Volume-activated Cl\textsuperscript{-} current in migrated nasopharyngeal carcinoma cells

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Abstract: The transwell chamber migration assay and the patch-clamp technique were used to investigate the volume-activated Cl\textsuperscript{-} current (I\textsubscript{Cl.vol}) in migrated nasopharyngeal carcinoma cells (CNE-2Z). 47% hypotonic solution activated a I\textsubscript{Cl.vol} in the migrated CNE-2Z cells. Compared with the control cells (non-migrated), the properties of this current and the sensitivity to Cl\textsuperscript{-} channel blockers were changed. The current density in migrated CNE-2Z cells was higher than that in non-migrated cells. The current was almost completely inhibited by extracellular application of adenosine-5'-triphosphate (ATP, 10 mmol/L), 5-nitro-2-3-phenylpropylamino benzoic acid (NPPB, 100 mmol/L) and tamoxifen (30 mmol/L) in all voltage steps applied. The inhibition of NPPB and tamoxifen on the current was stronger in migrated cells than that in non-migrated cells. The permeability sequence of the four anions was Br\textsuperscript{-} > Cl\textsuperscript{-} > I\textsuperscript{-} > Gluconate. The sequence was different from that of the non-migrated cells (I\textsuperscript{-} > Br\textsuperscript{-} > Cl\textsuperscript{-} > Gluconate). The results suggest that volume-activated chloride channels may be involved in the CNE-2Z cell migration.

Key words: tumor cells; cultured; chloride channels; cell migration; patch-clamp techniques
nasopharyngeal carcinoma cell (CNE-2Z).

1 MATERIALS AND METHODS

1.1 Cell culture. The poorly differentiated nasopharyngeal carcinoma cells (CNE-2Z) were cultured by a method described previously[3]. CNE-2Z cells were grown in culture medium (RPMI 1640 with 10% new-born 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.

1.2 Transwell migration assay. Transwell chambers (Costar Corporation, Cambridge, MA) equipped with polycarbonate membrane (12 µm pore size) were precoated with 10 µl fibronectin (0.5 mg/ml) on the bottom side. Cells were added onto the top side of the membrane and then incubated in culture media at 37°C. After 8 h culture, cells on the upper surface were removed using cotton swabs, and the transwell membrane with migrated cells was fixed on glass coverslips for electrophysiological experiments.

1.3 Patch-clamp experiments. Whole cell currents of CNE-2Z cells were recorded at room temperature (20–24°C) using the patch-clamp technique previously described by us[10], with a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). An agar bridge was used to connect the reference electrode (Ag-AgCl wire) to the bath solution. Cells were held at the Cl⁻ equilibrium potential (0 mV) and then stepped to ±40, 0 and ±80 mV for 200 ms with 4 s interval between pulses throughout the experiments. All current measurements were made 10 ms after the onset of each voltage pulse. The pulse generation and current analysis were carried out with the EPC software package (CED, Cambridge, UK). Currents were normalized by cell membrane capacity. The permeability ratios (P_X/P_Cl) of various anions (X⁻) relative to that of Cl⁻ were calculated using the modified Goldman-Hodgkin-Katz equation:

$$P_X/P_Cl = ([Cl^-]_s \times \exp (-\Delta V_{rev} F/RT) - [Cl^-]_i)/[X^-]_s$$

where [Cl⁻]_s and [Cl⁻]_i are the Cl⁻ concentration in the normal and the substituted bath solutions, [X⁻]_s is the concentration of the substituted anion, ΔV_{rev} the difference of the reversal potentials for Cl⁻ and X⁻, F the Faraday constant, R the gas constant and T the absolute temperature.

The percentage of inhibition of the hypotonic-activated Cl⁻ current was calculated using the following equation:

$$\frac{(Current_{I_{Cl, hyp}} - Current_{test, hyp})}{(Current_{I_{Cl, hyp}} - Current_{iso})} \times 100\%,$$

where Current_{iso} is the background current under isotonic condition, Current_{I_{Cl, hyp}} the peak hypotonic-activated current and Current_{test, hyp} the current recorded after inhibitor treatment.

1.4 Solutions and chemicals. The pipette solution contained (in mmol/L): 70 N-methyl-D-glucamine chloride (NMDG, Cl⁻), 1.2 MgCl₂, 10 HEPES, 1 EGTA, 140 D-mannitol, and 2 ATP. The isotonic bath solution contained (in mmol/L): 70 NaCl, 0.5 MgCl₂, 2 CaCl₂, 10 HEPES, and 140 D-mannitol. Solution osmolality was measured with a freezing-point osmometer (OSMOMAT 030; Gonotec, Germany). The osmolality of pipette and isotonic bath solutions was adjusted to 300 mOsmol/L with D-mannitol. The hypotonic bath solution was obtained by omitting the D-mannitol from the solution, giving an osmolality of 160 mOsmol/L (47% hypotonicity). In anion substitution experiments, 70 mmol/L NaCl in the hypotonic solution was replaced by equimolar of NaI, NaBr or sodium gluconate. The pH of the pipette and bath solutions was adjusted to 7.25 and 7.4, respectively, with Tris base. All chemicals were obtained from Sigma.

1.5 Statistics. Values were expressed as mean ±SD (number of observations). ANOVA was used to test for significant differences and P<0.05 was taken to be significant.

2 RESULTS

2.1 Volume-activated Cl⁻ current in migrated CNE-2Z cells

Whole cell currents in migrated and non-migrated (control) CNE-2Z cells were recorded. The results indicated that the time course of activation of $I_{Cl,vol}$ was similar in both of the cells. When the cells were bathed in isotonic solution, the currents were small and stable (data not show here), but a large current was activated after the cells had been exposed to a 47% hypotonic solution for 1–2 min. The current reached a stationary value in 3–5 min after activation and showed a mild outward-rectification property. There was no time-dependent inactivation at potentials applied (±40 and ±80 mV). The current-voltage relationship demonstrated that the hypotonic-activated current reversed at a voltage close to the calculated equilibrium potential for Cl⁻ (about 0 mV). During the experiments, the cells were swollen when bathed in the hypotonic solution unless the isotonic solution had been returned to the bath.

However, the density of $I_{Cl,vol}$ was significantly different between the migrated and non-migrated CNE-2Z cells.
The outward and inward currents in response to +80 mV and −80 mV were 68.6 ± 3.7 and −58.9 ± 3.3 pA/pF (n = 23), respectively, in migrated cells. These were much larger than those recorded from non-migrated cells, 46.8 ± 4.3 and −43.6 ± 2.1 pA/pF (n = 31, P < 0.01) (Fig. 1).

2.2 Anion permeability of volume-activated Cl− channel

When I_{Cl.vol} was activated and reached the peak, the hypotonic solution containing 70 mmol/L of Cl− was replaced with solution containing equimolar I−, Br−, or gluconate. The anion substitution shifted the reversal potential. The permeability ratios of P_i/P_{Cl−}, P_{Br−}/P_{Cl−}, and P_{gluconate}/P_{Cl−} calculated from the shifts in reversal potential using the modified Goldman-Hodgkin-Katz equation, were 0.92 ± 0.02, 1.09 ± 0.04, and 0.39 ± 0.03 (n = 7), resulting in the sequence of anion permeability of Br− > Cl− > I− > gluconate. However, in non-migrated cells, the sequence was I− > Br− > Cl− > gluconate with permeability ratios of 1.16 ± 0.05, 1.13 ± 0.03, and 0.37 ± 0.03, respectively (n = 5, Fig. 2).

2.3 Effects of Cl− channel blockers on the volume-activated Cl− current

As shown above, hypotonic bath solution activated a Cl− current in the migrated CNE-2Z cell. To further characterize its properties, the effect of Cl− channel blockers ATP, NPPB, and tamoxifen on the current was observed. Figure 3A shows the inhibitory effect of extracellular ATP (10 mmol/L) on I_{Cl.vol} in migrated and non-migrated cells. In both groups, extracellular application of ATP almost completely inhibited I_{Cl.vol} in a reversible manner, but the inhibition of ATP on the outward current was stronger than that on the inward current (P < 0.01). There was no significantly difference (P > 0.05) between the two groups in the effects of ATP.

NPPB (100 µmol/L), another Cl− channel blocker, could also completely inhibit both the inward and outward components of I_{Cl.vol} in migrated cells (Fig. 3B, P < 0.01). However, in non-migrated cells, NPPB inhibited only about half of outward and inward currents, which was significantly different from that in migrated cells (P < 0.01).

The effects of the chloride channel inhibitor tamoxifen (30 µmol/L) on I_{Cl.vol} were also studied (Fig. 3C). In migrated cells, the current was inhibited rapidly and completely (P < 0.01). The outward and inward currents were affected almost equally. However, in non-migrated cells, the effects of tamoxifen on the currents varied greatly. Tamoxifen (30 µmol/L) inhibited the currents completely.

Fig. 1. Volume-activated Cl− current in CNE-2Z cells. Typical current traces of a migrated cell recorded under isotonic bath condition (Iso) and at the peak of hypotonic response (Hypo) are shown in A and B. Voltage was held at 0 mV and then stepped to ±40, 0 and ±80 mV. The current-voltage (I-V) relationships of migrated cells (n = 23) and non-migrated cells (control, n = 31) are presented in C.

Fig. 2. Comparison of anion permeability of the volume-activated Cl− channel between migrated and control CNE-2Z cells. The data in the figure represents the means±SD of 5 non-migrated (control) and 7 migrated cells.
in some cells, but weakly in some other cells even though its concentration was increased to 40 µmol/L (data was not shown). Mean inhibition on the currents in non-migrated cells was smaller than that in migrated cells ($P<0.01$).

3 DISCUSSION

Cl⁻ channels are important for cell volume regulation in many kinds of cells\[7\]. We have previously reported that volume-activated Cl⁻ channels play a key role in regulatory volume decrease (RVD) of ciliary epithelial cells\[8\] and CNE-2Z cells\[5, 9\] and are involved in the control of cell cycle progress in CNE-2Z cells\[6\]. It has also been reported that volume-activated Cl⁻ currents may contribute to volume changes required for glioma cell migration through brain tissue\[3, 4\]. The results in this study suggest that volume-activated Cl⁻ channels may play an important role in migration of CNE-2Z cells.

In our experiments, exposure of migrated CNE-2Z cell to a hypotonic solution swelled the cells and activated a current with the properties similar to that recorded in ciliary epithelial cells\[10, 11\]. The current reversed at a potential

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Fig. 3. Inhibition of volume-activated Cl⁻ currents by Cl⁻ channel blockers. 10 mmol/L ATP (A), 100 mmol/L NPPB (B) and 30 mmol/L tamoxifen (C, tam) inhibited volume-activated Cl⁻ currents in both migrated cells ($n=5, 9$ and $5$, respectively) and non-migrated cells (control, $n=8, 14$ and $12$, respectively). Hypo: 47% hypotonic solution.
close to the equilibrium potential of Cl\(^-\) (about 0 mV) and could be inhibited by chloride channel blockers NPPB, tamoxifen and ATP. Substitution of Cl\(^-\) by gluconate decreased the current and shifted the reversing potential. The evidence indicate that the current induced by the hypotonic solution is the volume-activated Cl\(^-\) current.

Comparing the volume-activated Cl\(^-\) current of the migrated CNE-2Z cell with that of its counterpart, the non-migrated cell, indicated that the expression level and some properties of the current might be modulated by cell migration in CNE-2Z cells or the current might be differentially expressed in CNE-2Z cells with various migrating ability. The current density in migrated CNE-2Z cells was higher than that in non-migrated cells. This implicates that expression of the current was upregulated by cell migration or in the cells with higher migration ability and the current may be involved in the control of cell migration. Apart from the change in current density, the sensitivity of the current to the chloride channel blockers, NPPB and tamoxifen, was also changed. The blockers were more effective on the current of the migrated cells than on that of non-migrated cells. Furthermore, the permeability sequence of the current changed from I\(^-\) > Br\(^-\) > Cl\(^-\) > gluconate in non-migrated cells to Br\(^-\) > Cl\(^-\) > I\(^-\) > gluconate in migrated cells. These results suggest the possibility of modulation of volume-activated Cl\(^-\) channels during cell migration or the possibility of expression of more than one Cl\(^-\) channel in CNE-2Z cells. Cell migration may induce the modulation of the chloride channels or activate the expression of some other chloride channels that are silenced or expressed at a low level before migrating. It has been reported that there were three types of volume-activated chloride channels in ciliary epithelial cells and the expression of these channels was different between the two cell types of the ciliary epithelium\(^{[12]}\). Antisense knock-down of MDR1\(^{[13]}\), CIC-3\(^{[14]}\) or pICln\(^{[15]}\) gene expression inhibited the volume-activated chloride currents. In A6 renal epithelial cells, four different types of volume-activated chloride channels were identified and classified\(^{[16]}\). All the evidence suggests the presence of more than one volume-activated chloride channel, although the molecular identity of the volume-activated chloride channel(s) remains to be determined\(^{[17,18]}\).

In conclusion, we have found that migrated CNE-2Z cells expressed volume-activated Cl\(^-\) currents and the expression of the currents was upregulated by migration. The results suggest that volume-activated Cl\(^-\) channels may be involved in cell migration in CNE-2Z.

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