Roles of volume-activated Cl^- currents and regulatory volume decrease in the cell cycle and proliferation in nasopharyngeal carcinoma cells

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Abstract. *Objectives:* Previously it has been shown, that the volume-activated plasma membrane chloride channel is associated with regulatory volume decrease (RVD) of cells and may play an important role in control of cell proliferation. We have demonstrated that both expression of the channel and RVD capacity are actively regulated in the cell cycle. In this study, we aimed to further study the role of the volume-activated chloride current and RVD in cell cycle progression and overall in cell proliferation. *Materials and methods:* Whole-cell currents, RVD, cell cycle distribution, cell proliferation and cell viability were measured or detected with the patch-clamp technique, the cell image analysis technique, flow cytometry, the MTT assay and the trypan blue assay respectively, in nasopharyngeal carcinoma cells (CNE-2Z cells). *Results:* The Cl^- channel blockers, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and tamoxifen, inhibit the volume-activated chloride current, RVD and proliferation of CNE-2Z cells in a dose-dependent manner. Analysis of relationships between the current, RVD and cell proliferation showed that both the current and RVD were positively correlated with cell proliferation. NPPB (100 μM) and tamoxifen (20 μM) did not significantly induce cell death, but inhibited cell proliferation, implying that the blockers may inhibit cell proliferation by affecting cell cycle progression. This was verified by the observation that tamoxifen (20 μM) and NPPB (100 μM) inhibited cell cycle progress and arrested cells at the G_0/G_1 phase boundary. *Conclusions:* Activity of the volume-activated chloride channel is one of the important factors that regulate the passage of cells through the G_1 restriction point and that the Cl^- current associated with RVD plays an important role in cell proliferation.

INTRODUCTION

Cell proliferation is a fundamental property of tissue growth and cell reproduction. Central to cell proliferation is the cell cycle. Continual unchecked proliferation of a particular cell clone forms the basis of oncogenesis. Initiation of mitogenesis by mitogenic agents involves a complex series of molecular events preceding DNA synthesis (Berridge 1995). These events include transient changes in membrane ionic permeabilities and intracellular cation concentrations. It

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has been established that changes in intracellular concentrations of Ca^{2+} , H^+ and K^+ play a critical role in the transition between the quiescent state G_0 or the early G_1 stage and S phase where DNA replication takes place (Berridge 1995; Woodfork *et al.* 1995; Xu *et al.* 1996). Chloride channels have also been reported to be involved in cell proliferation control (Schumacher *et al.* 1995; Voets *et al.* 1995; Phipps *et al.* 1996; Schlichter *et al.* 1996; Pappas & Ritchie 1998; Rouzaire-Dubois & Dubois 1998; Shen *et al.* 2000; Nilius 2001; Wondergem *et al.* 2001; Chen *et al.* 2002b; Wang *et al.* 2002a; Zheng *et al.* 2003; Jiang *et al.* 2004; Wang *et al.* 2004).

Based on their gating mechanisms, there are five classes of chloride channel, including volume-activated chloride channels (Nilius & Droogmans 2003). An apparently ubiquitous response to swelling in vertebrate cells is the activation of a volume-sensitive Cl^- current (Strange *et al.* 1996; Okada 1997; Lang *et al.* 1998; Wang *et al.* 2000). The outflow of Cl^- ions through the Cl^- channel and of K^+ through a separate channel leads to a decrease in cell volume, named regulatory volume decrease (RVD). Our previous work demonstrates that expression of the volume-activated chloride currents and RVD capacity are cell cycle dependent (Chen *et al.* 2002b; Wang *et al.* 2002b). Both RVD capacity and the current level change significantly when cells progress through the cell cycle suggesting that the volume-activated chloride current and RVD may play important roles in modulation or control of cell cycle progression. Modulation of the chloride current and RVD may interfere with the cell cycle or even stop its progress. Involvement of volume-activated chloride currents in cell proliferation has been reported from other laboratories (Voets *et al.* 1995; Shen *et al.* 2000; Wondergem *et al.* 2001); however, the relationships between volume-activated chloride currents, RVD, the cell cycle and cell proliferation have not been well defined.

In this study, the role of the volume-activated chloride current and RVD in cell cycle progression, and overall in cell proliferation, have been considered and the relationship between these parameters has been investigated.

MATERIALS AND METHODS

Cell cultures

Cells of the poorly differentiated nasopharyngeal carcinoma (CNE-2Z) were routinely seeded at a density of $1.3 \times 10^4/\text{cm}^2$ and were grown in 25-cm² plastic tissue culture flasks in RPMI 1640 medium with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 (Chen *et al.* 2002a; Mao *et al.* 2005). They were subcultured every 2 days.

Preparation of cells for volume measurements and current recordings

Cells cultured in the flasks were trypsinized, centrifuged and re-suspended in RPMI 1640 medium with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells of the suspension were plated on round coverslips 22 mm diameter (150 µl/coverslip), which were located in 35-mm tissue culture dishes, and were then incubated at 37 °C for 2–3 h before volume regulation experiments or current recordings were taken.

Cell cycle monitoring by flow cytometry

In this study, the proportion of cells in G_0/G_1 , S and G_2/M phases was obtained by analysing cells for their different DNA content based on propidium iodide (PI) staining (Abu-Absi & Srienc 2000; Chen *et al.* 2002a). Cells were collected, washed twice with phosphate-buffered saline (PBS) and were fixed in 70% ethanol at –20 °C for 30 min. Before analysis, they were pelleted from

the ethanol solution, washed with PBS twice, re-suspended in 0.5 ml of RNase A (50 µg/ml in PBS) and were incubated at 37 °C for 30 min. PI from a stock of 1 mg/ml of distilled water was added to the cell suspension, to a final concentration of 50 µg/ml. The cells were stained for 15 min and then were analysed by flow cytometry with system software (EPICS XL, Coulter Co., Hialeah, FL, USA). For each cell population, 10 000–20 000 cells were analysed. Data were deconvoluted mathematically by the flow cytometer system software and the percentage of cells in each cell cycle phase was quantified. Flow cytometric analysis was performed in at least three independent experiments of each group.

Volume measurements

Cell volume was measured in the method described previously (Wang *et al.* 2002b; He *et al.* 2004). Coverslips with the prepared cells were stuck on the base of the recording chamber with non-melting hydrocarbon multipurpose grease and thus mounted on an inverted Leica microscope stage (Leitz DMIL; Leica Mikroskopie und Systemen GmbH, Wetzlar, Germany). The bath with a volume of 0.5 ml was perfused continually by solutions with a flow rate of 4 ml/min. All experiments were carried out at room temperature (20–24 °C). Cell images were captured every 30 s by a CCD digital camera (Mono CCD625, Leica, Germany) and were stored directly onto the computer. Each image was then analysed by Quantimet Q500MC image processor and analysis software (Leica, Germany). Cell volume was calculated using the equations of $V = 4/3 \times S \times (S/\pi)^{1/2}$, where S is the area (µm²). The regulatory volume decrease (RVD) was calculated with the equation of $RVD (\%) = (V_{\max} - V_{\min}) \div (V_{\max} - V_0) \times 100\%$, where V_0 is the cell volume in isotonic solution before hypotonic shock, $V_{\max}$ is the peak volume in hypotonic solution, $V_{\min}$ is the volume before return to isotonic solution.

Whole cell current recording

Whole cell currents of single CNE-2Z cells were recorded using the patch clamp technique (Wang *et al.* 1998; Chen *et al.* 1999) with a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). The patch-clamp pipettes were made from standard wall borosilicate glass capillaries with an inner filament on a two-stage vertical puller and gave a resistance of 4–6 MΩ when filled with the pipette solution. Electrode and whole cell capacitance were determined by adjusting and minimizing capacity transients in response to a 20-mV voltage step using the amplifier functions, following instructions of the amplifier manual. Once the whole cell configuration was established, cells were held at the chloride equilibrium potential (0 mV), and then were stepped repeatedly for 200 ms pulses to 0, ±40 and ±80 mV, with 4 s interval between steps. Command voltages and whole cell currents were recorded simultaneously by computer via a laboratory interface (CED 1401, Cambridge, UK) with a sampling rate of 3 kHz. Voltage pulse generation, data collection and current analysis were performed by the computer using the EPC software package (CED, Cambridge, UK). In analysis of data collected, all current measurements were made at 10 ms after onset of each voltage step. Cell size was monitored by the image system during experiments, but this was not used to monitor RVD (RVD was measured in separate experiments). Experiments were carried out at room temperature (20–24 °C).

Evaluation of cell proliferation

Cell proliferation was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Plumb *et al.* 1989). CNE-2Z cells were cultured in 96-well culture plates for 24 h (seeded at a density of 2500 cells/well), with culture volume of 100 µl/well. The culture medium was removed and replaced with 100 µl of fresh culture medium without (control) or with chloride channel blockers, NPPB and tamoxifen, or with solvents (used for preparing

tamoxifen and NPPB; solvent control) methanol or dimethyl sulphoxide (DMSO), respectively. Cells were then cultured for 72 h. Culture media with or without various blockers were renewed at 48 h. Cell proliferation was detected daily. The stock solution of MTT (5 mg/ml) was added to each well-being assayed to equal one tenth the original culture volume (10 µl MTT stock solution added to a 100 µl culture volume/well), and were incubated at 37 °C for 4 h (for converting MTT into dark blue, water insoluble formazan by mitochondrial dehydrogenase enzymes in the cells). Medium was removed, 100 µl of 0.1 N HCl isopropanol was added to each well and the plates were shaken to uniformly solubilize the formazan. Absorbance (expressed in optical density, OD) of each well at 570 nm, was measured using an automated plate reader (Labsystems multiskan MS, type 352, Finland). Plates were read within 30 min of adding acidified isopropanol. For the cell proliferation assay, eight culture wells were seeded for each group in one experiment and this was repeated at least three times.

Detection of cell viability

Cell viability was detected by the trypan blue assay. The method is based on the principle that live cells do not take up the dye, whereas dead cells do. Cells ready for detection were collected and suspended in PBS. Cell suspension (0.2 ml) was mixed with 0.5 ml of 0.4% trypan blue (in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic) and 0.3 ml of PBS and were stained for 10 min. Stained and unstained cells were counted with a haemocytometer. Cell viability (%) was calculated with the equation, cell viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100.

Solutions and chemicals

The solution in patch-clamp pipettes contained (in mM): 70 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1.2 MgCl₂, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 EGTA, 140 D-mannitol and 2 ATP. The isotonic bath solution contained (in mM): 70 NaCl, 0.5 MgCl₂, 2 CaCl₂, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 140 D-mannitol. Osmolarity in the pipette and isotonic bath solutions was measured by depression of freezing point using an osmometer (Osmomat 30, Gonotec, Berlin, Germany) and was adjusted to 300 mosmol/l with D-mannitol. The 47% hypotonic bath solution was obtained by omitting D-mannitol from the solution, giving an osmolarity of 160 mosmol/l (47% hypotonicity, compared to the isotonic solution). pH of the pipette and bath solutions was adjusted to 7.25 and 7.4, respectively, with Tris-base. NPPB and tamoxifen were dissolved with DMSO and methanol in concentrations of 100 mM and 50 mM, respectively. They were diluted to the indicated final concentrations using corresponding solutions for different experiments. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Statistics

Data were expressed as the mean ± standard error (number of observations). A analysis of variance and Student's *t*-test (SPSS version 11.5; Chicago, IL, USA) were used to analyse the data and differences were considered significant at $P < 0.05$. All experiments were repeated at least three times.

RESULTS

Inhibition of CNE-2Z cell proliferation by Cl⁻ channel blockers

The chloride channel blocker, NPPB, had been applied in three concentrations (50, 100 and 200 µM). Results indicated that cell proliferation was inhibited by NPPB in a dose-dependent

Figure 1. Inhibition of CNE-2Z cell proliferation by NPPB and tamoxifen. Cells were plated at a density of 2500 cells/well in 96-well culture plates and were cultured for 24 h. They were then cultured in medium without (control) or with indicated concentrations of the chloride channel blockers NPPB and tamoxifen for 3 days. The OD (optical density) value, which represented relative cell number, was determined daily using the MTT method (a and c). Percentage inhibition was obtained by comparing OD value of the culture treated with blockers for 3 days to that of the control culture (b and d). Data in (b) and (d) were then fitted by the equation $y = y_0 + (a - y_0)/(1 + (x/x_0)^b)$. Each point in the figures represents the means $\pm$ standard error of three experiments.

manner. Figure 1a shows the time-dependent changes of relative cell number, which is expressed as the OD (optical density) value. In the control culture, into which no additives were contributed, cell numbers grew exponentially in the time period observed. However, in the cultures to which NPPB was added, cell population growth was significantly inhibited, effects of NPPB detectable 24 h after treatments. As treatment time progressed, suppression of cell population growth became more obvious. Furthermore, inhibition was dose-dependent (Fig. 1b). On the third day (72 h) after NPPB treatment, cell proliferation was suppressed by $8.5 \pm 5.4\%$, $51.0 \pm 3.5\%$ and $100 \pm 1.6\%$ (in three experiments), when 50 μM , 100 μM or 200 μM NPPB was added to the cultures, respectively. The IC_{50} (the inhibition concentration at which the inhibition was 50%) in 72 h was 98 μM .

Proliferation of CNE-2Z cells was also inhibited by the chloride channel blocker, tamoxifen, in a dose-dependent manner. Compared to control culture (no additives), in which cell population expanded exponentially over the 3 days of observation, cell number growth rate was significantly slowed in cells treated with tamoxifen (Fig. 1c). Cell proliferation was blocked by $16.7 \pm 9.8\%$, $73.4 \pm 5.7\%$ or $88.0 \pm 3.6\%$ at 72 h, when 10 μM , 20 μM or 30 μM of tamoxifen was added to the cultures, respectively. Figure 1d presents the relationship between percentage inhibition of

Figure 2. Effects of chloride channel blockers on CNE-2Z cell viability. CNE-2Z cells were prepared as described in Fig. 1 and then were treated with chloride channel blockers, tamoxifen and NPPB, for 3 days. Cell viability was assessed daily by trypan blue assay. Each point in the curves represents the means $\pm$ standard error (three experiments).

cell proliferation and concentration of tamoxifen in the medium. The IC_{50} of tamoxifen was 15.6 μ M.

In the experiments, DMSO and methanol were used to prepare NPPB and tamoxifen solutions. Highest concentrations of DMSO and methanol in test solutions were 0.2% and 0.06%, respectively. To exclude the possibility that inhibition of proliferation was caused by DMSO or methanol, their effects on cell proliferation were tested. Results demonstrated that DMSO (0.2%) and methanol (0.1%) did not significantly affect cell proliferation.

Effects of Cl^- channel blockers on cell viability

As described above, chloride channel inhibitors significantly reduced the proliferation of CNE-2Z cells. Inhibition of cell proliferation could be caused by interference with cell cycle progression, but it could also be the result of decreased cell viability. Therefore, the viability of control cells and cells treated with chloride channel blockers was examined with the trypan blue assay.

Results showed that cell viability was high in the control group during the period of culture (72 h). Cell viability was $97.5 \pm 0.2\%$ ($n = 8$, in three experiments) when sampled at 72 h. In cells treated with different chloride channel blockers, cell viability varied between blockers and between concentrations applied.

Tamoxifen did not significantly affect cell viability when applied in the concentrations of 10–20 μ M (Fig. 2a). When tamoxifen concentration was increased to 30 μ M, cell viability declined slightly from $97.5\% \pm 0.2\%$ (control) to $91 \pm 0.2\%$ ($n = 8$, $P < 0.01$). For cells treated with 50 and 100 μ M of NPPB, cell viability was not significantly different from that of those in the control culture, at all time points examined (at 24, 48 and 72 h after treatment). At a higher concentration (200 μ M), NPPB decreased cell viability slightly (from $97.5 \pm 0.2\%$ to $92.5 \pm 0.1\%$ at 72 h after treatment, $n = 8$, $P < 0.01$) (Fig. 2b). The results indicate that chloride channel blockers inhibit cell number population growth rather than killing cells at low concentrations, but at high concentrations the blockers can cause cell death.

Arrest of cell cycle progression by Cl^- channel inhibitors

Nasopharyngeal carcinoma cells had been plated at a density of 2.5×10^5 cells per 25 cm^2 culture flask and cultured for 24 h. They were then incubated in the medium without (control) or with

Figure 3. Effects of chloride channel blockers on the distribution of cell cycle phases. Cells were cultured in medium (a) without (control) or with chloride channel blockers (b) 100 μ M NPPB or (c) 20 μ M tamoxifen for 2 days. Flow cytometric analysis was used to detect distribution of cells in different cell cycle phases. First peak in histograms represents cells in G_0 and G_1 phases. The second peak represents the cells in G_2 and M phases. Between are cells in S phase. Note the increase of cells at G_0/G_1 state and the decrease of cells in S and M phases in (b) and (c).

Table 1. Distribution of cell cycle stages of CNE-2Z cells

Treatment	24 h			48 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	M
Control	46.1 ± 1.8	41.1 ± 1.5	12.8 ± 0.5	53.5 ± 1.2	36.3 ± 1.3	10.2 ± 0.6
NPPB	$59.0 \pm 1.6^{**}$	$31.5 \pm 1.2^{**}$	$9.5 \pm 1.3^*$	$73.2 \pm 1.5^{**}$	$23.2 \pm 1.1^{**}$	$6.3 \pm 0.6^{**}$
Tamoxifen	$59.8 \pm 1.3^{**}$	$28.1 \pm 1.1^{**}$	12.1 ± 1.8	$72.5 \pm 2.3^{**}$	$21.3 \pm 1.6^{**}$	$6.2 \pm 0.4^{**}$

CNE-2Z cells were plated at a density of 2.5×10^5 per 25 cm^2 culture flask and were cultured for 24 h. Cells were then incubated in medium without (control) or with chloride channel blockers, NPPB (100 μ M) or tamoxifen (20 μ M). Flow cytometric analysis was carried out at 24 h and 48 h after treatments. Data are shown as percentage of total number of cells analysed and are the mean $\pm$ standard error of five experiments. $^*P < 0.05$; $^{**}P < 0.01$ (versus control).

chloride channel blockers, at concentrations that could significantly inhibit cell proliferation but did not affect cell viability. The applied concentrations of tamoxifen and NPPB were 20 μ M and 100 μ M, respectively. Flow cytometric analysis had been carried out 24 h and 48 h after treatments.

Results are presented in Fig. 3 and Table 1. The data show that in the control group (no additives), $46.1 \pm 1.8\%$ ($n = 4$) and $53.5 \pm 1.2\%$ ($n = 4$) of CNE-2Z cells were distributed in G_0/G_1 phase of the cell cycle when sampled at 24 h and 48 h, respectively. However, the G_0/G_1 cell population increased significantly when cells were treated with tamoxifen and NPPB. In the NPPB group, cells in G_0/G_1 increased in number to $59.0 \pm 1.6\%$ ($n = 4$, $P < 0.01$, versus control) and to $73.2 \pm 1.5\%$ ($n = 5$, $P < 0.01$, versus control) at 24 and 48 h after treatment, respectively. Paralleling the increasing number of cells at G_0/G_1 phase, there were less cells in S and G_2/M . Compared to the control group, changes of cell distribution at various stages were significant.

Effects of tamoxifen on cell cycle progression were similar to those of NPPB. Most of the cell population arrested significantly in G_0/G_1 ($59.8 \pm 1.3\%$ at 24 h and $72.5 \pm 2.3\%$ at 48 h; $P < 0.01$, versus corresponding control value) (Table 1 and Fig. 3c).

Figure 4. Inhibition of volume-activated chloride currents by NPPB. Typical whole cell currents for CNE-2Z cells are presented in (a) and (b). The cell was held at 0 mV and then was stepped in 200 ms pulses to 0, ± 40 and ± 80 mV with an interval of 4 s between voltage pulses. Exposure of the cell to 47% hypotonic solution activated a volume-sensitive chloride current (a) that was inhibited by extracellular application of 200 μM NPPB (b). Figure 4c shows dose-dependent inhibition of the current (at +80 mV). Figure 4d shows correlation between cell proliferation and volume-activated chloride current. Data in the figures represent the mean $\pm$ standard error of six to eight experiments.

Relationship between volume-activated Cl^- current and cell proliferation

The preceding results indicated that chloride channel blockers, tamoxifen and NPPB, inhibited proliferation of CNE-2Z cells. The results suggest that the volume-activated chloride current is involved in cell proliferation. To study this further, we investigated the effect of the chloride channel blockers on the current, and we analysed the relationship between blockage of current and inhibition of cell proliferation.

Exposure to 47% hypotonic solution (see Materials and methods) activated a volume-sensitive chloride current in CNE-2Z cells. Extracellular application of chloride channel blocker NPPB inhibited the current in a reversible and dose-dependent manner. Figure 4 shows a typical sample of hypotonic-activated chloride current (at 0, ± 40 and ± 80 mV; 4a) and inhibition of the current by 200 μM NPPB (4b). The results indicate that effects of NPPB are voltage-independent. NPPB inhibited both inwards and outwards components of the volume-activated currents. Figure 4c reveals the dose-dependent inhibition of volume chloride current by NPPB with an IC_{50} of 96.9 μM .

Extracellular application of chloride channel inhibitor, tamoxifen, also suppressed chloride currents induced by the 47% hypotonic challenge, at all the voltage steps applied (0, ± 40 and ± 80 mV). A typical example of responses is shown in Fig. 5a and b. Similar to NPPB, inhibition

Figure 5. Relationship between volume-activated Cl^- current and cell proliferation. A typical sample of volume-activated chloride current (at 0, ± 40 and ± 80 mV) induced by 47% hypotonic solution (a) and, inhibition of the current by 30 μM of tamoxifen (b). (c) Dose-dependent inhibition of the current (at +80 mV) by tamoxifen. (d) Correlation between cell proliferation and volume-activated chloride current. Data in the figures represent the means $\pm$ standard error of 6–10 experiments.

of volume-activated chloride currents by tamoxifen was voltage-independent, reversible, and was dose-dependent ($\text{IC}_{50} = 16.9 \mu\text{M}$, Fig. 5c).

To analyse the relationships between volume-activated chloride current and cell proliferation, percentage inhibition of cell proliferation (measured at 72 h after treatments) was plotted against the corresponding percentage blockage of volume-activated chloride current (Figs 4d and 5d). Analysis of the data indicated that cell proliferation was positively correlated to volume-activated chloride current ($r = 0.99$, $P < 0.01$). A similar correlation was obtained after omitting the data from the highest concentration of NPPB (200 μM) or tamoxifen (30 μM), in which cell viability was significantly decreased.

Relationship between RVD and cell proliferation

It is well recognized that cell volume increases significantly during the cell cycle. This implies that cell volume regulation mechanisms may be involved in control of the cell cycle and cell proliferation.

Our data indicated that under isotonic conditions, cell volume was stable. When exposed to a 47% hypotonic challenge, cell volume increased significantly. However, this initial swelling could not be maintained. The continuous hypotonic challenge activated a regulatory volume decrease (RVD). After the initial swelling, cell volume declined significantly while cells were

Figure 6. Correlation between cell proliferation and RVD. Figure 6a and b show dose-dependent inhibition of RVD induced by 47% hypotonic stimulation by chloride channel blockers, NPPB and tamoxifen, respectively. (c and d) Relationship between RVD and cell proliferation. Percentage inhibition of cell proliferation (measured at 72 h after blocker treatments) plotted against corresponding percentage blockage of RVD. Data indicate that CNE-2Z cell proliferation is linearly correlated to the capacity of RVD.

still bathed in hypotonic solution; cell volume recovered by $52.8 \pm 5.6\%$ ($P < 0.01$) in 20 min. The RVD could be inhibited by extracellular application of the chloride channel blockers, NPPB and tamoxifen. Including the blockers in bath solutions prevented the appearance of RVD or significantly attenuated the RVD response. NPPB at 50, 100 and 200 μM inhibited RVD by $15.7 \pm 2.5\%$ ($n = 25$, $P < 0.01$), $57.1 \pm 3.1\%$ ($n = 28$, $P < 0.01$) and $90.1 \pm 3.5\%$ ($n = 35$, $P < 0.01$), respectively. Tamoxifen at 10, 20 and 30 μM inhibited RVD by $22.7 \pm 2.5\%$ ($n = 30$, $P < 0.01$), $61.2 \pm 3.5\%$ ($n = 29$, $P < 0.01$) and $75.4 \pm 4.5\%$ ($n = 32$, $P < 0.01$), respectively. Fitting the data to the curve provided an IC_{50} of 92.2 μM for NPPB (Fig. 6a) and 15.9 μM for tamoxifen (Fig. 6b).

Comparing inhibition of RVD with inhibition of cell proliferation by NPPB and tamoxifen (Figs 6c and 6d) indicated that cell proliferation was correlated with RVD ($r = 0.99$, $P < 0.01$). A similar correlation was obtained after omitting the data from the highest concentration of NPPB (200 μM) or tamoxifen (30 μM), in which cell viability significantly decreased.

In this study, responses in the absence and presence of chloride channel blockers were measured in the same individual cells. Control experiments were also carried out by exposure of cells to identical hypotonic challenge twice in the absence of blockers. There was no significant difference between the two responses in the control group.

DISCUSSION

Volume-activated chloride currents, RVD and cell proliferation

The volume-activated chloride current has been reported to be expressed in many cell types and is involved in many cell functions, including cell volume regulation (Lang *et al.* 1998; Jentsch *et al.* 2002; Nilius & Droogmans 2003). We have previously reported that the volume-sensitive chloride current was detectable under isotonic conditions (Chen *et al.* 2002b; Sun *et al.* 2005) when addition of Cl^- channel blockers inhibited the current and increased basal cell volume. The results indicated that some volume-sensitive chloride channels are open under this physiological condition and are involved in the regulation of basal cell volume. An association of volume-activated chloride current with cell proliferation has been reported (Voets *et al.* 1995; Shen *et al.* 2000; Wondergem *et al.* 2001) but how the current affects cell proliferation is not clear.

In this study, an association between volume-activated chloride current and cell proliferation has been demonstrated. Treating CNE-2Z cells with chloride channel blockers, tamoxifen and NPPB, inhibited cell proliferation. Tamoxifen was originally classified as an anti-oestrogen and has been widely used in anticancer treatment. In recent years, tamoxifen has been demonstrated to be a potent blocker of the volume-activated chloride channel (Wu *et al.* 1996; Mitchell *et al.* 1997; Shen *et al.* 2000; Wondergem *et al.* 2001). In this study, tamoxifen was shown to inhibit the volume-activated chloride current, RVD and cell proliferation, as results suggest. This was further demonstrated by the effects of NPPB; NPPB is a conventional chloride channel blocker. Similar to tamoxifen, it was shown in this study that NPPB suppressed RVD, the volume-activated chloride current and cell proliferation in CNE-2Z cells, but suppression of the three measured variables by 200 μM NPPB was significantly greater than that by 30 μM tamoxifen.

Comparing inhibition of cell proliferation to inhibition of volume-activated chloride current or that of RVD indicates that cell proliferation proceeds as a function of the current; cell proliferation is positively correlated with the level of volume-activated chloride current or RVD. Furthermore, the IC_{50} values for NPPB and tamoxifen determined in this study are remarkably similar for each of the three measured variables, indicating that inhibition of RVD and of cell proliferation are most likely to be a direct consequence of inhibition of volume-activated Cl^- current, and that a certain amount of current and RVD are required for maintaining normal cell volume and proliferation.

Regulatory volume decrease associated with channel- and transport-mediated ion fluxes are known to be sensitive to temperature (Souza & Boyle 2001; Ernest *et al.* 2005). Energy-dependent transporters typically show much larger temperature dependence than channel-mediated diffusion (Ernest *et al.* 2005). We have demonstrated previously that outflow of Cl^- during RVD was mainly through chloride channels in CNE-2Z cells (Wang *et al.* 2002b). In this study, experiments to monitor changes in cell volume and chloride currents were mainly carried out at room temperature (20–24 °C). To observe the effect of temperature on RVD and currents, we carried out experiments at different temperatures. It was shown that the responses at room temperature were similar to those at 37 °C in CNE-2Z cells (data not shown), consistent with those in ciliary epithelial cells (Walker *et al.* 1999), corneal epithelial cells (Capó-Aponte *et al.* 2005) and glioma cells (Ernest *et al.* 2005).

Cell viability, cell cycle and cell proliferation

As discussed above, chloride channel blockers inhibited the volume-activated chloride current, RVD and cell proliferation, suggesting a correlation between these processes. How did the inhibition of volume-activated chloride current and RVD interfere with the cell proliferation in

CNE-2Z cells? Cell proliferation could be inhibited by decreasing cell viability (or killing cells) or by arresting cell cycle progression.

In this study, cell viability was determined by the trypan blue assay. When cells were treated with 50–100 μM NPPB or 10–20 μM tamoxifen, cell viability was similar to that of control cultures, but the cell proliferation rate had decreased significantly. This indicated that decline of cell growth was not due to change of cell viability at these concentrations. At higher concentrations, NPPB and tamoxifen both blocked cell proliferation almost completely, while cell viability decreased slightly. This suggests that decrease of cell viability could be one of the factors that affect cell proliferation in these cases. However, how the chloride channel blockers affected the cell viability was unclear. It has been reported that tamoxifen could induce cell apoptosis (Cameron *et al.* 1997; El Etreby *et al.* 2000; Mandlekar & Kong 2001).

The chloride channel blockers, in low concentrations, did not alter viability of CNE-2Z cells, but decreased the level of cell proliferation. This suggests that the blockers could affect cell proliferation *via* mechanisms other than killing the cells. Modulation of cell cycle progress can alter cell proliferation. In this study, cell cycle progression was monitored by flow cytometry. The results showed that it was interfered with by chloride channel blockers, tamoxifen and NPPB. Tamoxifen and NPPB arrested cells in the G_0/G_1 boundary (cells distributed in G_0/G_1 phase increased significantly). The results indicate that chloride channel blockers could suppress cell population growth by interfering with cell cycle progression. We have previously demonstrated that both the expression of volume-activated chloride currents and RVD capacity are cell cycle dependent in CNE-2Z cells (Chen *et al.* 2002b; Wang *et al.* 2002b). Expression of the currents and RVD capacity are high in G_1 , lower in S and elevated again during mitosis. This suggests that both volume-activated chloride currents and RVD are well regulated during the cell cycle. High expression of volume-activated chloride channels and high RVD capacity in G_1 are required for cells to pass through the G_0/G_1 restriction point. We have also demonstrated that the sensitivity of cells to chloride channel inhibitors changes during the cell cycle. G_1 cells were found to be more sensitive to tamoxifen than S cells (Luo *et al.* 2007) raising the possibility that the properties of chloride channels are modified in the cell cycle.

Possible mechanisms of regulating cell cycle progression and proliferation by volume-activated chloride currents and RVD

As shown above, chloride channel blockers inhibited the volume activated chloride current and cell cycle progression. How current is involved in control or regulation of cell cycle progression is, at present, not clearly understood.

The volume-activated chloride current may help cells to pass the restriction point in G_1 phase. Volume-activated chloride current or RVD may serve as one of the effective means of maintaining proper concentrations of cyclin/cyclin dependent kinase (CDK) and other important factors needed for regulating cell cycle progression (Nilis 2001). Activation of the current allows cells to conduct RVD. Outflow of water following ion efflux may be necessary for maintaining high concentration of critical elements required for controlling progress through the restriction point.

The activated chloride channel may work as a pathway for some important substrates, needed for control of the cell cycle, to enter cells. Volume-activated chloride channels are highly permeable to some organic molecules, for example, some amino acids (Baumgarten & Feher 2001). It has been demonstrated that diverse substrates can be transported into malaria-infected erythrocytes *via* a pathway showing functional characteristics of a chloride channel (Kirk *et al.* 1994). These substrates may be important for synthesizing cyclin/CDKs and other compounds needed for controlling cell cycle progression or needed for cell population increase.

Volume-activated chloride currents may modulate cell cycle progression *via* cell messengers. The efflux of Cl^- may alter intracellular and extracellular Cl^- concentrations that may affect the activities of further ion channels or transporters and thus affect cell cycle progression and cell proliferation. In addition, as mentioned above, open chloride channels may allow entry or exit of special molecules. Such entities could then affect progression of the cell cycle and cell proliferation. It has been reported that cell swelling induces ATP release from cells during the RVD process (Hazama *et al.* 1998; Okada *et al.* 2001; Hisadome *et al.* 2002; Nilius & Droogmans 2003). ATP could activate further channels or could act on a purinergic receptor to induce Ca^{2+} release from intracellular stores, which in turn could affect cell functions.

The volume-activated chloride current may be involved in control of cell proliferation *via* RVD; RVD plays key roles in regulation of cell population size. This has been demonstrated to be one of the important factors that regulate cell cycle progression and cell proliferation (Abu-Absi & Srienc 2000; Cooper 2000; Rouzaire-Dubois *et al.* 2000), and RVD is cell cycle dependent (Wang *et al.* 2002b). Cells increase in volumes throughout inter-phase, with most dividing cells doubling in size between one mitosis and the next. Mitogen-induced swelling may be necessary to provide the intracellular space needed to accommodate newly synthesized macromolecules and organelles, and the increasing cell volume itself may produce membrane changes that serve as positive feedback signals inducing further proliferative activity. In yeast, cell size is one of the key factors controlling cell cycle progression (Cooper 2000). In mammalian cells, cell size affects the length of the G_1 phase (Abu-Absi & Srienc 2000) and inhibition of RVD as a consequence of inhibition of chloride currents would facilitate cell swelling, which might be expected to increase proliferation by accelerating cells through the cell cycle. However, suppression of RVD by chloride channel blockers inhibited cell proliferation in this study. How can this contradiction be explained?

It is known that the restriction point, which controls progression from G_1 to S, divides the G_1 phase into two sub-phases, G_1 post-mitotic phase ($G_{1\text{pm}}$) and G_1 pre-synthetic phase ($G_{1\text{ps}}$). The length of $G_{1\text{ps}}$ varies greatly; after having passed the restriction point, larger cells may proceed almost immediately into S, while smaller cells may linger in $G_{1\text{ps}}$ for up to 10 h before beginning the transition into the S (Abu-Absi & Srienc 2000). These facts indicate that increase in cell volume facilitates cell progress into S from $G_{1\text{ps}}$. We have observed a remarkable increase in cell size in late G_1 phase, down-regulation of volume-activated chloride current and RVD at the G_1 /S border and S phase and have postulated that the volume set point rises significantly, resulting in deactivation of RVD mechanisms and dramatic increase of cell volume. However, before the $G_{1\text{ps}}$ -S transition, cells must pass through the restriction point to allow $G_{1\text{pm}}$ - $G_{1\text{ps}}$ transition. We have shown previously that RVD capacity is high in G_1 phase and it is suggested that RVD mechanisms could be important in maintaining levels of intracellular compounds that are required for cells to pass through the restriction point (Wang *et al.* 2002b). Once RVD is blocked by chloride channel blockers, cells may stop at $G_{1\text{pm}}$.

Activation of chloride channels may affect cell proliferation by changing intracellular pH. Cell swelling leads to cytosolic acidification (Hallows *et al.* 1994; Lang *et al.* 1998; Shen *et al.* 2002), which has been explained by the exit of HCO_3^- through the volume-activated chloride channels (Weiss & Lang 1992), by release of H^+ from acidic intracellular compartments (Lang *et al.* 1998), and by enhancement of $\text{Cl}^-/\text{HCO}_3^-$ exchange due to decreasing intracellular Cl^- activity (Livne & Hoffmann 1990).

In conclusion, this study has demonstrated that chloride channel blockers inhibited volume-activated chloride currents and RVD, arrested cell cycle progression and suppressed cell proliferation. There was positive correlation between the current, RVD and cell proliferation. The results indicate that current and RVD play important roles in the cell cycle progression and in cell proliferation control.

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