Cell cycle-dependent expression of volume-activated chloride currents in nasopharyngeal carcinoma cells


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Cell cycle-dependent expression of volume-activated chloride currents in nasopharyngeal carcinoma cells. Am J Physiol Cell Physiol 283: C1313–C1323, 2002. First published June 26, 2002; 10.1152/ajpcell.00182.2002.—Patch-clamping and cell image analysis techniques were used to study the expression of the volume-activated Cl– current, I_{Cl(vol)}, and regulatory volume decrease (RVD) capacity in the cell cycle in nasopharyngeal carcinoma cells (CNE-2Z). Hypotonic challenge caused CNE-2Z cells to swell and activated a Cl– current with a linear conductance, negligible time-dependent inactivation, and a reversal potential close to the Cl– equilibrium potential. The sequence of anion permeability was I– > Br– > Cl– > gluconate. The Cl– channel blockers tamoxifen, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and ATP inhibited I_{Cl(vol)}. Synchronous cultures of cells were obtained by the mitotic shake-off technique and by a double chemical-block (thymidine and hydroxyurea) technique. The expression of I_{Cl(vol)} was cell cycle dependent, being high in G1 phase, downregulated in S phase, but increasing again in M phase. Hypotonic solution activated RVD, which was cell cycle dependent and inhibited by the Cl– channel blockers NPPB, tamoxifen, and ATP. ATP. The expression of I_{Cl(vol)} was closely correlated with the RVD capacity in the cell cycle, suggesting a functional relationship. Inhibition of I_{Cl(vol)} by NPPB (100 μM) arrested cells in G0/G1. The data also suggest that expression of I_{Cl(vol)} and RVD capacity are actively modulated during the cell cycle. The volume-activated Cl– current associated with RVD may therefore play an important role during the cell cycle progress.

ion channels; volume regulation; cancer cells

CL– CHANNELS whose activity displays cell cycle dependence have been reported in ascidian embryos (2, 31), ascidian eggs (8), human B lymphocytes (3), and glioma cells (30). A maxi-Cl– channel had a substantially higher incidence in highly proliferating cells than in quiescent cells (14), and a voltage-activated Cl– channel (29) was expressed specifically in glioma cells but not in normal cells. Cl– channel blockers inhibited cell proliferation in many types of cells, including pulmonary artery endothelial cells (32), neuroblastoma cells (21), lymphocytes (24, 21), Schwann cells (19), microglial cells (22), liver cells (38), and cervical cancer cells (25), although they enhanced cell proliferation in some cases (9, 37). These observations suggest an important role for Cl– channels in the cell cycle and cell proliferation.

An apparently ubiquitous response to swelling in vertebrate cells is the activation of a Cl– current. The outflow of Cl– through the Cl– channel and of K+ through a separate channel leads to a decrease in cell volume, named regulatory volume decrease (RVD). It has been reported that a voltage-gated Cl– channel in ascidian embryos was modulated by both the cell cycle clock and cell volume (31). Inhibition of Cl– currents by channel blockers arrested cells in G0/G1 phase (24). Moreover, a volume-sensitive Cl– channel has been reported to be associated with human cervical carcinogenesis. The Cl– current can only be induced by hypotonicity in human cervical cancer cells but not in normal cervical epithelial cells (6, 25). It has also been reported that the magnitude of a volume-activated Cl– current was lower in differentiated cells than that in proliferating cells (33). In liver cells, volume-sensitive currents could only be activated in dividing cells but not in nondividing cells (38). All this evidence indicates that the volume-activated Cl– current is associated with the cell cycle and cell proliferation. However, the expression of the volume-activated Cl– current in the cell cycle has not yet been well defined. In this study, the expression of volume-activated Cl– currents and its relation with RVD capacity during cell cycle progression have been investigated, and the roles of the current and RVD in the cell cycle and cell proliferation are discussed.

METHODS

Cell Culture

The poorly differentiated nasopharyngeal carcinoma cells (CNE-2Z) (5, 15) were grown in culture medium (RPMI 1640 medium with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere.
of 5% CO₂ and were subcultured every other day. For electrophysiological and RVD experiments, cells, which had been cultured for 48 h and reached about 80% confluence, were harvested, resuspended in culture medium, plated onto 12-mm circular glass coverslips, and incubated for 2 h before experiments.

Cell Synchronization

Mitotic detachment. Synchronized cells were obtained by the “mitotic shake-off” (sometimes called “mitotic detachment”) method. Terasima and Tolmach (28) first made use of the fact that mammalian cells, grown as monolayer, are less well attached to the growing surface when they round up during mitosis (cells in other stages in a adherent cell line are well attached to the culture surface). Gentle agitation of the growth medium will thus remove the mitotic cells, which can then be used as a starting point for a synchronous population. This method can provide highly synchronous cultures of mitotic cells (10, 12, 13). CNE-2Z cells were incubated at a density of 1.5 × 10⁴ cells/cm² in culture medium in 175-cm² plastic tissue culture flasks at 37°C using a humidified atmosphere of 5% CO₂ for 24 h. After cell attachment and before the mitotic cells were harvested, the cultures were washed to remove unattached dead cells. The culture medium was removed and the cells were washed once with buffered saline solution (BSS) without Ca²⁺ and Mg²⁺. After the BSS solution was removed, 15 ml of culture medium were added to the flask. The flasks were shaken by hand for 2–3 min. Cell suspension was collected, pelleted at 80 g for 5 min, and resuspended in prewarmed culture medium. In this way, mitotic cells mainly in metaphase were harvested. The cell suspension was plated onto glass coverslips (for electrophysiological and RVD experiments) or into flasks (for flow cytometric analysis) and incubated at 37°C in the humidified 5% CO₂ incubator. Current recordings, RVD experiments, or flow cytometric analyses were carried out at the indicated time points after incubation.

Double chemical block. Double-block technique (10) was used to synchronize cells in S phase. The cells were arrested at G1/S boundary by the inhibitors of DNA synthesis, thymidine and hydroxyurea, and then allowed to enter S phase by removing the inhibitors. The cells were incubated in 2 mM thymidine for 14 h, washed, and allowed to grow in normal medium for 10 h and were then blocked by hydroxyurea (2 mM) for 14 h. The cells were harvested, resuspended in normal medium with hydroxyurea (2 mM) or washed twice with normal medium (no inhibitors), and resuspended in the normal medium (to release cells from block). The suspended cells were plated onto coverslips or flasks and incubated at 37°C in the humidified 5% CO₂ incubator. Current recordings, RVD experiments, or flow cytometric analysis were carried out at the indicated time points after incubation.

Electrophysiological Recordings

Whole cell currents of single CNE-2Z cells were recorded using the patch-clamp technique previously described (4) with a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The experiments were performed at room temperature (20–24°C). The patch-clamp pipettes were manufactured from standard wall borosilicate glass capillaries with an inner filament (Clark Electromedical Instruments, Kent, UK) on a two-stage vertical puller (PB-7, Narishige, Tokyo, Japan) and gave a resistance of 4–5 MΩ when filled with the pipette solution. The junction potential was corrected when the pipette entered the bath. The access resistance was <8 MΩ and was compensated. An agar bridge was used to connect the reference electrode (Ag-AgCl wire) to the bath solution. The command voltage and whole cell currents were recorded simultaneously on a computer via a laboratory interface (CED 1401, Cambridge, UK) with a sampling rate of 3 kHz. The voltage pulse generation, data collection, and current analysis were performed by the computer using the EPC software package (CED). Throughout the whole cell recordings the cells, unless otherwise indicated, were held at the Cl⁻ equilibrium potential (EC₅₀; 0 mV) and then stepped to +40 and +80 mV, with a 200-ms or 1-s duration for each step and 4-s intervals between steps. The cells were continuously cycled through the voltage protocol. In the analysis of the data collected, all current measurements were made at 10 ms after the onset of each voltage step. Cell size was monitored by the imaging system during the experiments.

The permeability ratios (Pₛ/P₅) of various anions (X⁻) relative to that of Cl⁻ were calculated by using the modified Goldman-Hodgkin-Katz equation

\[ Pₛ/P₅ = \left[ [Cl⁻]_n \exp(-\Delta V_{rev}/RT) - [Cl⁻]_s/[X^-]_n \right] \]  

where [Cl⁻]ₙ and [Cl⁻]ₗ are the Cl⁻ concentration in the normal and the substituted bath solutions, [X⁻]ₙ is the concentration of the substituted anion, ΔVₕ is the difference of the reversal potentials for Cl⁻ and X⁻, and Fₚ is the Faraday constant. R is the gas constant, and T is the absolute temperature.

Measurements of Cell Volume

Cell volume was monitored and measured during the patch-clamp and RVD experiments. Cell images were captured by a charge-coupled device digital camera (Mono CCD625, Leica, Germany) that was connected to the microscope (Leitz DML; Leica Mikroskopie und Systeme, Germany) with a method reported previously (34). The acquisition of the cell images was controlled by the Quantiﬁmet Q500MC image processor and analysis software (Leica). The cell volume was calculated from cell diameters (d) with the equation 4/3π(1/2d)³. RVD was determined by subtracting two cell volumes: Vₘₐₓ was the peak volume, and Vₘᵢₐₜ is the volume at 20 min under hypotonic condition. Cell volume was standardized to the time point before the solution change for the calculation of RVD.

Cell Capacitance

Capacitance of pipettes was measured before breaking the patch, and capacitance of CNE-2Z cells was determined once the whole cell configuration was achieved. Capacitance reading was obtained by adjusting and minimizing the capacity transients in response to a 2-mV voltage step using the amplifier functions, following the instruction of the amplifier manual (EPC-7, List Electronic, Germany). The capacitance increased during the cell cycle progression, along with increase of cell size. The capacitance was 31.2 ± 2.0 pF in G₁ phase (n = 15), 42.3 ± 3.8 pF in S phase (n = 12), and 48.1 ± 3.1 pF in M phase (n = 9). In this study, currents were normalized to cell capacitance.

Cell Cycle Analysis

Cell preparation and analysis followed the methods described by Holmes and Al-Rubeai (13). Cells were ﬁxed in 70% ethanol at −20°C for 30 min and stored at this temperature until required for analysis. Before analysis, cell were pelleted from the ethanol, resuspended in a RNase A solu-
tion (50 μg/ml in PBS), and incubated at 37°C for 30 min. Propidium iodide (PI) from a stock of 1 mg/ml in distilled water was added to a final concentration of 50 μg/ml, and the cells were stained for 15 min, after which they were analyzed by the flow cytometer and its system software (EPICS XL; Coulter). The proportion of cells in G0/G1, S, and G2/M phases were obtained by analyzing cells for their different DNA content on the basis of PI staining. Around 10,000 cells were analyzed. Frequency distribution-DNA content histograms were drawn. Data were deconvoluted mathematically by the flow cytometer system software, and the percentage of cells in each cell cycle phase was quantified.

Solutions and Chemicals

The pipette solution contained (in mM) 70 N-methyl-d-glucamine Cl− (NMDG-Cl), 1.2 MgCl₂, 10 HEPES, 1 EGTA, 140 d-mannitol, and 2 Na₂ATP. The isotonic bath solution contained (in mM) 70 NaCl, 0.5 MgCl₂, 2 CaCl₂, 10 HEPES, and 140 d-mannitol. The osmolarity in the pipette and isotonic bath solutions was measured by depression of the freezing point using an osmometer (Osmomat 30; Gonotec) and adjusted to 300 mosmol/l with d-mannitol. The hypotonic bath solution was obtained by omitting the d-mannitol from the solution, giving an osmolarity of 160 mosmol/l (47% hypotonicity). In anion substitution experiments, 70 mM NaCl in the hypotonic solution was replaced by equimolar of NaI, NaBr, or sodium gluconate.

Hypertonic bath solution was obtained by adding 140 mM d-mannitol into the isotonic bath solution, giving an osmolarity of 440 mosmol/l (47% hypertonicity). The pH of the pipette and bath solutions was adjusted to 7.25 and 7.4, respectively, with Tris base. BSS solution without Ca²⁺ and Mg²⁺ was used to wash cells contained (in mM) 125 NaCl, 5 KCl, 10 NaHCO₃, 10 HEPES, 5 glucose, and 20 sucrose, pH to 7.4 with 3 M NaOH.

Cell cycle blockers thymidine and hydroxyurea were dissolved in phosphate-buffered saline (PBS) and diluted to the final concentrations of 2 mM with culture medium. For Cl− channel block experiments, ATP was dissolved in distilled water, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) in dimethyl sulfoxide (DMSO), and tamoxifen in methanol, at concentrations of 100, 30, and 50 mM, respectively, and diluted to final concentrations of 10 μM, 100 μM, and 30 μM, respectively, with hypotonic bath solutions. The final concentration of methanol in the tamoxifen solution was 0.06% and DMSO in the NPPB solution was 0.33%. The pH of the final solutions was adjusted to 7.4. All chemicals were purchased from Sigma (Poole; Dorset, UK).

Statistics

Data are expressed as means ± SE (number of observations) and, where appropriate, have been analyzed using Student’s t-test and ANOVA. All experiments were repeated at least three times.

RESULTS

Hypotonic-Activated Cl− Currents in CNE-2Z Cells

The whole cell currents of unsynchronized CNE-2Z cells were recorded. Cells were clamped at 0 mV in the whole cell configuration and stepped from 0, ± 40, and ± 80 mV. As shown in Fig. 1A, currents in response to all voltage pulses were small and stable when the cell was bathed in isotonic solution. However, large currents were activated when the cell was exposed to a 47% hypotonic solution. In most cases, the currents appeared 1–2 min after application of the hypotonic solution, reached a peak in 3–5 min, and remained elevated for the duration of exposure to the hypotonic solution. The increase in current was partially reversible upon return to control (isotonic) condition. The residual swelling-activated current could be eliminated by perfusing the cells with hypertonic (47%) solution (Fig. 1A). The cells swelled apparently under the hypotonic conditions and remained swollen until the bath solution was changed back to isotonic conditions. Similar results were obtained in 30 cells with a mean current of 4.3 ± 0.9 pA/pF under isotonic conditions and 46.8 ± 4.3 pA/pF (peak current) in hypotonic solution at the +80 mV step.

Typical current traces under isotonic and 47% hypotonic conditions are shown in Fig. 1, B and C. The current exhibited an almost linear current-voltage (I-V) relationship; the outward and inward currents in response to ±80 mV were 47 ± 4.3 and -43 ± 4.5 pA/pF (n = 30), respectively. Under the voltage steps of ±40, and ±80 mV, the current exhibited negligible time-dependent inactivation. There was still no obvious time-dependent inactivation at more depolarizing potentials up to +120 mV (data not shown). The I-V relationship demonstrated that the hypotonic-activated current reversed at a voltage close to the calculated EC₅₀ (−0.9 mV), with a mean value of −2.4 ± 1.0 mV (n = 16; Fig. 1D). In these experiments, there was no K⁺ present either in the electrode or bath solutions. The concentrations of Cl⁻ inside and outside the cells were almost equal, giving a value of −0.9 mV for EC₅₀, very close to the experimental reversal potential. The equilibrium potentials for Na⁺ and Ca²⁺ were both predicted to be greater than +200 mV. Thus the data strongly support the hypothesis that the hypotonic-activated current was carried primarily by Cl⁻. This was further confirmed by the anion substitution experiments.

Permeability of Volume-Sensitive Cl⁻ Channel

When the volume-sensitive currents were activated and had reached the peak, the hypotonic solution containing 70 mM of Cl⁻ was replaced with solution containing equimolar I⁻, Br⁻, or gluconate. The anion substitution shifted the reversal potential. The permeability ratios of Pf/PCl, PBr/PCl, and Pgluconate/PCl, calculated from the shifts in reversal potential by using the modified Goldman-Hodgkin-Katz equation (Eq. 1), were 1.16 ± 0.05, 1.13 ± 0.03, and 0.37 ± 0.03 (n = 5), resulting in the sequence of anion permeability of I⁻ > Br⁻ > Cl⁻ > gluconate (Fig. 2).

Pharmacology of the Volume-Sensitive Current

The results above suggest that hypotonic bath solution activated a Cl⁻ current in the CNE-2Z cell. To characterize its properties further, the effect of Cl⁻ channel blockers tamoxifen, NPPB, and ATP on the
current were studied in unsynchronized cells. Cells were exposed to a 47% hypotonic solution to activate Cl⁻ currents. When the currents had reached their peaks, the cells were perfused with hypotonic solution containing the indicated inhibitors tamoxifen, NPPB, or ATP. Figure 3A shows the time course of the inhibitory effects of 10 mM extracellular ATP on the whole cell currents activated by 47% hypotonic solution. Extracellular application of 10 mM ATP almost completely inhibited the volume-activated Cl⁻ currents in a reversible manner. The I-V relationships under isotonic and hypotonic conditions and ATP treatments are shown in Fig. 3B. Both inward and outward currents were suppressed, but the effects of ATP on the currents were slightly voltage dependent. The inhibition of ATP on the outward currents was stronger than that on the inward currents. ATP inhibited 90.1 ± 2.1% of the inward current induced by −80 mV (n = 12, P < 0.01) and 98.0 ± 0.9% of the outward current induced by −80 mV (P < 0.01; Fig. 3G). The difference between them was significant (P < 0.01).

NPPB (100 μM) also reversibly inhibited both the inward and outward components of the volume-sensitive currents (Fig. 3, C and D); 51.1 ± 7.0% of the inward current at −80 mV step and 51.1 ± 7.1% of outward current at −80 mV step were blocked (n = 14; P < 0.01). The results indicated that the effects of NPPB were voltage independent (Fig. 3G).

The effects of tamoxifen on the volume-activated Cl⁻ currents varied greatly among cells. The blockage of the current by 30 μM tamoxifen ranged from 35 to 99%, giving a mean value of 69.3 ± 10% (at −80 mV; n = 12). The remaining current could be inhibited by 10 mM ATP. The typical example of responses is shown in Fig. 3E. Figure 3F represents the relationships between voltages applied and the mean currents of tamoxifen treatments. It seems that the blockage of outward components was slightly stronger than that of inward current (Fig. 3G), but the inhibition of inward current (65.0 ± 8.9%; at −80 mV) was not significantly different from that of outward current (69.3 ± 10%; at −80 mV) (n = 12; P >
VOLUME-ACTIVATED Cl⁻ CURRENT AND CELL CYCLE

Fig. 2. Anion permeability of the volume-activated Cl⁻ channel. When the hypotonic-activated current reached a peak and leveled off, the 47% hypotonic solution containing 70 mM Cl⁻ was substituted by hypotonic solution containing equimolar I⁻, Br⁻, or glucuronate. The permeability ratios (P_X, P_Cl⁻) of various anions (X⁻) relative to that of Cl⁻ were calculated by using the modified Goldman-Hodgkin-Katz equation, P_X/P_Cl⁻ = ([Cl⁻]exp(−ΔVrev/RT) − [Cl⁻]exp(−ΔVrev/RT))/[X⁻], where [Cl⁻]exp, [Cl⁻]exp, and [Cl⁻]exp, are the Cl⁻ concentration in the normal and the substituted bath solutions, [X⁻], is the concentration of the substituted anion, ΔVrev is the difference of the reversal potentials for Cl⁻ and X⁻, F is the Faraday constant, R is the gas constant, and T is the absolute temperature. Data represent means ± SE (n = 5).

0.05). Of those blockers used, ATP was the most effective (Fig. 3G).

In these experiments, NPPB solution contained 0.33% DMSO, and tamoxifen solution contained 0.06% methanol. The effects of DMSO and methanol on the currents were tested. DMSO or methanol at these concentrations did not significantly affect the currents (data not shown).

Expression of Volume-Sensitive Cl⁻ Current in the Cell Cycle

To investigate the expression of the volume-sensitive currents at different stages of the cell cycle, CNE-2Z cells were synchronized by the mitotic shake-off technique and by the double chemical-block technique as described in METHODS. As demonstrated by flow cytometry, highly synchronized cells were obtained (Table 1). In the unsynchronized cells, which were grown in growth medium for 48 h and had reached 80% confluence, 57, 32, and 11% of the population were distributed in G0/G1, S, and G2/M phases, respectively. Among the shake-off cells sampled at 4 h after reincubation in the normal medium, 85% of them were in G0/G1. Both thymidine and hydroxyurea inhibit DNA synthesis and arrest cells on the G1/S border (10). Flow cytometric analysis demonstrated that 86% of the blocked cells had progressed into S phase 4 h after having been released from the block. M-phase cells were distinguishable from the cell population by their condensed chromosomes visible under the phase-contrast microscope.

The Cl⁻ currents activated by 47% hypotonic solution of the shake-off cells sampled at 4 h of incubation, which represented those of G1 phase (85% of cells in G1 phase), were large. The currents showed the properties of linear conductance and negligible time-dependent inactivation (Fig. 4A, X). The mean value was 63.2 ± 2.6 pA/pF at +80 mV and −56.7 ± 2.6 pA/pF at −80 mV (n = 15; Fig. 4B, X). The currents were inhibited by ATP, NPPB, and tamoxifen, similar to those of unsynchronized cells.

To confirm this finding and to validate the mitotic shake-off technique, we used an alternative approach to isolate G1 cells: visual identification of cells as they emerged from M phase. M phase is characterized by the appearance of condensed chromosomes and is clearly visible under phase contrast microscopy (Fig. 5). The nuclear material, which was condensed during metaphase and divides into two in anaphase (Fig. 5, 0 and 5 min), becomes diffuse (Fig. 5, 8 min). The septum dividing the cells becomes more visible (Fig. 5, 18 min), and as the two daughter cells begin to separate their morphology alters to become more spherical (Fig. 5, 25 min). The cells now enter G1 phase. When these newborn G1 cells were exposed to 47% hypotonic solution, the volume-sensitive Cl⁻ current was activated; the outward and inward currents in response to ±80 mV voltage pulses were 59.6 ± 4.8 and −50.1 ± 4.3 pA/pF (n = 7), respectively. These currents were not significantly different from those of the shake-off cells sampled 4 h after reincubation (85% of cells in G1 phase as demonstrated by flow cytometry).

The hypotonic-activated Cl⁻ currents declined dramatically in the shake-off cells sampled at 10 h of incubation, when 71% of cells had reached S phase. The peak current (30.1 ± 3.1 pA/pF at +80 mV; n = 13) was much smaller than that of cells sampled at 4 h (P < 0.01), although the properties of the currents were similar. The results indicated that expression of volume-sensitive channels of cells in S phase was inhibited or the machinery for activation of the channels had been depressed, compared with that of cells in G1 phase.

Downregulated expression of the volume-sensitive currents in S phase was further demonstrated by the results obtained from the cells synchronized by double chemical-block (by 2 mM thymidine and hydroxyurea). As shown in Fig. 4A, Y and 4B, Y, cells sampled at 4 h after having been released from the block expressed a hypotonic-activated Cl⁻ chloride current with properties similar to those of shake-off cells. The mean current was 29.6 ± 2.1 pA/pF at +80 mV (n = 12). It was not significantly different from that of shake-off cells recorded at 8 h of incubation but smaller than that of shake-off cells detected at 4 h of incubation (G1 phase). Flow cytometry analysis demonstrated that the double-block cells were distributed predominately in S phase (86% of the population) at 4 h after release from the block. Only 7% were in G0/G1 and 7% in G2/M phases. As for the cells that had not yet been released from the chemical block (arrested on the border of G1/S), their hypotonic-activated Cl⁻ currents were similar to those of cells sampled at 4 h after having been released from the block, with a mean value of 34.8 ± 1.9 pA/pF (n = 13).

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The chemicals (thymidine and hydroxyurea) used to arrest cells could have direct effects on the volume-activated Cl$^-$ currents. However, the experiments of acute application of thymidine and hydroxyurea excluded this possibility. Addition of these chemicals to the bath, at the same concentration (2 mM) as for arresting cells, did not significantly affect the volume-activated currents.

For the cells in M phase, selected by their condensed chromosomes when viewed under phase-contrast microscopy, currents were also recorded. Perfusing the cells with a 47% hypotonic solution activated a Cl$^-$ current, showing properties similar to that of the shake-off cells and with a peak value of 48.4 ± 4.3 pA/pF at +80 mV ($n=9$; Figs. 4A, Z, 4B, Z). The current was smaller than that of shake-off cells at 4 h ($P<0.01$) but larger than that at 8 h and that of cells released from the double chemical block ($P<0.05$). Figure 4C summarizes the expression of the volume-activated currents of the three groups of cells, shake-off cells at 4 h (nominal G1 phase), chemical-block cells 4 h after release (nominal S phase), and M cells. Figure 4D
Linking I_{Cl,vol} to the Cell Cycle

Block of the volume-activated Cl\(^{-}\) current by NPPB (100 \(\mu\)M) arrested cells in G0/G1 phase. Flow cytometric analysis showed that, in the control group (no additives), 53.5 \(\pm\) 1.2\% (\(n = 4\)) of CNE-2Z cells were distributed in G0/G1 phase of the cell cycle, 36.3 \(\pm\) 1.3\% in S phase, and 10.2 \(\pm\) 0.6\% in G2/M phase, when sampled at 48 h. However, G0/G1 population increased significantly when cells were treated with NPPB (100 \(\mu\)M). Cells in G0/G1 phase increased to 70.5 \(\pm\) 1.3\% (4 experiments, \(P < 0.01\) vs. control), and cells in S phase and G2/M phase decreased to 23.2 \(\pm\) 1.1\% and 6.3 \(\pm\) 0.6\%, respectively, at 48 h after treatment.

DISCUSSION

This study demonstrates that CNE-2Z cells respond to hypotonic shock by activating a volume-sensitive current. Four lines of evidence suggest that the current is carried by Cl\(^{-}\). First, only the Cl\(^{-}\) flow was consistent with the current direction under the applied voltages and the designated pipette and bath solutions. Second, the membrane reversal potential for the current was close to the equilibrium potential for Cl\(^{-}\). Third, substitution of Cl\(^{-}\) with I\(^{-}\), Br\(^{-}\), or gluconate shifted the reversal potential, resulting in a permeability sequence of I\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) > gluconate, consistent with the reported properties of the volume-activated Cl\(^{-}\) current (24, 38). Fourth, the current was inhibited by the conventional Cl\(^{-}\) channel blockers NPPB (see Refs. 18 and 27), tamoxifen (27, 38, 39), and extracellular ATP (11, 36).

The volume-activated Cl\(^{-}\) current has been reported in many cell types and has the characteristics of ATP dependency, voltage sensitivity showing outward rectification and time-dependent inactivation at strong depolarizing potentials (see Refs. 18 and 27). However, the degree of outward rectification and time-dependent inactivation varies among experiments or cell types. It has been reported that ionic strength difference across the plasma membrane affected the time-dependent inactivation (38). Decrease of the ionic strength difference led to a smaller degree of time-dependent inactivation. In our experiments, the absence of significant time-dependent inactivation may result from the experimental conditions of equal ionic strength between bath and pipette solutions. Similar currents have been recorded by our group in pigmented and nonpigmented ciliary epithelial cells (4, 16, 35) under the same recording conditions. However, we cannot exclude the

Table 1. Cell cycle stage distribution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1,%</th>
<th>S,%</th>
<th>G2/M,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57 (\pm) 1.5</td>
<td>32 (\pm) 2.1</td>
<td>11 (\pm) 1.6</td>
</tr>
<tr>
<td>Shake off (4 h)</td>
<td>85 (\pm) 1.3</td>
<td>9 (\pm) 3.6</td>
<td>6 (\pm) 2.7</td>
</tr>
<tr>
<td>Shake off (10 h)</td>
<td>21 (\pm) 1.7</td>
<td>71 (\pm) 1.5</td>
<td>8 (\pm) 1.3</td>
</tr>
<tr>
<td>Chemical block (4 h)</td>
<td>7 (\pm) 0.5</td>
<td>86 (\pm) 1.3</td>
<td>7 (\pm) 1.1</td>
</tr>
</tbody>
</table>

Control cells were unsynchronized cells that were grown in growth medium for 48 h and had reached 80% confluence. Cells were synchronized by a mitotic shake-off technique and by a double chemical-block technique and then incubated in normal growth medium for the times indicated, as described in METHODS. Cell phase was determined by flow cytometry. Data were obtained from 5 experiments and are shown as means \(\pm\) SE.

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This study demonstrates that CNE-2Z cells respond to hypotonic shock by activating a volume-sensitive current. Figure 6B shows the correlation between RVD capacity and volume-activated Cl\(^{-}\) currents. RVD was plotted against the corresponding current (evoked at \(+80\) mV step) of the G1 group (shake-off cells at 4 h of incubation), S-group (chemical block cells at 4 h after release), and M cells (selected under microscope). Fitting the data, by linear regression, resulted in a positive correlation between the two factors (RVD and current), with a linear correlation coefficient \(r = 0.99\) (\(P < 0.01\)).

RVD was measured in the CNE-2Z cells. The data show that the 47% hypotonic solution swelled the cell and activated RVD. Cell volume was recovered by 54 \(\pm\) 5.7\% (\(n = 36\)) in unsynchronized cells. The Cl\(^{-}\) channel blockers tamoxifen, NPPB, and ATP inhibited the RVD by 62 \(\pm\) 5.5\% (\(n = 26\)), 58 \(\pm\) 6.2\% (\(n = 18\)), and 95 \(\pm\) 1.9\% (\(n = 21\)), respectively. Similar to the volume-sensitive Cl\(^{-}\) current, RVD capacity was cell cycle dependent. The cells in G1 phase exhibited the highest, and the cells in S phase the lowest, RVD capacity among the three groups of cells (G1, S, and M) investigated. The RVD capacity was 71.5 \(\pm\) 4.3\% (21 cells in 6 experiments), 34 \(\pm\) 2.9\% (18 cells in 5 experiments), and 56.5 \(\pm\) 3.5\% (19 cells in 5 experiments), respectively, in the G1 group (shake-off cells sampled after 4 h of incubation), S group (chemical-block cells sampled at 4 h after release), and M cells (selected under microscope by their condensed chromosomes).

The relationship between the volume-sensitive Cl\(^{-}\) current and RVD was analyzed by comparing current level and RVD capacity. As shown in Fig. 6A, Cl\(^{-}\) channel blockers NPPB, tamoxifen, and ATP inhibited both the volume-sensitive current and RVD. The percentage of RVD inhibition was a function of current inhibition by NPPB, tamoxifen, and ATP. The inhibition of RVD was linearly correlated with the blockage of the volume-sensitive current (\(r = 0.98; P < 0.01\)). Furthermore, RVD capacity was a function of the volume-activated Cl\(^{-}\) current. Figure 6B shows the correlation between RVD capacity and volume-activated Cl\(^{-}\) currents. RVD was plotted against the corresponding current (evoked at \(+80\) mV step) of the G1 group (shake-off cells at 4 h of incubation), S-group (chemical block cells at 4 h after release), and M cells (selected under microscope). Fitting the data, by linear regression, resulted in a positive correlation between the two factors (RVD and current), with a linear correlation coefficient \(r = 0.99\) (\(P < 0.01\)).
possibility that lack of time-dependent inactivation and outward rectification in the CNE-2Z cell and the differing properties of the currents between cells may arise from differential expression of different types of Cl⁻ channels. It has been reported that there were three types of volume-activated Cl⁻ channels in ciliary epithelial cells and that the expression of these channels was different between the two cell types of the ciliary epithelium (40). Antisense knockdown of MDR1 (35), ClC-3 (36), or pICln (4) gene expression inhibited the volume-activated Cl⁻ currents. All this evidence suggests the presence of more than one volume-activated Cl⁻ channel, although the molecular identity of the volume-activated Cl⁻ channel(s) has not yet been determined (7, 27).

In synchronized cells, the expression of the volume-activated Cl⁻ current was actively modulated during the cell cycle. The shake-off cells, sampled at 4 h after reincubation (85% cells in G1 phase), expressed a high level of the current, but the expression of the current in
cells sampled at 10 h (71% cells in S phase) decreased significantly. The results implied that the expression of the volume-activated Cl\textsuperscript{-}/H\textsubscript{11002} current was high in G\textsubscript{1} phase and then was downregulated in S phase. This was verified further by the results obtained from the double DNA block studies. Cells blocked by thymidine and hydroxyurea and sampled 4 h after having been released from the chemical block possessed low levels of the volume-activated Cl\textsuperscript{-} current. Both thymidine and hydroxyurea block DNA synthesis (a feature of S phase) and thereby prevent cells from progressing into S phase, arresting cells at the G\textsubscript{1}/S border (10). Once released from the block, cells will progress into the S phase. Data showed that 86% of cells were in S phase sampled at 4 h after released from the block. The volume-activated Cl\textsuperscript{-} current was downregulated

Fig. 5. Cell images during division. M-phase cells are distinguished by their condensed chromosomes visible under the phase contrast microscope. In anaphase, the 2 sets of chromosomes begin to pull apart (0 and 5 min) and a septum begins to form between them (8 min). The 2 newly formed daughter cells begin to separate (18 min) and, as they do so, become visible as independent cells (25 min) and their shape becomes more spherical. These newly born cells were taken from patch-clamp studies as representatives of G\textsubscript{1} phase.

Fig. 6. Correlation between volume-activated Cl\textsuperscript{-} currents and regulatory volume decrease (RVD). A: correlation between inhibition of RVD and the Cl\textsuperscript{-} current (at +80 mV) by ATP (10 mM), tamoxifen (30 \textmu M), and NPPB (100 \textmu M). Both RVD and the current were activated by 47% hypotonic bath solution and measured in parallel experiments. B: correlation between RVD and the volume-activated Cl\textsuperscript{-} current at different stages of the cell cycle. Both RVD and the current were measured in 4 groups of cells in parallel. The mean value of RVD was then plotted against that of the currents in corresponding groups. The straight lines in A and B were obtained by fitting the data with a linear regression equation, \( y = b + ax \).
while cells progressed through the G1/S border and into the S phase. The downregulation of expression of a Cl– channel in S phase (30) has also been reported. As the cells progressed into M phase, the expression of the volume-activated Cl– current was upregulated again.

The cell cycle dependent expression of the volume-activated Cl– current suggests that the current may play an important role in cell cycle progress and cell proliferation. It has been reported that inhibition of the current by Cl– channel blockers suppressed cell proliferation and arrested cells in G0/G1 phase (24). Our data confirm this finding. Cells, when exposed to 100 μM NPPB, which blocks around 50% of the volume-activated Cl– current, were arrested in G0/G1 phase. How does the current affect the cell cycle progress and cell proliferation? Our results demonstrate that, as with the volume-activated Cl– current, RVD capacity was also cell cycle dependent, and expression of the volume-activated Cl– current was closely correlated to the RVD capacity during the cell cycle. Both the current and RVD were high in G1, low in S, and upregulated in M phase. Furthermore, the blockage of the current and RVD was positively correlated. This suggests that the volume-activated Cl– current may regulate the cell cycle progress and proliferation via alterations in RVD.

The eukaryotic cell cycle consists of four separate phases: G1, S, G2, and M phases. The nondividing cells exit the cell cycle at G1 phase into either a quiescent (G0) state or a terminally differentiated state. The restriction point, which controls progression from G1 to S, divides the G1 phase into two subphases, the G1 postmitotic phase (G1pm) and the G1 presynthesis phase (G1ps). The high level of the volume-activated Cl– channel activity and RVD in G1 may ensure the concentration of critical factors needed for controlling progression through the restriction point, as suggested by Nilius (17). Having passed through the restriction point, cells can progress into the next phase. However, mature cells must grow before they divide to maintain normal cell size. It is thought that the purpose of the G1ps period is to allow cells to grow so that, later in the cycle, the cell can focus its energy on other cellular processes such as DNA replication in S phase and reorganization of the cellular infrastructure during mitosis (1). It has been demonstrated that the length of G1ps phase varies greatly. After having passed the restriction point, larger cells may proceed almost immediately to the S phase, whereas smaller cells may linger in G1ps for up to 10 h before beginning the transition to the S phase (1). We have observed a remarkable increase in cell size in late G1 phase (data not shown) and the downregulation of the volume-activated Cl– current and RVD at the G1/S border and S phase. The lower level of the current and RVD capacity may facilitate cell growth and help the maintenance of large cell size.

In this study we have also observed the upregulation of the expression of the volume-activated Cl– current and RVD in M phase. It is known that one important cell cycle checkpoint that maintains integrity of the genome occurs toward the end of mitosis. This checkpoint monitors the alignment of chromosomes on the mitotic spindle, thus ensuring that a complete set of chromosomes is distributed accurately to the daughter cells.

The volume-activated Cl– current may not only affect the cell cycle progression via RVD but also interfere with the cell cycle or cell proliferation via undefined mechanisms. The Cl– currents can influence the intracellular pH or the pH in various organelles. The cell proliferation can also be affected by intracellular pH (19a). Thus it has been postulated that the volume-activated Cl– current may affect cell proliferation by changing cellular pH (17).

In conclusion, this study has demonstrated that CNE-2Z cells express volume-activated Cl– currents. The expression of these currents was actively modulated during the cell cycle and closely correlated to the RVD capacity. The results suggest that the current may play an important role in the cell cycle progression and proliferation, but how the change of the current expression affects the cell cycle progression remains to be elucidated.

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