as EIPA inhibition of NHE1 almost completely eliminated DNA replication and Ca oscillations are essential for S phase transition (7, 55), we are probably not observing a cumulative effect but rather an effect of inhibiting two individually essential mechanisms.

As already noted, the very different response of Na and Cl substitution on proliferation led us to speculate that Cl might exert its effects on cell cycle progression via Na-independent mechanisms. Substituting Cl with MSA, which has a medium permeability in several Cl channels (5, 8, 17, 24, 60), had a much smaller effect on proliferation than substituting with gluconate and glucuronate, which generally have very low permeabilities (5, 8, 17, 24, 60). This indicates that anion permeability rather than Cl concentration per se is the dominating factor regulating proliferation. With calculated Nernst potentials for Cl of -19.3, -26.2, and -23.9 mV after 0, 8 h and 16 h in serum, respectively, and a measured Em between -60 and -70 mV, a decrease in Cl permeability should hyperpolarize Em. In accordance with this notion, the decrease in Cl permeability from G1 (8 h) to S phase (16 h) was accompanied by hyperpolarization of Em. Em plays a central role in regulating cell cycle progression, primarily by changing the driving force for Ca uptake (see Refs. 7 and 33), and Cl channel dependency of Ca oscillations has been observed in T-lymphocytes (64). Our studies further underline the importance of Cl conductance for Ca uptake, during the cell cycle as Cl substitution increased [Ca]. This increase is in line with an increased inward driving force for Ca due to a general hyperpolarization of Em as a consequence of decreased anion permeability in the presence of glucuronate or MSA. Notably, the effect of Cl substitution on Ca was identical to the effect on cell proliferation, hence, dependent on the overall permeability of the substituting anion: Substituting with MSA did not change [Ca]; at 50% substitution while 50% substitution with the less permeable glucuronate significantly increased [Ca]. Of course, Cl can affect proliferation by...
other means than $E_m$. However, Cl$^-$ is not functioning as a messenger as $[\text{Cl}^-]_i$ did not change between G1 and S phase cells and pH$_i$ was insensitive to Cl$^-$ substitution.

It must be emphasized that the effect of Cl$^-$ substitution is markedly increased between 76% and 95% substitution, indicating an additional Cl$^-$-sensitive mechanisms with very high threshold to Cl$^-$ substitution. This effect could be speculated to involve the regulation of Cl$^-$-dependent enzymes important for proliferation (15). As MSA only had an effect on proliferation at 95% Cl$^-$ substitution, i.e., during the secondary effect, it could be speculated that MSA only functions during this secondary mechanism.

This raises the question of which Cl$^-$ channel(s) regulate cell cycle progression. Of course any change in channel Cl$^-$ permeability will also affect $E_m$. In our hands, neither niflumic acid nor DIDS—both of which inhibit CaCC in ELA cells (31)—affected cell proliferation. DIDS also inhibits VRAC in ELA cells, however, only at positive $E_m$ (31), which is not relevant in these studies. The VRAC inhibitors NS3728 and tamoxifen both potently inhibited ELA cell proliferation. It must be emphasized though that these experiments were performed in the presence of serum and as the antagonists are hydrophobic, the free concentration is possibly not identical to the given concentration, e.g., up to 75% binding of NS3728 to serum has been described (51), and albumin binding to niflumic acid has also been extensively described (26–29, 53). Hence, these pharmacological data should be treated with some caution, and we will not speculate on the molecular target. For this reason the cell cycle pattern of VRAC expression and isotonic VRAC activity (both of which are increased in S phase compared with G1) in ELA cells (30), indicating that VRAC is not responsible for S phase hyperpolarization, is not necessarily in disagreement with pharmacological data.

Both ClC-2 and CLIC1 were downregulated in the plasma membrane in S phase, correlating with the downregulation of Cl$^-$ permeability, and we hypothesize that altered activity of these channels may underlie the observed changes. In accordance with this view, ClC-2 regulates proliferation of porcine arterial smooth muscle cells (11) and CLIC1 was found to be exclusively expressed in the plasma membrane in G2/M phase of CHO cells (62).

This leaves us with the final question of the possible role of the VRAC upregulation in S phase which we previously demonstrated (30). We speculate that VRAC upregulation serves to prepare the cells for volume challenges as a result of nucleic acid and protein synthesis in the S-phase, and may also allow the cells to compensate for volume disturbances resulting from Ca$^{2+}$ oscillations during G1-S (see Refs. 23 and 55).
Collectively, the data indicate that Cl\(^{-}\) channels play multiple roles in cell cycle progression in ELA cells: CLIC1 and CIC-2 downregulation at the plasma membrane in G1\(_s\) S serves to hyperpolarize \(E_m\) and facilitate Ca\(^{2+}\) oscillations, while VRAC upregulation in the S-phase secures the volume-regulatory capacity necessary for further cell cycle progression.

In conclusion, the cellular content of Na\(^{+}\), Cl\(^{-}\), and water content, yet not of K\(^{+}\), is altered during cell cycle progression in ELA cells, and substitution of Na\(^{+}\) with NMGD\(^{+}\) or Cl\(^{-}\) with impermeable anions, as well as inhibition of VRAC or NHE1, inhibits cell proliferation. Moreover, total Cl\(^{-}\) conductance is reduced and \(E_m\) hyperpolarized in S-phase compared with G1. Finally, Cl\(^{-}\) replacement with impermeable, yet not with permeable, anions, depolarizes \(E_m\) and increases [Ca\(^{2+}\)]\(_{cyt}\). We suggest that in ELA cells, Cl\(^{-}\) may regulate proliferation through at least two mechanisms: hyperpolarization of \(E_m\) in the G1-S transition, possibly through CLIC1 and ClC-2 intercalization, facilitating dynamic Ca\(^{2+}\) signaling; and VRAC-mediated protection of cell volume regulation during S-phase and further cell cycle progression.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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