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Volume-Sensitive Chloride Channels are Involved in Maintenance of Basal Cell Volume in Human Acute Lymphoblastic Leukemia Cells

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Abstract Chloride channels are expressed ubiquitously in different cells. However, the activation and roles of volume-activated chloride channels under normal isotonic conditions are not clarified, especially in lymphatic cells. In this study, the activation of basal and volume-activated chloride currents and their roles in maintenance of basal cell volume under isotonic conditions were investigated in human acute lymphoblastic leukemia Molt4 cells. The patch-clamp technique and time-lapse image analysis were employed to record whole-cell currents and cell volume changes. Under isotonic conditions, a basal chloride current was recorded. The current was weakly outward-rectified and volume-sensitive and was not inactivated obviously in the observation period. A 47% hypertonic bath solution and the chloride channel blockers NPPB and tamoxifen suppressed the current. Exposure of cells to 47% hypotonic bath solution activated further the basal current. The hypotonicity-activated current possessed properties similar to those of the basal current and was inhibited by NPPB, tamoxifen, ATP and hypertonic bath solution. Furthermore, extracellular hypotonic challenges swelled the cells and induced a regulatory volume decrease (RVD). Extracellular applications of NPPB, tamoxifen and ATP swelled the cells under isotonic conditions and inhibited the RVD induced by hypotonic cell swelling. The results suggest that some volume-activated chloride channels are activated under isotonic conditions, resulting in the appearance of the basal chloride current, which plays an important role in the maintenance of basal cell volume in lymphoblastic leukemia cells. Chloride channels can be activated further to induce a regulatory volume recovery when cells are swollen.

Keywords Chloride channel · Basal chloride current · Cell volume regulation · Lymphoblastic leukemia cell · Regulatory volume decrease · Chloride channel blocker

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Introduction

Chloride channels are expressed ubiquitously in different cells and tissues. Accumulated data demonstrate that chloride channels are involved in a wide range of biological functions, including epithelial fluid secretion, cell-volume regulation, neuroexcitation, smooth-muscle contraction and acidification of intracellular organelles, among others (Lang et al. 1998; Zhou et al. 2005). Our recent experimental results and the data presented by other laboratories indicate that chloride channels play important roles in the cell cycle, cell proliferation, apoptosis and migration (Chang et al. 2006; Chen et al. 2007; d'Anglemont de Tassigny et al. 2008; Guan et al. 2006; Lang et al. 1998, 2007; Mao et al. 2007, 2009; Wondergem et al. 2001; Zuo et al. 2009). There are at least six types of chloride channels, including volume-activated

(or swelling-activated) chloride channels, the ClC chloride channel family, cystic fibrosis transmembrane conductance regulator (CFTR), calcium-activated chloride channels, intracellular chloride channels and ligand-gated chloride channels (Jentsch et al. 2002). As for the volume-activated chloride channels, their molecular identities are still a matter of debate (Duan et al. 1997; Jentsch et al. 2002; Lang et al. 1998; Mao et al. 2009; Xiong et al. 2010).

In many cells, volume-activated chloride channels are involved in the regulatory volume decrease (RVD) process induced by hypotonic challenge (Lang et al. 1998; Wang et al. 2002b). When cells are swollen under hypotonic conditions, the volume-activated chloride channels, as well as potassium channels, are activated. Activation of these channels leads to outflows of Cl^- and K^+ and an accompanying efflux of water, resulting in a partial or complete recovery of cell size. However, in isotonic conditions, the activities and the activation mechanisms of the swelling-activated chloride channels are far from clarified.

Blood cells are bathed in plasma and frequently receive hypotonic or hypertonic challenges due to the intake of large amounts of water or the dehydration induced by heavy sweating. Hypotonic challenges may activate the swelling-activated chloride channels. However, under normal isotonic conditions, are the channels activated to induce a basal chloride current in blood cells? If yes, are the properties of the basal chloride current different from those of the swelling-activated chloride current? Is the basal chloride current involved in maintaining basal blood cell volume in normal isotonic conditions? In this study, the activities of the basal chloride current, the involvement of the basal chloride current in regulation of basal cell volume under isotonic conditions and the properties of the basal chloride current and the swelling-activated chloride current were investigated in human acute lymphoblastic leukemia Molt4 cells.

Materials and Methods

Cell Culture

Molt4 is a cell line derived from the human acute lymphoblastic leukemia. Cells were routinely grown in 25-cm^2 culture flasks with RPMI-1640 medium containing 10% newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured every 2 days. To prepare cells for current recordings and volume measurements, cells were collected; resuspended in RPMI-1640 medium with 10% newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin; plated on 22-mm round coverslips (150 μl/coverslip); and incubated at 37°C for 2–3 h before carrying out the experiments.

Solutions

The isotonic bath solution contained (mM) 70 NaCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 140 D-mannitol. The pipette solution consisted of (mM) 70 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 140 D-mannitol and 2 ATP. The osmolarity of pipette and isotonic bath solutions was adjusted to 300 mOsmol/l with D-mannitol and measured with a freezing-point osmometer (OSMOMAT 030; Gonotec, Berlin, Germany). The 47% hypertonic solution and the 47% hypotonic solution were obtained by adding 140 mM D-mannitol into or omitting D-mannitol from the isotonic bath solution, respectively. The pH of bath and pipette solutions was adjusted to 7.4 and 7.25, respectively, with 1 M Tris base.

Chloride Channel Blockers

The chloride channel blockers 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), tamoxifen and ATP were purchased from Sigma (St. Louis, MO). NPPB, tamoxifen and ATP were dissolved in dimethyl sulfoxide (DMSO), methanol and distilled water at concentrations of 100, 20 and 100 mM, respectively, and diluted to the indicated final concentrations with isotonic or 47% hypotonic bath solution.

Whole-Cell Current Recordings

Whole-cell currents were recorded with 5–10 MΩ pipettes and an EPC-7 patch-clamp amplifier (Heka, Lambrecht/Pfalz, Germany) at 20–24°C. Membrane potential was held at the Cl^- equilibrium potential (0 mV) and stepped to 200-ms voltage pulses of 0, ±40 and ±80 mV repeatedly, with 4-s intervals between pulses. Using a laboratory interface (CED 1401; CED, Cambridge, UK), voltages and currents were recorded in computers. Currents were measured at 10 ms after onset of voltage steps.

Volume Measurements

Cell images were captured every 60 s by a CCD camera (Mono CCD625; Leica, Heidelberg, Germany) at 20–24°C and analyzed with Scion (Frederick, MD) software. Cell volume was computed from cell diameters. Changes in cell volume induced by different treatments were calculated using the formula $(V_{TEST} - V_{CTRL})/V_{CTRL} \times 100\%$, where V_{CTRL} is the basal cell volume under isotonic conditions and V_{TEST} is the cell volume after various treatments. The level of RVD was calculated using the equation RVD $(\%) = (V_{max} - V_{min})/(V_{max} - V_0) \times 100\%$, where V_0 is the cell volume in the isotonic solution before hypotonic shocks, V_{max} is the peak volume in the hypotonic solution and V_{min} is the volume before returning to the isotonic

solution. Cell volume was standardized to the time point before change of solutions.

Statistical Analysis

Data were expressed as the mean ± standard error (with n indicating the number of observations). ANOVA was used to analyze the data, and differences were considered significant at $P < 0.05$.

Results

Characterization of Basal Chloride Currents

The whole-cell patch-clamp technique was used to record the basal chloride current under isotonic conditions. Cells were held at 0 mV and cycled though the 200-ms pulses of 0, ±40 and ±80 mV with 4-s intervals between pulses. In the current recording experiments, there was no potassium in the bath and electrode solutions. As shown in Fig. 1, a weak basal current was recorded under the isotonic condition. The current was not inactivated obviously in the observation period and reversed at a voltage close to the calculated equilibrium potential for Cl^- (about 0 mV) (Fig. 1a, b). Analysis of the current (I)–voltage (V) relation indicated that, in most cases, a weak outward rectification was observed at the voltages applied. In this study, recordings were made at 20–24°C. The effect of temperature on the properties of the basal current was tested. It was shown that the properties of the current recorded at 35–37°C were not significantly different from those recorded at 20–24°C.

The basal current was volume-sensitive. Exposures of cells to a 47% hypertonic solution shrank the cells and suppressed the basal current significantly (Fig. 1c). The basal current at +80 mV was inhibited significantly by 41.1 ± 8.9% ($n = 6$) when the isotonic bath solution was replaced by the 47% hypertonic solution.

Pharmacological Properties of Basal Chloride Currents

To study further the pharmacological characteristics of the basal current recorded under normal isotonic conditions, the effects of extracellular applications of chloride channel blockers on the current were observed. As shown in Fig. 1d, e, the basal current could be inhibited by the chloride channel blockers NPPB and tamoxifen, respectively. Extracellular application of 100 μM NPPB or 20 μM tamoxifen decreased significantly the basal current at +80 mV by 34.0 ± 7.3% ($n = 5$, $P < 0.01$) or 20.1 ± 5.9% ($n = 5$, $P < 0.01$), respectively. The action of NPPB and tamoxifen on the basal chloride current was voltage-independent. The inhibition of the inward current by these blockers was not significantly different from that of the outward current.

Activation of Hypotonicity-Induced Currents

As shown above, a volume-sensitive basal chloride current was activated in the isotonic solution in Molt4 cells. Would the chloride current be activated further when the cells were swollen by extracellular hypotonic challenges? To answer this question, the effects of a 47% hypotonic bath solution on the chloride current were recorded. Cells were held at 0 mV and cycled though the 200-ms pulses of 0, ±40 and ±80 mV with 4-s intervals between pulses. Under isotonic conditions, the current was weak and relatively stable (Fig. 2a–c). However, a strong current was activated in about 1 min when the cells received a 47% hypotonic challenge (Fig. 2a, b, d). Activation of the current reached its maximum in 3–5 min and remained in a relatively stable plateau before further treatments. The hopotonicity-activated current was weakly outward-rectified, with a mean density of −88.1 ± 18.4 pA/pF at −80 mV and 122.7 ± 19.9 pA/pF at +80 mV ($n = 19$, Fig. 2b). There was no significant time-dependent inactivation at the voltages applied (0, ±40 and ±80 mV) (Fig. 2d). Increasing the voltage to ±120 mV did not change the current properties significantly (data not shown).

Further studies indicated that the hypotonicity-activated current is volume-sensitive. When the current reached a plateau, the 47% hypotonic bath solution was replaced with the 47% hypertonic solution. As shown in Fig. 3, the current was suppressed by the hypertonic treatment. Analysis of the images acquired during the experiments indicated that cells were swollen by hypotonic stimulation and shrank by hypertonic treatment.

Pharmacological Properties of Hypotonicity-Induced Currents

The pharmacological properties of the current activated by the 47% hypotonic solution were investigated using the chloride channel blockers NPPB, tamoxifen and ATP. When maximal activation was obtained, different chloride channel blockers were added to the hypotonic solution and the effects of the chloride channel blockers on the hypotonicity-activated chloride current were recorded and analyzed.

As shown in Fig. 4a, b, the hyptonicity-activated current was inhibited by the chloride channel blocker NPPB. Extracellular application of 100 μM NPPB inhibited the inward current recorded at −80 mV by 88.9 ± 9.5% and the outward current obtained at +80 mV by 92.1 ± 2.4%, respectively ($n = 5$, $P < 0.01$). The difference in NPPB inhibition between the inward and outward currents was not significant ($P > 0.05$).

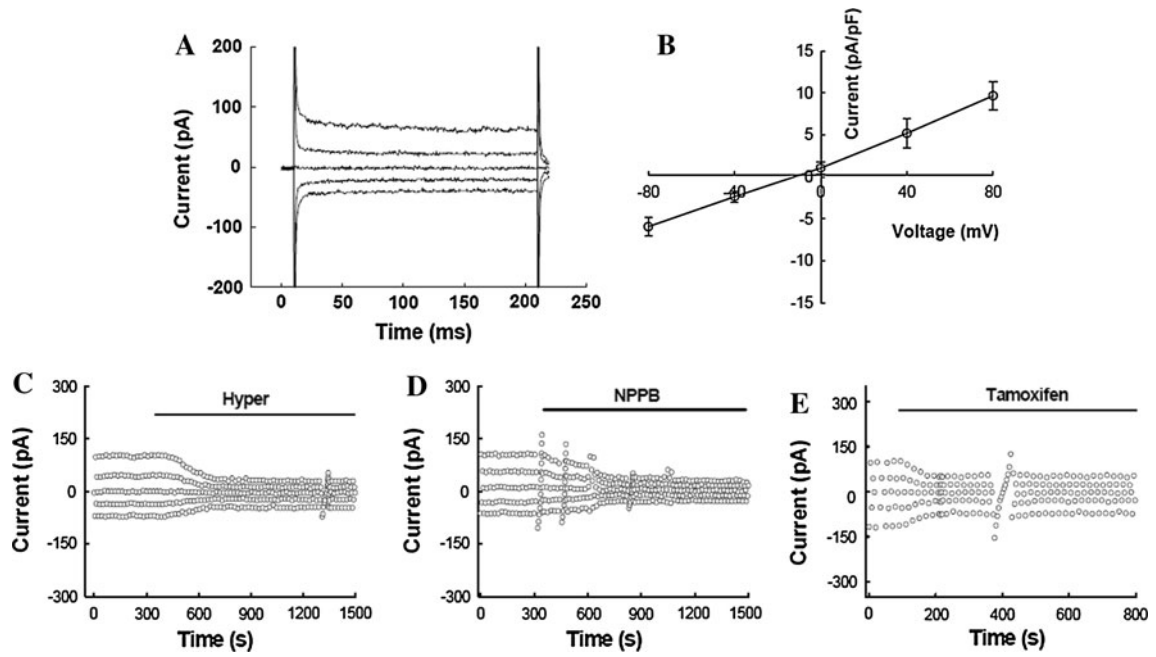

Fig. 1 Properties of basal chloride currents recorded under isotonic conditions in human acute lymphoblastic leukemia cells. Whole-cell currents were recorded by the patch-clamp technique under isotonic conditions. Current traces were elicited by 200-ms voltage pulses from a holding potential of 0 mV to 0, ±40 and ±80 mV with 4-s intervals between pulses. **a** Representative basal chloride current recorded in the isotonic solution. **b** *I–V* relation (mean ± standard error of 18 cells). Extracellular applications of 47% hypertonic solution (*Hyper*, **c**) and the chloride channel blockers NPPB (100 μM, **d**) and tamoxifen (20 μM, **e**) inhibited the basal current

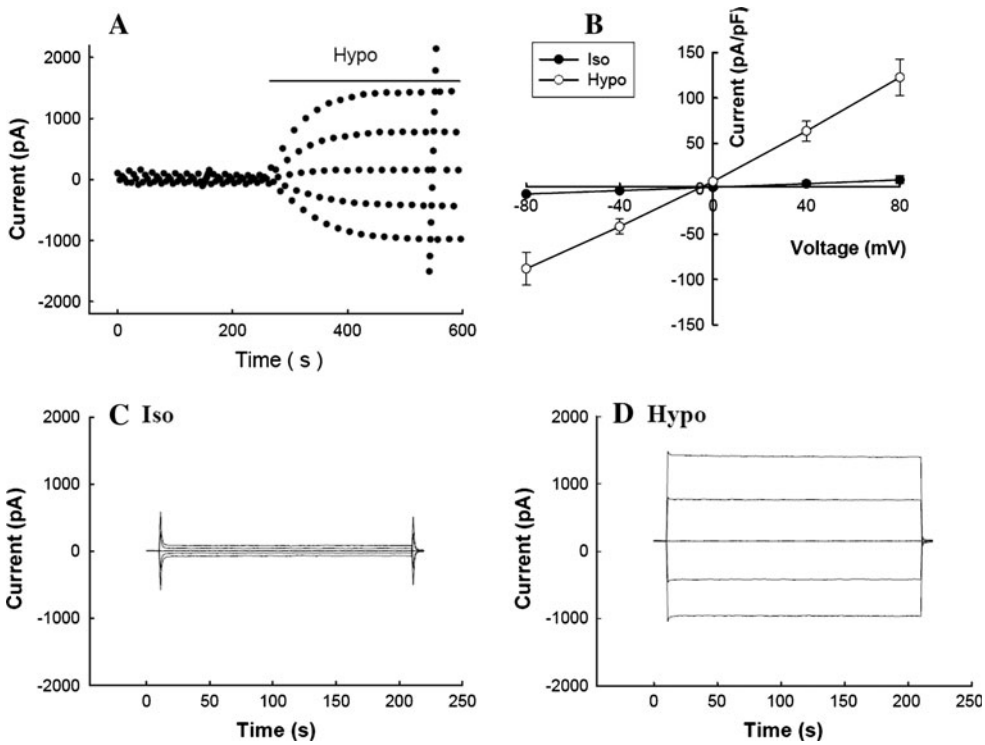

Fig. 2 Chloride currents activated by 47% hypotonic bath solution in human acute lymphoblastic leukemia cells. Current traces were elicited by 200-ms voltage pulses from a holding potential of 0 mV to 0, ±40 and ±80 mV with 4-s intervals between pulses. **a** Typical time course of activation of volume-activated chloride current induced by 47% hypotonic bath solution (*Hypo*). **b** *I–V* relation (mean ± standard error of 19 cells). Typical current traces recorded in isotonic (*Iso*) and 47% hypotonic bath solutions are shown in **c** and **d**

Similar to the effects of NPPB, the chloride channel blocker tamoxifen could inhibit the hypotonicity-induced current in a voltage-independent manner (Fig. 4c, d). Extracellular application of 20 μM tamoxifen inhibited the current by 74.2 ± 6.2% at −80 mV and by 73.9 ± 8.4% at 80 mV ($n = 6$, $P < 0.01$).

Extracellular application of 10 mM ATP also inhibited the current induced by the hypotonic challenge (Fig. 4e, f).

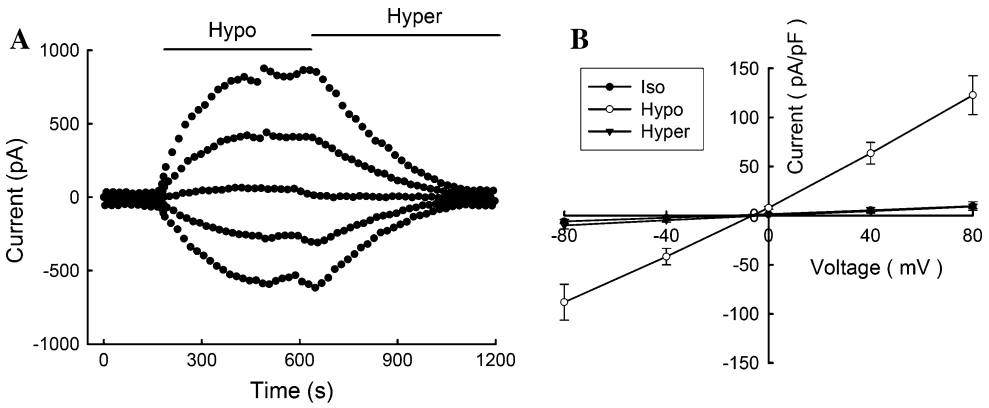

Fig. 3 Inhibition of the volume-activated chloride current by 47% hypertonic bath solution in human acute lymphoblastic leukemia cells. Current traces were elicited by 200-ms voltage pulses from a holding potential of 0 mV to 0, ±40 and ±80 mV with 4-s intervals between pulses. **a** Typical time course of activation of volume-activated chloride current induced by 47% hypotonic bath solution (*Hypo*) and inhibition of the current by cell shrinkage induced by 47% hypertonic bath solution (*Hyper*). **b** *I–V* relation (mean ± standard error of 19 cells)

However, the inhibitory properties of ATP were different from those of NPPB and tamoxifen. The inhibitory effect of ATP on the outward current was stronger than that on the inward current. ATP (10 mM) inhibited the inward current at −80 mV by 34.9 ± 3.2% and the outward current at +80 mV by 78.0 ± 2.6% ($n = 5$, $P < 0.01$). Furthermore, the onset of inhibition of the outward current was faster than that of inhibition of the inward current. The time difference between the onset of inhibition of the outward and inward currents was several seconds to 30 s and was significant. The mechanism of the difference is not clear.

Involvement of Volume-Sensitive Chloride Channels in the Maintenance of Basic Cell Volume Under Isotonic Conditions

The above data indicate that the basic chloride current recorded under isotonic conditions is volume-sensitive. The results imply that volume-activated chloride channels may play an important role in the maintenance of normal cell volume. This was confirmed by our experiments. Cells were bathed in the control isotonic solution for more than 5 min, and different chloride channel blockers were then added to the control solution. Time-lapse cell images were acquired and analyzed. The results showed that the cells were swollen by treatment with the chloride channel blockers NPPB, tamoxifen and ATP (Fig. 5). Cell volume was increased by 10.3 ± 1.5% ($n = 11$, $P < 0.01$), 9.3 ± 1.6% ($n = 15$, $P < 0.01$) and 8.1 ± 1.3% ($n = 13$, $P < 0.01$), respectively, by 100 μM NPPB, 20 μM tamoxfen and 10 mM ATP.

Hypotonicity-Induced RVD in Molt4 Cells

The capacity of RVD was measured in Molt4 cells. Cell volume was relatively steady in the isotonic solution. Cell volume increased rapidly with exposure to the 47% hypotonic solution. Cell swelling reached its maximum in a few minutes. However, cell volume decreased gradually toward the original level when cells were still bathed in the hypotonic solution (Fig. 6). Cell volume was recovered by 56.3 ± 4.6% ($n = 10$, $P < 0.01$) in 25 min under hypotonic conditions. When the hypotonic bath solution was replaced with the control isotonic solution, cells shrank to, or became even smaller than, their original volume.

Inhibition of RVD by Chloride Channel Blockers

To study the role of chloride channels in the regulation of cell volume, the effects of chloride channel blockers NPPB, tamoxifen and ATP on the RVD induced by hypotonic stimulation were studied. Cells were pretreated with the chloride channel blockers for 5 min and then exposed to the 47% hypotonic solution for 25 min in the presence of chloride channel blockers before returning them to the isotonic control solution (Fig. 6). The results indicated that RVD was suppressed by the chloride channel blockers.

As shown in Fig. 6, the hypotonicity-induced RVD was suppressed by extracellular application of the chloride channel blockers NPPB (100 μM), tamoxifen (20 μM) and ATP (10 mM). Similar to the responses in the control group, cells were swollen rapidly when exposed to the hypotonic solution. The degree of swelling in the cells treated with chloride channel blockers was similar to or slightly larger than that in control cells. However, the level of regulatory recovery of cell volume in the hypotonic condition was decreased significantly when treated with the chloride channel blockers. In the NPPB group, the degree of RVD was 9.3 ± 3.5% ($P < 0.05$, $n = 10$). Compared with the RVD in the control cells (56.3 ± 4.6%), the RVD in the NPPB group was inhibited by

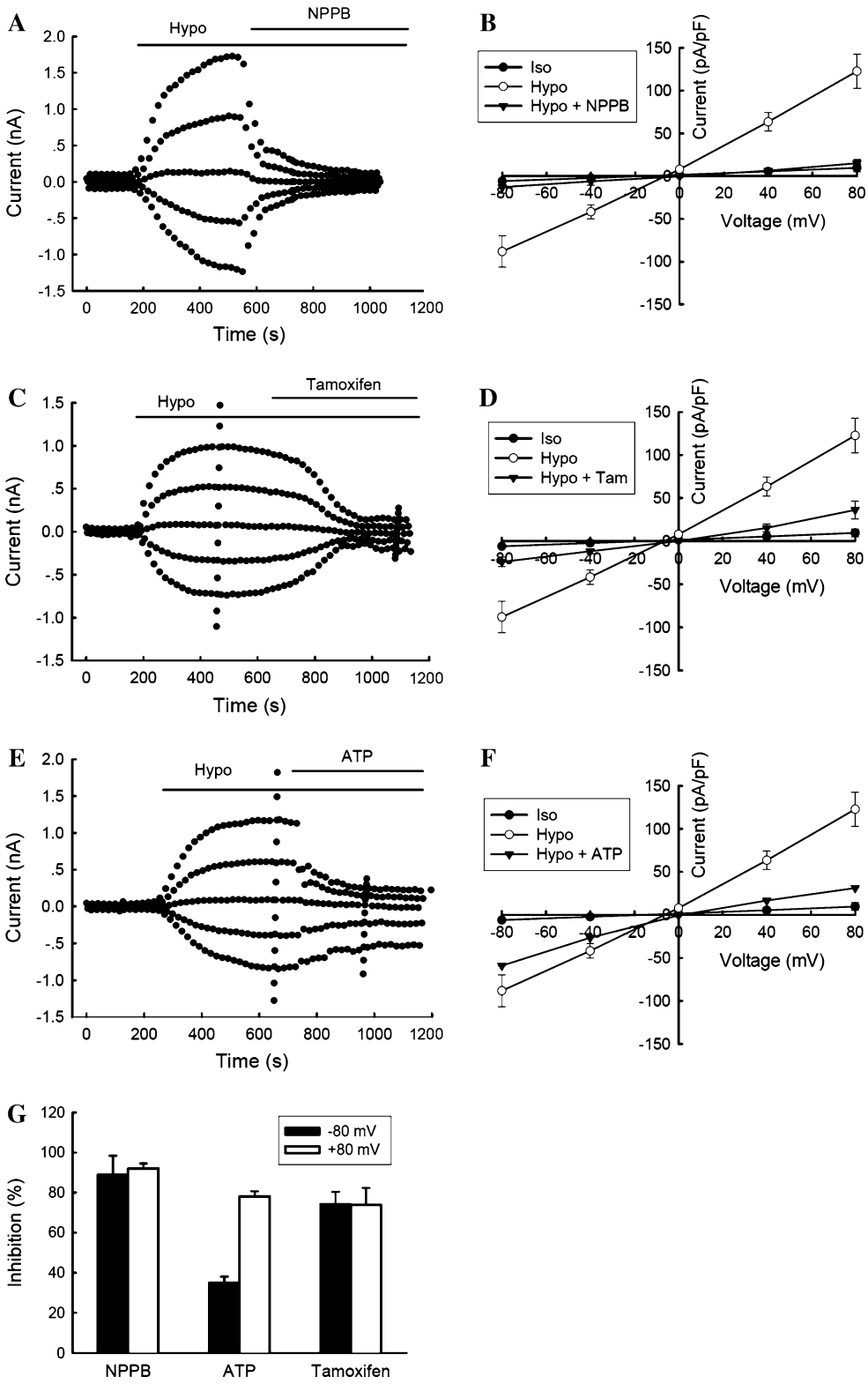

Fig. 4 Inhibition of volume-activated chloride current by chloride channel blockers NPPB, tamoxifen and ATP in human acute lymphoblastic leukemia cells. Current traces were elicited by 200-ms voltage pulses from a holding potential of 0 mV to 0, ±40 and ±80 mV with 4-s intervals between pulses. Typical time courses of activation of volume-activated chloride current induced by 47% hypotonic bath solution (*Hypo*) and inhibition of the current by the chloride channel blockers NPPB (100 μM), tamoxifen (20 μM) and ATP (10 mM) are shown in **a**, **c** and **e** and *I–V* relations are shown in **b**, **d** and **f** (mean ± standard error of 5, 6 and 5 cells, respectively). Comparison of inhibition of the hyponicity-activated current by the chloride channel blockers at +80 and −80 mV are presented in **g**

83.4 ± 5.7%. In the tamoxifen group, the degree of RVD was 17.5 ± 5.5% ($P < 0.05, n = 16$). The RVD was inhibited by 68.9 ± 5.1% when cells were treated with 20 μM tamoxifen. In the ATP group, the degree of RVD was 25.6 ± 3.5% ($P < 0.05, n = 10$). The RVD was inhibited by 54.5 ± 3.8% when cells were treated with 10 mM ATP.

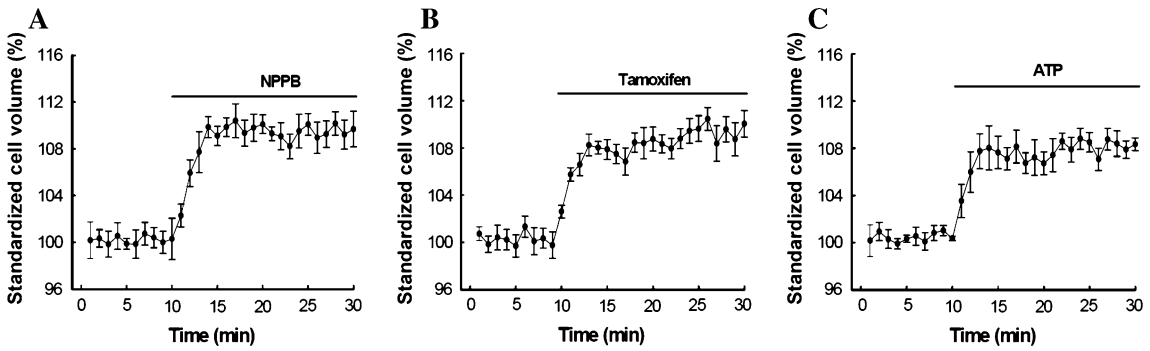

Fig. 5 Cell swelling induced by the chloride channel blockers NPPB, tamoxifen and ATP. Cells were bathed in isotonic bath solution and then exposed to the isotonic solution containing NPPB (100 μM, **a**), tamoxifen (20 μM, **b**) and ATP (10 mM, **c**). Data in **a–c** are mean ± standard error of 11, 15 and 13 cells, respectively

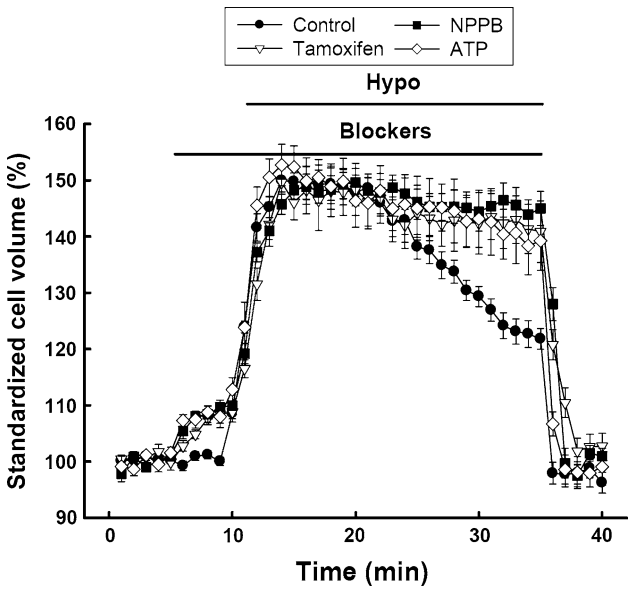

Fig. 6 Inhibition of hypotonicity-induced RVD by chloride channel blockers NPPB (100 μM), tamoxifen (20 μM) and ATP (10 mM) in human acute lymphoblastic leukemia cells. In the control group, cells were bathed in isotonic solution for 10 min, 47% hypotonic solution (*Hypo*) for 25 min and then isotonic solution for 5 min. In the treatment groups, cells were bathed in control isotonic solution for 5 min, isotonic solution containing chloride channel blockers for 5 min, 47% hypotonic solution with chloride channel blockers for 25 min and then isotonic solution for 5 min. Data are mean ± standard error of 10–16 cells

Discussion

In this study, we report for the first time the activation and activities of chloride channels under isotonic and hypotonic conditions and the roles of these channels in the maintenance and regulation of normal cell volume in human acute lymphoblastic leukemia Molt4 cells. Under the normal isotonic condition, a basal current was recorded. The current was not inactivated obviously in the observation period. In most recordings, the observation period was longer than 5 min. In some cells, the period was more than 30 min. Cells were quite small, and the cell capacitance was about 7–11 pF. The pipette solution would be completely equilibrated with the contents of the cell in 1–2 min, estimated with the method described by Pusch and Neher (1988). Thus, it is unlikely that the basal current is actually recorded under hypotonic conditions before the complete exchange of the contents of the cell with the pipette solution.

The basal current reversed at a voltage close to the calculated equilibrium potential for Cl^- (about 0 mV). In these experiments, K^+ was omitted from the electrode or bath solutions; thus, the recorded basal current was unlikely a K^+ current. The Cl^- concentration inside the cell (determined by the electrode solution) was almost equal to that outside the cell (determined by the bath solution), giving a value of −0.9 mV for E_{Cl}, which was very close to the experimental reversal potential of the recorded current. The equilibrium potentials for Na^+ and Ca^{2+} were both predicted to be greater than +200 mV. These data indicated that the basal current recorded in this study was carried mainly by Cl^-. The involvement of chloride in the basal current was further supported by our channel-blockage experiments. The basal current was inhibited significantly by extracellular application of the chloride channel blockers NPPB and tamoxifen.

Our further experiments indicated that the basal chloride current is volume-sensitive. Replacement of the isotonic bath solution with a hypertonic solution shrank the cells and suppressed the basal chloride current. This result suggests that the basal chloride current is induced by activation of volume-activated chloride channels. To study this further, the responses of cells to hypotonic challenges were observed. Exposure to 47% hypotonic bath solution swelled the cell and increased the chloride current greatly. The hypotonicity-activated chloride current shared the properties of weak outward rectification and negligible time-dependent inactivation with the basal chloride current. Furthermore, both

currents were inhibited by NPPB and tamoxifen and suppressed by the hypertonic solution. These data indicate strongly that activation of volume-activated chloride channels is responsible for both the basal chloride current and the hypotonicity-activated chloride current and suggest that some of the volume-activated chloride channels are activated under normal isotonic conditions.

What is the role of the basal chloride current? The sensitivity of the current to volume change implies that it may be involved in the maintenance of basal cell volume under normal isotonic conditions. This was confirmed by our volume-detection experiments. The results showed that cell volume was increased significantly after exposure of cells to the chloride channel blockers NPPB, tamoxifen and ATP under isotonic conditions. Our experiments also demonstrated that the capacity of RVD was attenuated by the chloride channel blockers under hypotonic conditions. These results indicate that volume-sensitive chloride channels play important roles in the maintenance of normal cell volume not only when encountering hypotonic challenges but also under isotonic conditions in lymphoblastic leukemia cells. The involvement of the volume-activated chloride current in the process of RVD induced by hypotonic stimuli has been demonstrated in many other cell types (Lang et al. 1998), but the roles and activation of the basal chloride current under isotonic conditions are not clear, especially in lymphoblastic leukemia cells. We demonstrated previously that cell volume, capacity of RVD and expression of chloride channels were cell cycle-dependent in nasopharyngeal carcinoma cells (Chen et al. 2002; Wang et al. 2002b). It was also shown by us and others that blockage of chloride channels arrested progress of the cell cycle and inhibited the proliferation of many cell types including nasopharyngeal carcinoma cells (Chen et al. 2007), cervical cancer cells (Shen et al. 2000), liver cells (Wondergem et al. 2001) and aortic smooth-muscle cells (Wang et al. 2002a), among others. These data suggest that chloride channels and cell volume regulation mechanisms play important roles in regulation of the cell cycle and cell proliferation. As the basal chloride current plays an important role in the maintenance of basal cell volume under normal isotonic conditions, as demonstrated in this study, the chloride channels responsible for the basal current may also be involved in the regulation of cell proliferation in lymphoblastic leukemia cells and may become the therapeutic target in treatments of lymphoblastic leukemia. The role of the basal chloride current in regulation of leukemia cell proliferation will be an objective of our next study.

The activation mechanism of the current is not clear. It has been demonstrated that ATP can be released from cells by an autocrine/paracrine mechanism under isotonic conditions (Lazarowski et al. 2000; Praetorius and Leipziger 2009). The maxi-anion channel has been proposed to be responsible for ATP release from cells (Bell et al. 2009; Dutta et al. 2004; Liu et al. 2008; Sabirov and Okada 2005; Shirley et al. 2009). We demonstrated previously that extracellular ATP of micromolar scale could activate a chloride current in nasopharngeal carcinoma cells (He et al. 2004). These data imply that ATP, one of the most important signaling molecules, may be involved in activation of the basal chloride current. We will investigate the activation mechanism of the basal current in our next study.

In conclusion, this study demonstrates that volume-sensitive chloride channels can be activated to induce a basal chloride current under isotonic conditions. The basal chloride current plays an important role in the maintenance of basal cell volume in human acute lymphoblastic leukemia Molt4 cells and may be a potential target in the treatment of the lymphoblastic leukemia.

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