Cl-channel blockers inhibit cell proliferation and arrest the cell cycle of human ovarian cancer cells

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Summary

Objective: To investigate the role of chloride channels in cell proliferation and cell cycles of human ovarian cancer cell line A2780. *Methods:* Chloride channel blockers were used to observe the effects of chloride channels on A2780 cells with MTT assay and flow cytometry. *Results:* NPPB (100 μM) significantly inhibited the cell proliferation and affected the cell cycle, which increased the percentage of cells in the G1 phase, and reduced it in the S phase. NFA (100 μM) and TAM (30 μM) had similar inhibitory effects. Glibenclamide (100 μM), however, had no effect on cell proliferation or cycle. Moreover, chloride channel blockers could inhibit Ca^{2+} influx in these cells. *Conclusion:* Chloride channels, voltage-gated chloride channels, and volume-sensitive chloride channels especially, play an important role in the cell proliferation and cycle of A2780 cells. It is likely that the influence of chloride channels on cell proliferation and cell cycle is mediated by a Ca^{2+}-dependent mechanism.

Key words: Chloride channels; Cell proliferation; Cell cycle; Ovarian cancer.

Introduction

Chloride channels are ubiquitous transmembrane proteins which have been implicated in salt and fluid movements across epithelia, cell differentiation and migration, cell volume regulation and intracellular organelle acidification [1-4]. According to their gating mechanisms, there are, from the functional point of view, five classes of chloride channels, including voltage-gated chloride channels (CLCs), cystic fibrosis transmembrane conductance regulator (CFTR), volume-regulated anion channels (VRACs) (also named volume/swelling-sensitive/-activated chloride channels), calcium-activated chloride channels (CLCAs), and glycine or γ-aminobutyric acid (GABA) activated channels which mainly form synaptic channels. Cl-channels are also the main targets for many drugs that change cellular function to produce beneficial effects or to cause toxicity. Cl-channel blockers inhibit cell proliferation in many types of cells, including pulmonary artery endothelial cells and liver cells [5,6]. Diverse types of Cl-channels have also been documented in tumor cells. Accumulating evidence supports the essential role of plasma membrane chloride channels in tumor cell proliferation control [7-10]. Tumor cells of unlimited proliferation are usually accompanied by an abnormal cell cycle. Lots of evidence has indicated that chloride channel activity plays an important role in cell cycle progression. For example, volume-activated Cl-currents displayed cell cycle-dependent expression in nasopharyngeal carcinoma cells which was high in the G1 phase, downregulated in the S phase, but increased again in the M phase [8]. Pharmacological blockage of VRAC causes proliferating cervical cancer cells to arrest in the G0/G1 phase, demonstrating that activity of this channel is critical for G1/S checkpoint progression [11]. These observations suggest an important role for chloride channels in cell proliferation and cell cycles.

Ovarian cancer, one of the most gynecological malignant epithelial tumors, is characterized by its high potential for unlimited proliferation and metastasis. There is a need to explore new therapeutic targets to have a better understanding of the mechanisms involved in the unlimited proliferation of ovarian carcinoma. The factors correlated with the proliferation of ovarian cancer, however, are poorly understood. Up to date, there are no data available as to whether Cl-channels are related to the cell proliferation and cell cycle progression of ovarian cancer. In this study, we investigated the representative Cl-channel expressions and the role of Cl-channels in cell proliferation and cell cycles of human ovarian cancer cell line A2780.

Materials and Methods

Reagents. 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), niflumic acid (NFA), glibenclamide, tamoxifen (TAM), propidium iodide (PI), and MTT were purchased from Sigma, MO, USA. Trizol reagent, dNTP and M-MLV reverse transcriptase were obtained from Takara, Japan. Blockers were prepared in stocks (100- to 1000-fold concentrated) in DMSO. The final concentration of DMSO in electrophysiological studies, cell proliferation and cell cycle experiments never exceeded 0.2%. We have tested that this concentration has no effect on current measurements, cell proliferation and cell cycle. Blockers were diluted to the desired final concentrations using corresponding solutions for different experiments. Fura2/acetoxymethylester (Fura2/AM) was obtained from Molecular Probes, Inc., OR, USA.

Cell culture. Ovarian cancer cell line A2780 was obtained from Basic Medicine Research Institute, Qilu hospital, Shandong University, China. Cells were cultured in 90% RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS),

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100U/ml penicillin, 100 μg/ml streptomycin and maintained at 37°C in a humid atmosphere of 5% CO_2 in air.

RT-PCR. Total RNA was isolated from the cultured A2780 cell line using the Trizol reagent according to the manufacturer's procedure. mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase. The cDNA was used for subsequent PCR using primers specific for human CLC-3, CFTR and hCLCA-2. The sequences used were: CLC-3 primer, sense, 5,- GGCAGCATTAACAGTTCTACAC-3,; antisense, 5,-TTCCAGAGCCACAGGCATATGG-3,. hCLCA-2 primer, sense, 5, -CCAAAGGAGCATTGCAGGTC -3,; antisense, 5, -ATCTCCATGTGCCCCATACC-3,. CFTR primer, sense, 5,-ACTATTGCCAGGAAGCCATT-3,; antisense, 5,-CACCGGAACTCAAGCAAGTG-3,. _-actin primer, sense, 5,-AACTCCATCATGAAGTGTGA-3,; antisense, 5,- ACTCCTGCTTGCTGATCCAC-3,. Control reactions without reverse transcriptase were performed for each PCR amplification experiment.

Electrophysiology and solutions. The whole-cell mode of the patch-clamp technique was used to measure membrane potentials and membrane currents. Currents were monitored with an EPC-9 patch clamp amplifier (HEKA Electronks, Lambrecht, Germany). Patch eletrodes had a resistance of between 3 to 5 MΩ. The step protocol consisted of a 1s voltage step, applied every 15s from a holding potential of -40 mV to test from -100 to +100 mV with an increment of 20 mV. All experiments were performed at room temperature (22~26°C). The standard extracellular medium was a Krebs solution. VRACs were activated in A2780 cells by superfusing the cells with the same solution without mannitol, resulting in a 25% hypotonicity (240 ± 5 mOsm). To measure VRAC activity, the pipette solution contained (mM): CsCl 40, caesium aspartate 100, $MgCl_2$ 1, $CaCl_2$ 0.81, BAPTA 5, Na_2ATP 4 and Hepes 10. In this pipette solution, the free Ca^{2+} concentration was buffered at 25 nM. The pipette solution was adjusted to pH 7.2 with CsOH.

Proliferation assay. MTT assay was used to analyze the effect of different Cl-channel inhibitors on A2780 cell proliferation. One hundred microliters of cell suspension (1×10^5 cells/ml) was distributed into each well of flat-bottomed 96-well culture plates. After the 24 h incubations, 100 μl reagent solutions or media at the desired blocker concentrations were distributed into each well. The well containing only media served as a positive control. Two hundred microliters of the medium alone without cells and reagent were used as a negative control. The culture plate was incubated for 48 h. Thereafter, 20 μl of the MTT dye (5 mg/ml) was added into each well. Four hours later, 150 μl of DMSO was added to each well after discarding media. The absorbance (A) values of each well at 540 nm were read. The negative control well was used for zero absorbance. The percentage of proliferation was calculated using the background-corrected absorbance as follows: proliferation rate = A of experimental well/A of positive control well × 100%.

Colony formation assay. Cells were seeded at a number of 200 per well of flat-bottomed 6-well culture plates. After the 24 h incubations, the media was removed, and 2 ml reagent solutions or media at the desired blocker concentrations were distributed into each well. The well containing only media served as a control. The culture plate was incubated for two weeks and was found to display colonies. The emerging colonies were fixed with methanol and stained with Giemsa. The colony formation rate was calculated as follows: colony formation rate = colony numbers/seeded cell numbers × 100%.

Flow cytometry. The cells were incubated for 72 h with or without chloride channel inhibitors. Approximately 10^6 cells were fixed with 1 ml ice-cold 70% methanol for 30 min. After fixing, cells were pelleted by centrifugation to remove the fixatives and stained with PI at a final concentration of 50 μg/ml. The stained cells were stored at 4°C in the dark and analyzed within 2 h. The stained samples were measured on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The data were stored and analyzed using CellQuest software to assess cell cycle distribution patterns.

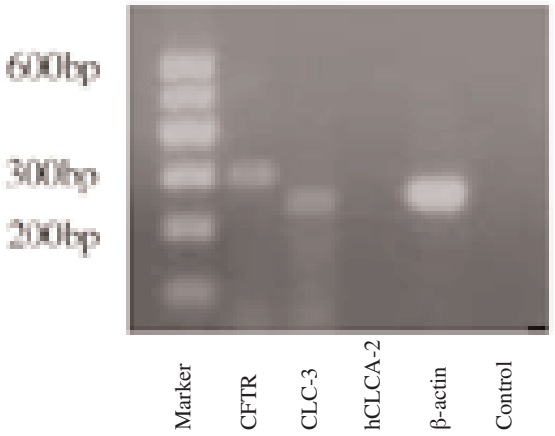

Figure 1. — RT-PCR-based detection of CLC-3, CFTR, and hCLCA -2mRNA in A2780 cells.

$[Ca^{2+}]i$ measurements. Cells were loaded with Fura2/AM for 1 h in the dark at room temperature by incubation with 10 μM membrane permeant Fura-2/AM in a physiological solution (PS) that contained 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4. The cells were then washed and resuspended in a Ca^{2+}-free PS that contained 145 mM NaCl, 1 mM MgCl2, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM EGTA, and pH 7.4. When appropriate, the cells were pretreated with Cl-channel blockers for 5 min before the initiation of Ca^{2+} influx. Ca^{2+} influx was measured by the changes in fluorescent signals as recorded by a LS-50B luminescent spectrometer at an excitation wave length of 380/340 nm and emission wave length of 510 nm.

Statistics. Data are presented as the mean ± standard error. The Student's *t*-test (SPSS version 12.0; Chicago, IL, USA) was used for statistical analyses and differences were considered significant at $p < 0.05$. All experiments were performed at least three times with representative data presented.

Results

RT-PCR assay

To determine whether Cl-channels are expressed in the A2780 cell line, RT-PCR analysis was performed using primers specific for CLC-3, CFTR, hCLCA-2 and β-actin. Figure 1 shows that PCR amplified 235-bp CLC-3, ~300-bp CFTR and 247-bp β-actin from total RNA isolated from A2780 cells. Employing the same strategy to examine the expression of hCLCA-2, no product was yielded on A2780 cells. No product was detected in the absence of reverse transcriptase yet.

Hypotonic cell volume-activated chloride channels in ovarian cancer cells

Because the VRAC has not yet been identified at the molecular level, we used whole-cell voltage-clamp recordings to detect whether volume-activated Cl-cur-

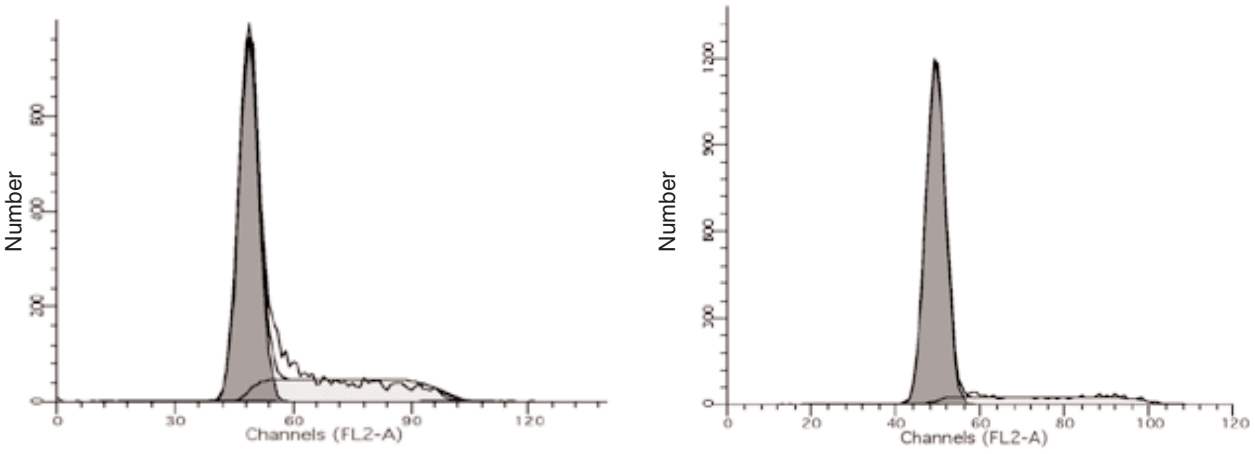

Figure 2. — The effect of different chloride channel blockers on the progression of the cell cycle. A) Cells were incubated without any chloride channel blockers. B) Cells were incubated in culture medium with 100 μM NPPB for 72 h. NPPB induced a significant increase in cells arrested in the G1 phase and a decrease in cells arrested in the S phase.

rents exist in A2780 cells. Membrane currents recorded during the step protocol applied to A2780 cells in isotonic solution were small and time independent. A hypotonic solution induced cell swelling which was accompanied by activation of large outwardly rectifying currents. The current-voltage relationship in hypotonic solution, obtained from the step protocols, reversed close to the theoretical equilibrium potential for Cl- (E_{Cl} = -25mV), indicating that the volume-regulated currents were carried mainly by Cl-. Results suggested that addition of NPPB (100 μM) inhibited the hypotonic volume-activated outward current along with the inward current of A2780 cells. Tamoxifen (30 μM) also inhibited the hypotonic volume-activated outward current and inward current of A2780 cells.

The effect of a variety of chloride channel blockers on cell proliferation and colony formation

Different channel inhibitors were used to examine the effects on A2780 cell proliferation. NPPB is a conventional non-selective chloride channel blocker. TAM is thought to exert its antiproliferative action by binding competitively to estrogen receptors (ERs) and thereby blocking the mitogenic effect of estradiol. However its effectiveness in the treatment of the estrogen-independent neoplasia indicates that TAM has other mechanisms underlying the antiproliferative action. In this study, human ovarian cancer A2780 cells did not express ERs [12], which was by using immunocytochemistry (data not shown). Tamoxifen has been demonstrated to be a specific high-affinity inhibitor of VRACs in several cell types, including endothelial cells [5]. TAM was found to specifically inhibit the volume-sensitive Cl-currents used as blockers of VRAC. Niflumic acid is considered to be able to inhibit the CLC family. CFTR is an ATP-binding cassette family, blocked by glibenclamide at 100 μM/L[13]. Results indicated that A2780 cell proliferation was inhibited by 100 μM NPPB, 30 μM TAM and 100 μM NFA. In contrast, 100 μM glibenclamide had no effect on cell proliferation (Table. 1). These data showed an important role of CLC and VRAC, but not CFTR, in the proliferation of A2780 cells.

Table 1. — *Effect of chloride channel blockers on absorbance values in cultured A2780 ovarian cancer cell lines*[a].

Control	100 ± 4
Glibenclamide (100 μM)	96 ± 6
NPPB (100 μM)	9.1 ± 0.6
Tamoxifen (30 μM)	23.4 ± 2.1
NFA (100 μM)	31.3 ± 3.4

[a] Data are presented as means ± SE in percentage (n = 8-20). Control with no inhibitor treatment is normalized to 100%.

In addition, 100 μM NPPB strongly reduced the number of colonies. A moderate decrease was also observed on treatment with NFA and TAM. The colony formation rate was 43.7 ± 1.2% in the control group (no additives), 10.6 ± 3.1%, 17.0 ± 2.3%, 21.2 ± 3.5% in the 100 μM NPPB, 30 μM TAM, and 100 μM NFA group, respectively. Compared to the control group, changes of colony formation rate at various groups were significant ($n = 6$, $p < 0.05$). Glibenclamide, however, had no effect on the number of the colonies compared to the control group. The colony formation rate was 41.5 ± 1.7% in the glibenclamide group ($n = 6$, $p > 0.05$).

The effect of chloride channel blockers on cell cycle

Results showed that NPPB at 100 μM significantly blocked the progression of the cell cycle with a 72-h treatment of A2780 cells. In NPPB-treatment cells, the proportion of G1 cells increased significantly to 84.19 ± 2.32% ($n = 8$, $p < 0.05$), while it decreased significantly to 12.14 ± 2.33% ($n = 8$, $p < 0.05$) in the S phase. NFA at 100 μM and TAM at 30 μM had a similar effect on the progression of the cell cycle to NPPB. On the other hand, no effect of 100 μM glibenclamide on cell cycles was observed (Table 2, Figure 2).

Table 2. — *Distribution of cell cycle phases of A2780 cells (%).*

Treatment	G1	S	G2/M
Control	55.41 ± 2.08	34.03 ± 2.45	9.05 ± 0.91
Glib	53.09 ± 3.21	36.89 ± 1.57	9.98 ± 0.86
NPPB	84.19 ± 2.32*	12.14 ± 2.33*	3.20 ± 0.67*
TAM	79.37 ± 3.05*	15.26 ± 1.61*	5.44 ± 0.97*
NFA	74.55 ± 2.09*	18.72 ± 1.93*	7.93 ± 0.89

A2780 cells were incubated in media without (control) or with chloride channel blockers, NPPB (100 μM), NFA (100 μM), TAM (30 μM) and Glibenclamide (100 μM). Flow cytometric analysis was carried out 72 h after treatments of A2780. Data are shown as means ± SE. n = 8,*$p < 0.05$ vs control.

The effect of chloride channel inhibitors on $[Ca^{2+}]i$

To further explore the mechanism of Cl-channel involvement in A2780 cell proliferation, we examined the effect of Cl-channel inhibitors on Ca^{2+} influx. Ca^{2+} influx was evoked by adding 0.5 mM $CaCl_2$ to the bathing medium. The addition of extracellular Ca^{2+} caused a rapid rise in cytosolic Ca^{2+} and the cytosolic Ca^{2+} level remained elevated for several minutes. Results displayed that 100 μM NPPB almost completely abolished the increase of $[Ca^{2+}]i$ evoked by the application of external Ca^{2+}. NPPB reduced the peak Ca^{2+} response by 39 ± 2.1% ($n = 5$, $p < 0.05$) while 100 μM NFA decreased the Ca^{2+} response by 28 ± 2.7% ($n = 6$, $p < 0.05$), and 30 μM TAM by 35 ± 1.7% ($n = 6$, $p < 0.05$).

Discussion

In this study we determined, for the first time, the presence of Cl-channel genes in ovarian cancer A2780 cells by using RT-PCR. Our data showed that A2780 cells expressed CLC-3 and CFTR genes. Furthermore we used whole-cell voltage-clamp recordings to find out if volume-activated Cl-currents exist in A2780 cells. Quite surprisingly, we had no way to detect the expression of hCLCA-2 genes using RT-PCR, which shows that the distributions of Cl-channels possess tissue-specific expression patterns.

Chloride channels have been implicated in the proliferation of normal as well as tumor cells. Chloride channels can control mouse cell proliferation and human mast cells [6, 14]. Volume-activated Cl-currents play an important role in human nasopharyngeal carcinoma cell proliferation [10]. In our study we observed the effect of diverse Cl-channel blockers on the proliferation of the human ovarian cancer cell line A2780. Our data showed NPPB, a non-selective inhibitor for chloride channels, NFA, a voltage-gated chloride channel inhibitor, and TAM, a volume-activated chloride channel blocker, obviously inhibited cell proliferation, whereas glibenclamide, a CFTR chloride channel blocker, had no effect on A2780 cell proliferation, indicating that CLC and VRAC were critical for A2780 cell proliferation, whereas CFTR channels might not be important. In addition to the inhibition of cell proliferation, Cl-channel activity has been tightly linked to the cell cycle. CLC-2 is regulated by cdc2/cyclinB activity, suggesting a mechanism for M phase activation [15]. The Cl-channel blocker NPPB inhibits progression through the cell cycle in synchronized NIH3T3 cells, further supporting a role in the cell cycle [16]. Our data indicated that glibenclamide had no effect on the A2780 cell cycle. NPPB, NFA and TAM, in contrast, could arrest the progression of the A2780 cell cycle. Distribution of the G1 phase obviously went up, and distribution of the S phase, surprisingly declined, thus demonstrating that Cl-channels modulated the cell cycle of A2780 cells. Taken together with the present study, Cl-channel blockers inhibited the proliferation and arrested the cell cycle of A2780 cells. This also suggests that Cl-channel blockers are a useful tool for studying the physiologic role of these channels.

How Cl-channels are involved in control or regulation of cell proliferation and cell cycle progression is, at present, not clearly understood. Cell proliferation can also be affected by intracellular pH [17]. Chloride channels can influence the intracellular pH or the pH in various organelles. CLC-3 is a representative member of the CLC family, and because it might be associated with VRAC, it attracts further interest in terms of testing whether it is involved in cell proliferation. A previous study revealed that CLC-3 chloride channels contribute to regulate intracellular pH [18]. Thus it has been postulated that CLC may affect cell proliferation by changing cellular pH. Furthermore, efficient concentrations of cyclin/cyclin dependent kinase (CDK) and other key factors used to control cell cycle progression need to be maintained by the volume-activated chloride current [7]. The volume-activated chloride current may facilitate cells to pass through the restriction point in the G1 phase. Having passed through the restriction point, cells can enter into the next cell cycle by virtue of helping cyclin/cyclin dependent kinase (CDK) even if the stimulation of growth factors is deficient. In addition, Ca^{2+} is an intracellular second messenger and correlated with a restriction point which controls progression from the G1 to the S phase [19]. The intracellular Ca^{2+} concentration which is involved in cell proliferation and cycle progression is controlled by Ca^{2+} entry pathways in the plasma membrane. Ca^{2+} affects cell proliferation by pathways of Ca^{2+}-dependent signal transduction. Blocking the Ca^{2+} signal transduction pathway can arrest the G1 phase resulting in cessation of cell progression [20, 21]. We found that NPPB, TAM and NFA abrogate the increase of $[Ca^{2+}]i$ evoked by external Ca^{2+}, indicating that chloride channel activity may regulate Ca^{2+} influx into ovarian cancer cells, and subsequently modulate the proliferation of these cells. It, however, is unknown how the chloride channels interact with the Ca^{2+} channels.

In summary, the present findings show that ovarian cancer cell line A2780 expresses CLC, VRAC and CFTR chloride channels. Chloride channels, voltage-gated chloride channels and volume-sensitive chloride channels especially, play an important role in cell proliferation and the cycle of ovarian cancer cells. It is likely that the Cl-channel activity may modulate cell volume and regulate Ca^{2+} influx into A2780 cells, therefore affecting the pro-

liferation and cycle of these cells. It should be worthwhile to investigate the possibility of using Cl-channel blockers as a new class of antineoplastic drugs.

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