Blockage of Volume-Activated Chloride Channels Inhibits Migration of Nasopharyngeal Carcinoma Cells

Jianwen Mao¹, Liwei Wang², Aihui Fan³, Jianhong Wang¹, Bin Xu¹, Tim J.C. Jacob⁴ and Lixin Chen²

¹Institute of Basic Medical Sciences, Guangdong Pharmaceutical University, ²Department of Physiology, Medical College, Jinan University, ³Laboratory of Cell Biology, Guangdong Medical College, ⁴Cardiff School of Biosciences, Cardiff University

Key Words

Chloride Channels • Cell Migration • Chloride Channel Blockers • Volume Regulation • Transwell Chamber

Abstract

Cell migration is crucial for tumor metastasis. Membrane ion channels may play a major role in tumor cell migration because the cells must undergo changes in shape and volume during migration. In the present study, we used the transwell migration assay, an in vitro model for cell migration, and the patch-clamp technique to investigate the role of the volume-activated Cl⁻ current ($I_{cl,vol}$) in the regulation of the migration of nasopharyngeal carcinoma CNE-2Z cells. 5-Nitro-2- (3-phenylpropylamino) benzoic acid (NPPB) inhibited the $I_{cl,vol}$ and the migration of CNE-2Z cells with almost identical dose-dependent pattern (IC_{50} of 98.1 μ M and 97.7 μ M for $I_{cl,vol}$ and cell migration, respectively). Extracellular adenosine triphosphate (ATP) also showed similar dose-dependent inhibitory effects on the currents and migration (IC_{50} of 1.07mM, and 1.11mM for $I_{cl,vol}$ and cell migration, respectively). Hypotonic treatments, which activated $I_{cl,vol}$, increased cell migration. Exposure to hypertonic solutions, which was shown to suppress

$I_{cl,vol}$, inhibited cell migration. Replacement of Cl⁻ with gluconate, which is relatively chloride channel-impermeable, impaired cell migration, whereas substitution of Cl⁻ by I⁻ and Br⁻, the chloride channel-permeable ions, did not significantly affect cell migration. Analysis of the effects of all the above treatments on $I_{cl,vol}$ and cell migration indicated that the inhibition of migration was positively correlated with the blockage of $I_{cl,vol}$ with a correlation coefficient (r) of 0.97, suggesting a functional relationship between $I_{cl,vol}$ and cell migration. These data suggest that the volume-activated Cl⁻ channels are involved in cell migration.

Copyright © 2007 S. Karger AG, Basel

Introduction

Among the major obstacles to the improvement of cancer treatment and prognosis is the metastasis of cancer cells. Cell migration is one of the key events in tumor metastasis and malignant transformation is frequently accompanied by enhanced mobility of tumor cells. Tumor cells must undergo several morphological changes in cell shape and volume as they navigate through the narrow, tortuous extracellular spaces [1]. Ion channels and trans-

porters have been reported to be involved in modulation of cell migration [2]. By maintaining cell volume, ion channels and transporters create an intracellular milieu that is required for the optimal operation of the cytoskeletal migration machinery. Several reports have shown that cell migration is modulated by Cl^- channels, which has been observed in transformed renal epithelial (MDCK-F) cells [3], glioma cells [4], monocytes [5] and neutrophils [6].

Multiple mechanisms are engaged in the regulation of cellular volume, including transport of ions, organic metabolites and osmolytes across the cell membrane. The overall regulation of cell volume is accomplished by two major mechanisms, namely regulatory volume decrease (RVD) and regulatory volume increase (RVI), through a cooperative machinery involving various transport or uptake systems such as Na^+ , K^+ and Ca^{2+} pumps, channels for Ca^{2+} , K^+ or Cl^- and co-transporters [7]. Most cells accomplish RVD by release of K^+ and Cl^- through activation of K^+ and/or anion channels. Volume-activated Cl^- currents ($I_{\text{cl,vol}}$) are believed to be essential for RVD [8]. The activation of $I_{\text{cl,vol}}$ by volume change and its influence on cell volume make it a prime component in determining the shape-volume changes required for cell migration. In our previous studies, we demonstrated that both the rate of RVD and the functional expression of the volume-activated Cl^- channel were up-regulated in migrated nasopharyngeal carcinoma CNE-2Z cells [9, 10]. Ranson et al. [11] showed that the glioma cell movement is accompanied by the spontaneous activation of $I_{\text{cl,vol}}$. Recently, CIC-3, the most plausible candidate for the molecular characterization of $I_{\text{cl,vol}}$, was revealed to be present in a polarization distribution on the edges of the lamellipodia during glioma cell migration [12], although migration of polymorphonuclear leukocytes in vivo was not altered in mice lacking CIC-3 [13]. The present study was designed to investigate further the effects of various Cl^- channel blockers, different osmotic treatments, and Cl^- ion replacement on both $I_{\text{cl,vol}}$ and cell migration in CNE-2Z cells and to analyze the relationship between $I_{\text{cl,vol}}$ and cell migration.

Materials and Methods

Chemicals, reagents, and solutions

The solution in patch-clamp pipettes contained 70mM N-methyl-D-glucamine chloride (NMDG-Cl), 1.2mM MgCl_2 , 10mM HEPES, 1mM EGTA, 140mM D-mannitol, and 2mM ATP. The isotonic bath solution contained 70mM NaCl, 0.5mM MgCl_2 ,

2mM CaCl_2 , 10mM HEPES, and 140mM D-mannitol. The osmolarity in pipette and isotonic bath solutions was measured by depressing the freezing point using an osmometer (Osmomat 30, Gonotec, Berlin, Germany) and was adjusted to 300mOsm/L with D-mannitol. The 47% hypotonic bath solution was obtained by omitting the D-mannitol from the solution, giving an osmolarity of 160mOsm/L (47% hypotonicity, compared with the isotonic solution). The 23% and 47% hypertonic solutions were obtained by adding D-mannitol to the isotonic solution, reaching an osmolarity of 370 and 440mOsm/L (23% and 47% hypertonicity, compared with the isotonic solution), respectively. The pH of pipette and bath solutions was adjusted with Tris base to 7.25 and 7.4, respectively. 5-Nitro-2- (3-phenylpropylamino) benzoic acid (NPPB) and adenosine triphosphate (ATP) were obtained from Sigma (St Louis, MO, USA). Stock solutions for Cl^- channel block experiments were 30mM NPPB in dimethyl sulfoxide (DMSO) and 100mM ATP in distilled water. They were diluted to the indicated final concentrations by using corresponding solutions for different experiments and were adjusted to pH 7.4. For Cl^- replacement experiments, solutions were made with the migration assay medium, based on RPMI 1640 without NaCl (Saier Corporation, Yixing, China), and the salts containing replacement ions (NaI, NaBr or sodium gluconate). Different osmotic solutions for the transwell assay were obtained by adjusting the amount of mannitol in media. All the chemicals were purchased from Sigma.

Cell culture

The CNE-2Z cell line, a poorly differentiated nasopharyngeal carcinoma cell line, was kindly provided by Professor Weiping Tang (Department of Pathology, Guangdong Medical College, China). The cell line was grown in 25cm² plastic tissue culture flasks in RPMI 1640 medium supplemented with 10% newborn calf serum, 100IU/ml of penicillin and 100μg/ml of streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 . The cells were sub-cultured every 2d. For electrophysiological experiments, the cells were trypsinized, centrifuged, and resuspended in the same culture medium, after they had been cultured for 48h and had reached about 80% confluence. The cell suspension was plated onto round coverslips with a diameter of 22mm (150μl/coverslip), which were located in 35mm tissue culture dishes, incubated at 37°C for about 2-3h before being used for current recording or cell volume determination.

Electrophysiological Recording

The whole cell current of single CNE-2Z cell was recorded by the patch-clamp technique with a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Patch-clamp pipettes were made from standard wall borosilicate glass capillaries with an inner filament on a two-stage vertical puller, giving a resistance of 4-6MΩ when filled with pipette solution. The electrode and the whole cell capacitance were determined by adjusting and minimizing the capacity transients in response to a 20mV voltage step using the amplifier functions, following the instruction of the amplifier manual. Once the whole-cell configuration was established, cells were held at the chloride equilibrium potential (0mV), and then stepped repeatedly to 200ms pulses of 0, ± 40 and ± 80mV, with a 4s interval between

Fig. 1. Effects of NPPB on the volume-activated chloride currents. Representative whole cell currents of CNE-2Z cells are presented in A and B. The single cell was held at 0mV and then stepped to 0, ± 40 , ± 80 mV for 200ms with an interval of 4s between voltage pulses. Exposure of the cell to the 47% hypotonic solution activated a volume-sensitive chloride current (A) that was inhibited by the extracellular application of 200 μ M NPPB (B). The dose-dependent inhibition of the current (at -80 mV) by NPPB is illustrated in C and the data were fitted by the equation $y = y_0 + a / (1 + \exp(-(x - x_0)/b))$. The data points represent the means $\pm$ SE of 9 experiments. The time course of a typical experiment is shown in D.

steps. The command voltages and the whole cell currents were recorded simultaneously on a computer via a laboratory interface (CED 1401, Cambridge, UK) with a sampling rate of 3kHz. The voltage pulse generation, data collection and current analysis were performed using the EPC software package (CED, Cambridge, UK). For the analysis of the data collected, all current measurements were made at 10ms after the onset of each voltage step. All experiments were carried out at room temperature (20 $\pm$ 2 $^{\circ}$ C).

Cell migration assay with transwell chambers

To determine the cell migration potential, the transwell chambers (Costar Corporation, Cambridge, MA) with 6.5mm diameter polycarbonate membrane filters of 12 μ m pore size were used to form dual compartments in a 24-well tissue culture plate. 10 μ l fibronectin (FN, 1mg/ml) was used to pre-cover the lower surface of the filter, which was then air-dried and used as a source of chemoattractant for cells navigating to cross the filter to the bottom side of the chamber. 600 μ l of RPMI 1640 medium containing 10% newborn calf serum was placed into the lower compartment of the well and 100 μ l of cell suspension in RPMI 1640 medium with 10% newborn calf serum at a density of 3×10^6 cells/ml (3×10^5 cells) was added to each of the upper compartments of the dual chambers. Various concentrations of blockers were added to both upper and lower compartments of the chambers. All assays were performed at 37 $^{\circ}$ C in humidified 5% CO₂ air for a period of 6h. Then, the transwell chambers were gently rinsed with phosphate-buffered saline

(PBS) and placed into other compartments of the 24-well culture plate containing 100 μ l of 0.25% trypsin-EDTA to be trypsinized for 5min, to which 200 μ l of RPMI 1640 medium was added (the total volume was 300 μ l). The cells that had migrated onto and attached to the lower surface of the filter were gently pipetted 5-7 times using a 200 μ l pipette and re-suspended in RPMI 1640 medium. The 300 μ l cell suspension was added to the upper compartment of another new transwell insert that was not precovered with FN. Inserts containing non-migrated or migrated cells were placed into the wells of another 24-well tissue culture plate, which contained 600 μ l of medium and 100 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5mg/ml) per well. After additional 4h incubation for converting MTT into dark blue, water insoluble formazan by mitochondrial dehydrogenase enzymes in cells, the inserts were put onto a piece of filter paper to drain off the medium on the upper compartment of the inserts. After 100 μ l of DMSO was added to each well to solubilize the formazan, the color solution was transferred to a separate well in a 96-well culture plate and the absorbance (expressed as optical density, OD) was then measured at 570nm. The percentage of migrated cells (migration rate) and the percentage of inhibition of cell migration by inhibitors were calculated based on the OD values measured. The rate of migration (%) = $OD_{mig} / (OD_{mig} + OD_{non}) \times 100$, where OD_{mig} is the OD value of migrated cells, OD_{non} is the OD value of non-migrated cells. The percentage of inhibition of cell migration by inhibitor (%) = $(mig-rate_{ctrl} - mig-rate_i) \div mig-rate_{ctrl} \times 100$, where $mig-rate_{ctrl}$ is the migration rate of the control cells and $mig-rate_i$ is the migration rate of the cells treated with

inhibitors. All experiments were repeated at least 3 times.

Detection of cell viability

Cell viability was detected by the trypan blue assay. Cells ready for detection were collected and suspended in PBS solution. 0.2ml of the cell suspension was mixed with 0.5ml of 0.4% trypan blue (in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic) and 0.3ml of PBS and stained for 10min. Stained (dead) and unstained (viable) cells were counted with a hemacytometer and cell viability was then obtained.

Statistical analysis

The data were expressed as the mean $\pm$ standard error (SE). ANOVA and/or Student's t-test were used to determine the significant differences among various treatments and $P < 0.05$ was considered significant.

Results

Effects of Cl⁻ channel blockers on the volume-sensitive Cl⁻ current

In this study, the effects of chloride channel blockers, NPPB and ATP, on the chloride current in CNE-2Z cells were investigated. A volume-sensitive chloride current with an almost linear current-voltage (I-V) relationship was activated by hypotonic (47%) stimulation, as demonstrated previously [14]. The current-voltage (I-V) relationship is nearly linear. Extracellular application of chloride channel blocker NPPB inhibited the current in a reversible and dose-dependent manner. Figure 1 illustrates the representative samples of the hypotonic-activated chloride current (at ± 40 , ± 80 mV) and the inhibition of the current by 200 μ M NPPB (Fig. 1A, B). The results indicated that the effect of NPPB was voltage-independent. NPPB inhibited both the inward and outward components of the volume-activated current. Figure 1C shows the dose-dependent inhibition of the volume chloride current at -80 mV by NPPB, with the IC_{50} value of 98.1 μ M. The time course of a typical experiment is shown in Figure 1D.

Extracellular application of chloride channel inhibitor ATP also suppressed reversibly the chloride currents induced by the 47% hypotonic challenge at all the voltage steps applied (± 40 , ± 80 mV). However, the effects of ATP on the currents were voltage dependent. The inhibition of ATP on the outward currents was stronger than that on the inward currents. ATP inhibited $82.3 \pm 4.6\%$ of the inward current induced by -80 mV ($n=9$, $P < 0.01$) and $94.6 \pm 5.0\%$ of the outward current induced by +80 mV ($P < 0.01$). The difference between them was significant ($P < 0.01$). The typical currents activated by 47% hypot-

Fig. 2. Effects of ATP on the volume-activated chloride currents. A and B show typical samples of the volume-activated chloride current (at 0, ± 40 , and ± 80 mV) induced by 47% hypotonic solution and the inhibition of the current by 10 mM of ATP, respectively. C is the dose-dependent inhibition of the current (at -80 mV) by ATP. The data points represent the means $\pm$ SE of 6 experiments.

onic stimulation and the responses to 10 mM ATP are shown in Figure 2A and 2B. Extracellular ATP inhibited the outward current induced by a -80 mV voltage step in a dose-dependent manner with an IC_{50} of 1.07 mM (Fig. 2C).

Fig. 3. Inhibition of CNE-2Z cell migration by NPPB. CNE-2Z cells (3×10^5 cells/0.1 ml) were plated onto the upper compartment of the transwell chamber and incubated with or without NPPB for 6 h at 37°C. The numbers of non-migrated and migrated cells were assessed by the MTT assay. A and B show, respectively, the migration rate and the inhibition of cell migration (percentage) under different treatments (various concentrations of NPPB). Each point in the figure represents mean $\pm$ SE from three different experiments.

Under isotonic conditions (before exposure to hypotonic solutions), the current recorded was small. Similar to that under hypotonic conditions, the current-voltage relation under isotonic conditions is linear. The resting conductance was inhibited by NPPB and ATP in a manner similar to that under hypotonic conditions.

Effects of Cl-channel blockers on CNE-2Z cell migration

Using a conventional transwell migration assay, our previous studies have shown that CNE-2Z cells have the ability to migrate across the filter membrane with

Fig. 4. Inhibition of CNE-2Z cell migration by ATP. CNE-2Z cells (3×10^5 cells/0.1 ml) were plated onto the upper compartment of the transwell chamber and incubated with or without ATP for 6 h at 37°C. The numbers of non-migrated and migrated cells were assessed by the MTT assay. A and B show, respectively, the migration rate and the inhibition of migration after treatments with different concentrations of ATP. The data are expressed as mean $\pm$ SE (three different experiments).

micropores, but the migration can be inhibited by 100 μM NPPB [10]. In this study we assessed the effect of various concentrations (50, 100 and 200 μM) of NPPB on CNE-2Z cell migration using the modified transwell migration system. $17.7 \pm 2.0\%$ ($n=8$) of the seeded cells migrated across the filter membrane in 6 h. The cell migration was inhibited by NPPB in a dose-dependent manner (Fig. 3A). Compared with the controls (treated with vehicle 0.2% DMSO), the migration rate of cells treated with NPPB was significantly reduced by $11.2 \pm 3.5\%$ ($n=3$, $P < 0.05$), $54.1 \pm 2.7\%$ and $73.8 \pm 3.4\%$ ($n=3$, $P < 0.01$) with 50, 100 and 200 μM of NPPB was used, respectively.

Fig. 5. Effects of ion substitutions on migration of CNE-2Z cells. Using NaCl-free RPMI 1640 media, 100mM NaI, NaBr, sodium gluconate or mannitol were used to substitute NaCl. CNE-2Z cells were suspended in the corresponding media and plated onto the upper compartment of the transwell chamber. The lower compartment of the transwell chamber was filled with the same solutions of various ion substitutions. Cells were incubated for 6 h at 37°C. The numbers of non-migrated and migrated cells were assessed by the MTT assay. Results are expressed as percentage of migrated cells over total cells including migrated and non-migrated cells (mean±SE) from three independent experiments.

Dose-response curve for NPPB is shown in Figure 3B (giving an IC_{50} of 97.7μM). Similarly, the CNE-2Z cell migration was also inhibited by the chloride channel blocker ATP in a dose-dependent manner (Fig. 4A and 4B) with an IC_{50} of 1.11mM.

To confirm further the inhibitory effects of chloride channel blockers on cell migration, the effect of another chloride channel inhibitor tamoxifen was tested. Compared with the control (no additives), cell migration was significantly inhibited by tamoxifen. Cell migration rate was decreased by 15.2±4.5% (n=3, P<0.05), 68.5±2.5% (n=3, P<0.01) and 78.0±3.7% (n=3, P<0.01) when 10μM, 20μM and 30μM of tamoxifen were added to the culture, respectively. The IC_{50} was 15.8μM.

Effects of ion replacement on CNE-2Z cell migration

As reported previously, based on electrophysiological recordings, the permeability sequence for the swelling-activated anion channel in CNE-2Z cells is $I^- > Br^- > Cl^- > gluconate$, and the swelling-activated current is signifi-

Fig. 6. Effects of various osmolarities on volume-activated chloride currents and cell migration. A shows the volume-activated chloride currents of a typical experiment under different osmotic conditions (at 0, ±40, and ±80mV). B presents the migration rate (mean±SE of 3 experiments) of the different osmotic groups. Cells were incubated in the media with different osmolarity during the transwell migration assay of 6h.

cantly reduced by replacing Cl^- with gluconate [14]. We next performed the transwell assays after replacing Cl^- ions with I^- , Br^- , or gluconate, to observe the effects of ion replacement on cell migration. As shown in Figure 5, substitution of chloride ions with the ions of similar permeant properties such as bromide and iodide (70mM NaBr or NaI in the NaCl-free medium) did not remarkably affect the migration rate of CNE-2Z cells (n=4, P>0.05). However, transwell migration of CNE-2Z cells was significantly reduced when NaCl was replaced by mannitol (70mM) or sodium gluconate (n=3, P<0.01), which are both relatively impermeant to swelling-activated anion channels. These results implicate that Cl^- fluxes across cell membrane are necessary for the CNE-2Z cell migration.

Fig. 7. The correlation between cell migration and the volume-activated chloride current in CNE-2Z cells A and B are the double y-axis plots of NPPB and ATP inhibitions of volume-activated Cl^- currents (at -80mV) and cell migration. The solid lines in the figures are the lines fitted to the migration data. NPPB or ATP inhibition of cell migration correspond well with the inhibition of the volume-activated Cl^- current. C is the plot of the inhibition of cell migration against that of the volume-activated Cl^- current by different treatments. The line in C was obtained by fitting the data with a linear equation. Data points are means $\pm$ SE from three to nine experiments.

Modulation of CNE-2Z cell migration after exposure to different osmolalities

As shown in our previous experiments [14], the hypotonic-activated current could be eliminated by perfusing the cells with 47% hypertonic solution. When the hypotonic-activated currents reached a peak and remained relatively stable for about 5min, the 47% hypotonic solution was replaced by hypertonic solutions. The current was partly inhibited by 10% and 23% hypertonic solutions and was completely abolished by the 47% hypertonic solution (Fig 6A).

To study further the relationship between the volume-activated chloride current and cell migration, we examined the effects of different osmolarities on the CNE-2Z cell migration, by activating or de-activating the swelling-activated chloride currents. CNE-2Z cells were suspended in solutions of various osmolarities (isotonic normal media and bath solutions, 47% hypotonic solution, 23% and 47% hypertonic solutions) for 5min and then added to the upper compartments of the transwell chamber. The lower compartment of the transwell chamber was filled with the same solutions of various osmolarities. The cells were incubated for 6h. The results showed that there was no significant difference in the cell migration rate between isotonic normal media and bath solution ($n=3$, $P>0.05$). However the migration rate of the cells treated with the 47% hypotonic solution was enhanced significantly ($n=3$, $P<0.01$) and the migration rate of the cells treated with the 47% hypertonic solution decreased significantly (Fig. 6B).

As for the change of cell volume, the volume was relatively stable when the cells were bathed in isotonic condition. When exposed to hypotonic challenges, cell volume increased significantly. However, the cells underwent a regulatory decrease (RVD) while they were still bathed in the hypotonic solutions. Cell volume recovered gradually as seen in our in our previous report [15].

Effects of different treatments on cell viability

To eliminate the possibility that the difference of migration rate in various treatments was caused by their effects on cell viability, cell viability was determined by the trypan blue assay after 6h of different treatments (the period of the migration assay). The results showed that none the above treatments (chloride channel blockers, various osmotic solutions, ion replacement) significantly affected the cell viability. The cell viability in different treatment groups was not significantly different from that of control cells.

Correlation between the volume-activated chloride current and cell migration

As shown above, both the volume-activated chloride current and the migration of CNE-2Z cells were inhibited by chloride channel blockers. We examined further the relationship between the migration and the volume-activated Cl⁻ current by comparing the dose-dependent curves for both events. The concentration dependence of NPPB or ATP inhibition of migration was in excellent agreement with the patch-clamp data for the volume-activated Cl⁻ current (Fig. 7A and B). Plots of the inhibition of cell migration against the inhibition of the volume-activated Cl⁻ current by various blockers and ion replacements indicated that the inhibition of the volume-activated chloride current was positively related to the inhibition of cell migration, with a correlation coefficient (*r*) of 0.97 (*P* < 0.01, Fig. 7C). The inhibition of the volume-activated Cl⁻ current and migration by different hyperosmolarities also correlate well, even though the experimental conditions are necessarily slightly different (in whole-cell patch clamp the intracellular osmolarity is clamped by the pipette solution).

Discussion

Our current understanding about the role of Cl⁻ channels in cell migration is limited. In the present study, we demonstrated that there is a positive correlation between the inhibition of cell migration and the inhibition of volume-activated Cl⁻ currents in CNE-2Z cells using both electrophysiology and cell migration approaches. We speculate that the membrane Cl⁻ currents and the volume regulatory Cl⁻ effluxes play an important role in controlling migration of nasopharyngeal carcinoma cells.

After osmotic cell swelling, the regulatory volume decrease (RVD) is the result of the concerted activities of K⁺ and Cl⁻ channels. The loss of osmolytes, following hypotonic challenge, induces a passive efflux of water with the consequent decrease in cell volume [8]. Although several anion channels, such as cAMP-activated, Ca²⁺-activated and basally active Cl⁻ channels, in addition to volume-activated Cl⁻ channels, are involved in extruding chloride in acute cell swelling, the volume-activated outwardly rectifying Cl⁻ channel plays the most important or particular role [16]. The volume-activated Cl⁻ channel has a typical selectivity order of I⁻ > Br⁻ > Cl⁻ > F⁻ > gluconate that corresponds to Eisenman's sequence I [17, 18]. Its activity can be blocked by several agents such as NPPB,

DIDS, A9C, niflumic acid, DCPIB, YM-198313 and tamoxifen and by extracellular ATP [7, 8, 19].

Channel-mediated cellular shape-volume changes also occur under isotonic conditions. For example, a dramatic loss (about 50%) of cellular Cl⁻ content was observed during the spreading of neutrophils stimulated by various chemoattractants [20]. In cultured astrocytes, the induction of morphological changes is accompanied by the activation of outwardly rectifying chloride channels [21]. Ca²⁺-sensitive K⁺ channels (IK1) and voltage-dependent K⁺ channels (Kv1.3 and Kv3.1) have been implicated in cell volume changes during migration of several types of cells [2]. We hypothesize that volume-activated Cl⁻ channels contribute to Cl⁻ flux, resulting in the shape-volume changes that are required for cell navigation through the narrow extracellular space during cell migration. Accordingly, pharmacological inhibition of volume-activated Cl⁻ channels should inhibit cell migration in a dose-dependent manner. The results of our *in vitro* migration assays support this notion. In the transwell migration assay, which mimics the three-dimensional spatial constraints encountered by migrating cells *in vivo*, NPPB and extracellular ATP inhibited both the volume-activated Cl⁻ currents and the transwell migration in CNE-2Z cells with almost identical dose-dependent patterns.

NPPB (normally at concentrations of 50–300 μM) and ATP (in the millimolar range) have been used as chloride channel blockers by many laboratories [14, 22–26]. NPPB is also a mitochondrial uncoupler at concentration of around 10 μM [27]. This latter effect may also impair cell migration. However, migration rate of cells treated with NPPB was only reduced by 11.2 ± 3.5% at the lowest concentration (50 μM) used in this study, at which the mitochondria activity should be significantly affected. This result suggests that NPPB inhibition of cell migration is caused mainly by acting on chloride channels. Our data with another chloride channel blocker, tamoxifen, also supports the role of chloride channels in cell migration. Consistent with our previous study [28], tamoxifen inhibited cell migration with an IC₅₀ of 15.8 μM.

However, the above results alone may not be sufficient to demonstrate that I_{cl,vol} plays an important role in cell migration because neither NPPB nor ATP are high affinity I_{cl,vol} blockers. They may slow the cell migration by inhibiting other types of Cl⁻ currents. In this study, we demonstrated that hypotonic treatments enhanced the migration of CNE-2Z cells, whereas exposure to hypertonic solutions inhibited cell migration. The volume-activated Cl⁻ channels were activated by hypotonic chal-

lenges, but deactivated by perfusing the cells with hypertonic solutions. These results suggest that the volume-activated chloride current is required for the process of cell migration. Hypotonic stimulations may facilitate changes of cell volume and shape by activating chloride currents and inducing RVD, which have been demonstrated by our previous experiments [15]. The enhanced ability to regulate cell volume, in turn, promotes cell migration. The hypertonic solutions may work oppositely to inhibit cell migration.

We also conducted ion replacement experiments to confirm that Cl^- flux is an important element of the cell migration machinery. In these studies, transwell migration was undisturbed when Cl^- was replaced by a permeant anion (I^- or Br^-) but greatly reduced when Cl^- was substituted by an impermeant anion (gluconate). The results suggest that anion flux across the cell membrane through anion channels or other transporters is important for cell migration. A long period of exposure of cells to a solution lacking Cl^- (gluconate-substituted solutions) can deplete the intracellular Cl^- [29]. Our previous experiments demonstrated that incubation of cells in Cl^- -free solutions (gluconate-substituted solutions) can deplete intracellular Cl^- , resulting in an inhibition of the volume-activated chloride current and a decrease of RVD capability [15]. The decrease of RVD capability will then impair cell volume change and reduce cell migration rate.

The positive relationship between the inhibition of $I_{\text{cl,vol}}$ and the inhibition of cell migration was established by analyzing the inhibitory effects of various blockers, different osmolarities and ion replacements on both events. All the present data support the notion that blockade of $I_{\text{cl,vol}}$ inhibits the ability of CNE-2Z cells to migrate across the transwell filters, suggesting that the activation of volume-activated Cl^- channels is required for the migration of CNE-2Z cells.

As shown above, chloride channel blockers inhibit the volume-activated chloride current and cell migration. It is not clear how the current change modulates cell migration. We speculate that the volume-activated chloride current is involved in the volume regulation of the migrating cells. The shape changes of the migrating cells are closely associated with locomotor activity and they are

inhibited by impairing volume regulatory mechanisms. According to the ion transport model for cell migration developed by Schwab [2], salt and osmotically obliged water uptake mediated by the parallel operation of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange as well as $\text{Na}^+-\text{HCO}_3^-$ cotransport at the front of the migrating cells contribute to the extension of the lamellipodium. Increased volume and membrane tension eventually trigger massive (Cl^- and K^+) ion efflux, resulting in a local shrinkage of the rear portion, which is similar to the process of regulatory volume decrease in the cell. Thus, RVD may be involved in cell migration via the RVD process happening in the rear part of cells, which is supported by our previous observations that the inhibition of RVD impaired cell migration [10]. The activation of $I_{\text{cl,vol}}$ is essential for RVD, therefore, $I_{\text{cl,vol}}$ may be activated by the increase in cell volume of migrating cells. Together with the activation of IK1 channel, which exhibits functional and structural polarization being distributed in the rear part of migrating cells [30], the activation of $I_{\text{cl,vol}}$ may induce local cell shrinkage at the rear end of migrating cells, supporting the cytoskeletal mechanism of migration. The inhibition of shrinkage of the rear part induced by the blockade of $I_{\text{cl,vol}}$ or Cl^- depletion impairs cell migration. We speculate that, similar to the IK1 channels, volume-activated Cl^- channels may also exhibit a polarized distribution to the rear part of migrating cells thereby facilitating cell migration. However, a recent study reveals that CIC-3, the most possible molecular candidate for $I_{\text{cl,vol}}$, shows a polarization distribution on the edges of the lamellipodia during glioma cell migration [13]. However, it is still controversial as to whether CIC-3 acts as a volume-activated chloride channel or a channel regulator [8].

Acknowledgements

We would like to thank Prof. Nie Sihuai (Guangdong Medical College) for the experimental direction. This work was supported by grants from the Wellcome Trust UK (056909/299/Z) and the Education Ministry of China (GJ9901).

References

- 1 Yamaguchi H, Wyckoff J, and Condeelis J: Cell migration in tumors. *Curr Opin Cell Biol* 2005;17:559-564.
- 2 Schwab A: Function and spatial distribution of ion channels and transporters in cell migration. *Am J Physiol Renal Physiol* 2001;280:F739-747.
- 3 Oberleithner H, Westphale, HJ, and Gassner, B: Alkaline stress transforms Madin-Darby canine kidney cells. *Pflugers Arch* 1991;419:418-420.
- 4 Soroceanu L, Manning TJ Jr, and Sontheimer H: Modulation of glioma cell migration and invasion using Cl^- and K^+ ion channel blockers. *J Neurosci* 1999;19:5942-5954.
- 5 Kim MJ, Cheng G, and Agrawal DK: Cl^- channels are expressed in human normal monocytes: a functional role in migration, adhesion and volume change. *Clin Exp Immunol* 2004;138:453-459.
- 6 Menegazzi R, Busetto S, Declava E, Cramer R, Dri P, and Patriarca P: Triggering of chloride ion efflux from human neutrophils as a novel function of leukocyte beta 2 integrins: relationship with spreading and activation of the respiratory burst. *J Immunol* 1999;162:423-434.
- 7 Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, and Haussinger D: Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 1998;78:247-306.
- 8 Sardini A, Amey JS, Weylandt KH, Nobles M, Valverde MA, and Higgins CF: Cell volume regulation and swelling-activated chloride channels. *Biochim Biophys Acta* 2003;1618:153-162.
- 9 Mao JW, Wang LW, Sun XR, Zhu LY, Li P, Zhong P, Nie SH, Jacob T, and Chen LX: Volume-activated Cl^- current in migrated nasopharyngeal carcinoma cells. *Sheng Li Xue Bao* 2004;56:525-530.
- 10 Mao JW, Wang LW, Jacob T, Sun XR, Li H, Zhu LY, Li P, Zhong P, Nie, SH, and Chen LX: Involvement of regulatory volume decrease in the migration of nasopharyngeal carcinoma cells. *Cell Res* 2005;15:371-378.
- 11 Ransom, CB, O'Neal, JT, and Sontheimer, H: Volume-activated chloride currents contribute to the resting conductance and invasive migration of human glioma cells. *J Neurosci* 2001;21:7674-7683.
- 12 McFerrin MB, and Sontheimer H: A role for ion channels in glioma cell invasion. *Neuron Glia Biol* 2006;2:39-49.
- 13 Moreland JG, Davis AP, Bailey G, Nauseef, WM, and Lamb FS: Anion channels including CLC-3 are required for normal neutrophil oxidative function, phagocytosis, and transendothelial migration. *J Biol Chem* 2006; 281:12277-12288.
- 14 Chen L, Wang L, Zhu L, Nie S, Zhang J, Zhong P, Cai B, Luo, H, and Jacob TJ: Cell cycle-dependent expression of volume-activated chloride currents in nasopharyngeal carcinoma cells. *Am J Physiol Cell Physiol* 2002; 283: C1313-1323.
- 15 Wang L, Chen L, Zhu L, Rawle M, Nie S, Zhang J, Ping Z, Cai K and Jacob TJ: Regulatory volume decrease is actively modulated during the cell cycle. *J Cell Physiol* 2002;193:110-119.
- 16 Okada Y: Cell volume-sensitive chloride channels. *Contrib Nephrol* 1998;123:21-33.
- 17 von Weikersthal SF, Barrand MA, and Hladky SB: Functional and molecular characterization of a volume-sensitive chloride current in rat brain endothelial cells. *J Physiol* 1999;516:75-84.
- 18 Jentsch TJ, Stein V, Weinreich F, and Zdebik, AA: Molecular structure and physiological function of chloride channels. *Physiol Rev* 2002;82:503-568.
- 19 Shibata H, Satoh TO, Ugawa T, Masuda N, Yanai-Inamura H, Abe A, Kondo Y, Kuramochi T, Akamatsu S, and Uchida W: Characterization of the pharmacology of YM-198313 on volume-regulated anion channels. *Biol Pharm Bull* 2005;28:1187-1191.
- 20 Menegazzi R, Busetto S, Dri P, Cramer R, and Patriarca P: Chloride ion efflux regulates adherence, spreading, and respiratory burst of neutrophils stimulated by tumor necrosis factor- α (TNF) on biologic surfaces. *J Cell Biol* 1996;135:511-522.
- 21 Lascola CD, Nelson DJ, and Kraig RP: Cytoskeletal actin gates a Cl^- channel in neocortical astrocytes. *J Neurosci* 1998;18:1679-1692.
- 22 Leaney JL, Marsh SJ, and Brown DA: A swelling-activated chloride current in rat sympathetic neurones. *J Physiol* 1997;15:501:555-564.
- 23 Al-Nakkash L, Iserovich P, Coca-Prados M, Yang H, and Reinach PS: Functional and molecular characterization of a volume-activated chloride channel in rabbit corneal epithelial cells. *J Membr Biol* 2004;201:41-49.
- 24 Barriere H, Rubera I, Belfodil R, Tauc M, Tonnerieux N, Poujeol C, Barhanin J, and Poujeol P: Swelling-activated chloride and potassium conductance in primary cultures of mouse proximal tubules. Implication of KCNE1 protein. *J Membr Biol* 2003;193:153-170.
- 25 Gosling M, Smith JW, Poyner DR: Characterization of a volume-sensitive chloride current in rat osteoblast-like (ROS 17/2.8) cells. *J Physiol* 1995;485:671-682.
- 26 Okada Y: Volume expansion-sensing outwardly rectifying Cl^- channel: fresh start to the molecular identity and volume sensor. *Am J Physiol* 1997;273:C755-789.
- 27 Lucas GL, Nanda A, Rotstein OD, and Grinstein S: The chloride channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells. *FEBS Lett* 1991;288:17-20.
- 28 Li H, Wang LW, Mao JW, He QF, Sun XR, Nie SH, Zhong P, Li P, and Chen LX: Roles of chloride channels in the migration of nasopharyngeal carcinoma cells at different stages of the cell cycle. *Chinese Journal of Physiology* 2005;21:1783-1786.
- 29 Smet PD, Simaels J, and Driessche WV: Regulatory volume decrease in a renal tubular cell line (A6) 1. Role of K^+ and Cl^- . *Pflugers Archiv* 1995;430:936-944.
- 30 Schwab A, Gabriel K, Finsterwalder F, Folprecht G, Greger R, Kramer A, and Oberleithner H: Polarized ion transport during migration of transformed Madin-Darby canine kidney cells. *Pflugers Arch* 1995;430:802-807.