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# Pharmacological and Biophysical Properties of Swelling-Activated Chloride Channel in Mouse Cardiac Myocytes

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## Abstract

**The present study was designed to observe the properties of swelling-activated chloride channel ($I_{Cl.swell}$) in mouse cardiac myocytes using patch clamp techniques. In whole-cell recordings, hypotonic solution activated a chloride current that exhibited outward rectification, weak voltage-dependent inactivation, and anion selectivity with permeability sequence of $I^- > Br^- > Cl^-$. The current was sensitive to $Cl^-$ channel blockers tamoxifen, NPPB and DIDS. In single-channel recordings, cell swelling activated a single channel current which showed outward rectification with open probability of 0.76 ± 0.08 and conductance of 38.1 ± 2.5 pS at +100 mV under [$Cl^-$] symmetrical condition. I-V relation revealed the reversal potential as expected for a $Cl^-$-selective channel. These results suggested that in mouse cardiac myocytes, swelling-activated, outward rectifying chloride channel with a single channel conductance of 38.1 ± 2.5 pS (at +100 mV under [$Cl^-$] symmetrical condition) underlies the volume regulatory $Cl^-$ channel.**

**Key Words: cardiac myocytes, cell volume regulation, swelling-activated $Cl^-$ channel, patch clamp**

## Introduction

Following cell swelling upon exposure to hypotonic solution, cells decrease volume and try to restore their original value by activating channel and transporter pathways that results in the net efflux of $K^+$ and $Cl^-$ and organic osmolytes, followed by obligatory water loss which will result in regulatory volume decrease (RVD).

Substantial evidence indicates that the activation of swelling-activated $Cl^-$ channel plays an important role in the process of RVD. It has been found and described in a number of different cell plasma membranes (10, 11, 19), including some mammalian cardiac myocytes (7, 14, 17, 20, 21). In addition to its volume regulatory role, this channel is also essentially involved in cell electrical activity in both physiological and pathological situations (2), as well as cell proliferation, cell differentiation, intracellular pH regulation, immunological response or cell cycle (16, 23) and apoptosis (13). Nevertheless, there is few investigation on the properties, especially the single channel properties of the swelling-activated chloride channel in mouse cardiac myocytes. The aim of this study was to investigate the pharmacological and biophysical properties of swelling-activated, volume-sensitive chloride channel ($I_{Cl.swell}$) in mouse cardiac ventricular myocytes.

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## Materials and Methods

*Cell Preparation*

Single ventricular myocytes were isolated from male mouse heart (2-4 months), as described previously (22). The heart was quickly isolated from mouse anesthetized with pentobarbital sodium and put into ice-cold, $Ca^{2+}$-free modified Tyrode's solution composed of (in mM): 133.5 NaCl, 4 KCl, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 10 HEPES, and 11 glucose (pH 7.4 adjusted with NaOH). The aorta was cannulated and mounted on Langendorff apparatus at 37°C. Heart was perfused with $Ca^{2+}$-free Tyrode's solution saturated with 100% $O_2$ for 5 min, then with the same solution containing bovine serum albumin (1 mg/ml) and collagenase (0.14 mg/ml; Worthington Co., Lakewood, NJ, USA) for 15-20 min. Finally, the ventricle was minced in KB solution containing (in mM) the following: 70 L-glutamic acid, 25 KCl, 20 taurine, 10 $KH_2PO_4$, 3 $MgCl_2$, 0.5 EGTA, 10 glucose, and 10 HEPES/NaOH (pH 7.4). The cell suspension was stored in KB solution at 4°C and provided for experiments within 6 h.

*Patch Clamp Recording*

Whole-cell and single-channel recording (cell-attached mode or inside-out mode) were performed at room temperature. The electrode had a resistance of 1.5-2.0 MΩ for whole-cell recording and of 4-6 MΩ for single-channel recording when filled with pipette solution. Data were obtained using an EPC-9 amplifier and Pulse software (HEKA Electroniks, Lambrecht, Germany). Current signals were low-pass filtered at 2.9 and 1.0 kHz using a four-pole Bessel filter and digitized at 10 and 4 kHz, in whole-cell and single-channel recording, respectively. Acquired data were analyzed using Origin 6.1 (Origin Lab Inc., Northampton, MA, USA). In all experiments, a grounded Ag/AgCl pellet electrode was placed in the perfusion solution. Whenever $Cl^-$ concentration in the bath solution was altered, a salt bridge containing 3M KCl in 2% agarose was used to minimize bath electrode potential variations.

In whole-cell recording, to observe the voltage and time dependency of current profile, step pulses were applied from a holding potential of -40 mV to test potentials of -100 to +100 mV in 20 mV increments after attaining the steady-state current level.

To obtain single channel I-V relations, the channel activity was measured at different levels of holding potential. Conductance and open probability ($P_o$) of single channel were calculated from data samples of 30-60 s duration in steady-state. $P_o$ was obtained from patches with only one open peak and one closed peak in the amplitude histogram from the ratio of area under the curve representing open events (fitted with a gaussian equation by a curve-fitting program using a Peak Fitting module in Origin 6.1).

*Solutions and Drugs*

For whole-cell recording, the pipette solution contained the following (in mM): 103 CsOH, 103 aspartic acid, 25 CsCl, 5 Mg-ATP, 0.3 $Na_3$-GTP, 5 EGTA, 10 HEPES and 30 mannitol (pH 7.4 adjusted with CsOH, 295 mOsm/kg $H_2O$). The isotonic bath solution contained the following (in mM): 85 *N*-methyl-D-glucamine-Cl (NMDG-Cl), 10 NaCl, 2 4-aminopyridine (4-AP), 2.5 $BaCl_2$, 0.33 $NaH_2PO_4$, 4 $MgCl_2$, 5 tetraethylammonium-Cl (TEA-Cl), 10 HEPES, 5.5 glucose, with pH adjusted to 7.4 by NMDG-OH and osmolarity adjusted to 305 mOsm/kg $H_2O$) by mannitol. Hypotonic bath solution was made by removing mannitol from the isotonic solution (220 mOsm/kg $H_2O$). Eight μM tetrodotoxin (TTX) and 5 μM nifidipine were routinely added to all bath solutions to block voltage-gated $Na^+$ channel and L-type $Ca^{2+}$ channel. For anion selectivity studies, 113 mM $Cl^-$ in bath solutions was decreased to 25 mM by replacing $Cl^-$ with gluconate.

For single-channel recording, the pipette solution contained the following (in mM): 85 NMDG-Cl, 10 NaCl, 2 4AP, 2.5 $BaCl_2$, 0.33 $NaH_2PO_4$, 4 $MgCl_2$, 10 HEPES, 5.5 glucose, 5 TEACl, 8 μM TTX, 5 μM nifidipine. With pH adjusted to 7.4 by NMDG-OH, and osmolarity adjusted to 305 mOsm/kg $H_2O$) by adding mannitol. Isotonic bath solution contained (in mM): 113 NMDG-Cl, 5 EGTA, 5 HEPES, 8 μM TTX, 5 μM nifidipine, with pH adjusted to 7.4 by NMDG-OH, and osmolarity adjusted to 305 mOsm/kg $H_2O$) by mannitol. Hypotonic bath solution (220 mOsm/kg $H_2O$) was made by removing mannitol from the isotonic solution and adding 5 MgATP, 0.3 $Na_3$-GTP in it. To evaluate the $Cl^-$ selectivity, the equal molar $Cl^-$ was replaced by gluconate in the bath solution. The osmolarity of all solutions was measured using a freezing-point depression osmometer (OM802, Vogel, Germany).

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents except for TTX (in distilled water) were first dissolved in dimethylsufoxide (DMSO) as stock solution, and then further diluted in the corresponding bath solution. The vehicle alone at the employed concentration (< 0.15%) never affected membrane currents.

*Statistical Analysis*

Data were expressed as means ± SEM, and n

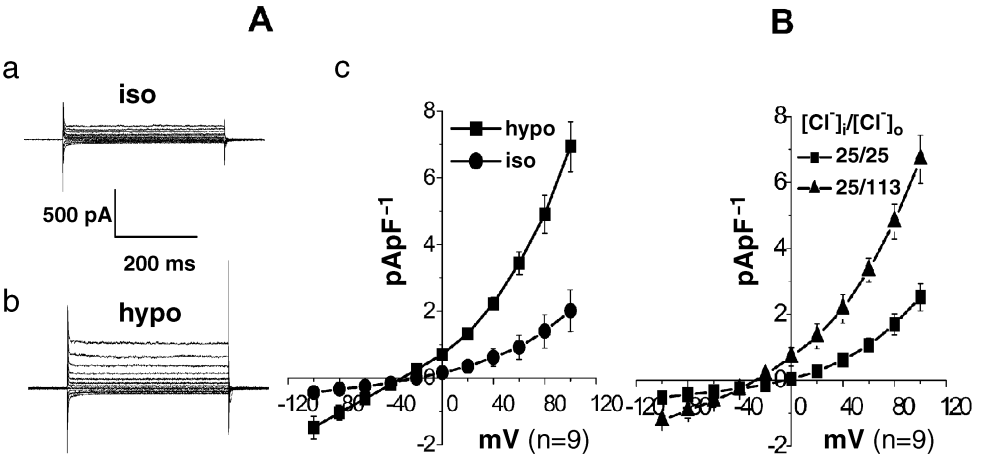


Fig. 1. Whole-cell recordings of swelling-activated $Cl^-$ currents in cardiac myocytes isolated from mouse. A: Representative current responses to step pulses (from -100 to +100 in 20-mV increments) during exposure to isotonic (a) and hypotonic (b) solution and I-V relation (c). Hypotonic data points (squres) at given voltages between 0 and +100 mV are significantly different from isotonic data points (circle points). B: I-V relationships of instantaneous currents recorded during cells were exposed to hypotonic solution with different extracellular $[Cl^-]_o$ concentration (113 mM and 25 mM). Each symbol represents the mean ± SEM (vertical bar) of 9 experiments.

represented the number of observations. Statistical differences of the data were evaluated by paired or unpaired Student's *t*-test or one-way ANOVA and post hoc multiple comparison tests. Differences between groups were considered significant when $P < 0.05$.

## Results

### *Whole-Cell Current of Swelling-Activated $Cl^-$ Channel in Mouse Cardiac Ventricular Myocytes*

To examine swelling-activated $Cl^-$ channel in mouse cardiac myocytes, whole-cell patch clamp recording was employed. Under isotonic conditions, the currents were small and stable. Cells swelled after superfusion with hypotonic bath solution (220 mOsm/kg $H_2O$) for about 15-30 min after perfusion. Meanwhile, swelling-activated currents increased gradually and reached a plateau level after around 20 min, and were reversible when returning to isotonic solution. Fig. 1A showed the current profile under isotonic (Fig. 1Aa), hypotonic (Fig. 1Ab) condition, and I-V relation (Fig. 1Ac). The currents exhibited moderate outward rectification. Current densities under isotonic and hypotonic condition were 2.08 ± 0.28 pA/pF and 7.21 ± 0.97 pA/pF, respectively at +100 mV. The $Cl^-$ selectivity of the channel was demonstrated by measuring the reversal potential ($E_{rev}$) at two different extracellular $Cl^-$ concentrations. The results were shown in Fig. 1B, when the extracellular $Cl^-$ concentration was reduced from 113 mM to 25 mM by replacing with gluconate, the reversal potential shifted from -34.5 ± 0.8 mV to -3.7 ± 1.2 mV. The results revealed a slope of 57 mV per 10-fold increase in extracellular $Cl^-$ concentration. This was close to the theoretical value for a $Cl^-$ selective channel. These data indicated that the swelling-activated currents were highly selective for $Cl^-$.

Anion selectivity of the channel was examined in the hypotonic solution in which $Cl^-$ ions were replaced with other anions. Anion permeability sequence for $I^-$, $Br^-$ and $Cl^-$ evaluated by the reversal potentials was 2.32 : 1.28 : 1 (n = 6), corresponding to Eisemman's sequence I. So the obtained permeability sequence from the reversal potential was $I^- > Br^- > Cl^-$.

### *Sensitivity to $Cl^-$ Channel Blockers of Swelling-Activated $Cl^-$ Channel in Mouse Cardiac Ventricular Myocytes*

Swelling-activated $Cl^-$ whole-cell currents were rapidly blocked by a carboxylate analogue $Cl^-$ channel blocker, NPPB (5-nitro-2- (3-phenylpropylamino)-benzoate). The addition of NPPB 100 μM in the bath solution inhibited both outward current (79.1 ± 2.5%) and inward current (77.1 ± 2.2%), respectively ($P < 0.01$) (Fig. 2A). The blockade effect was fully reversible.

Disulfonic stibene compounds are also effective blockers of a variety of cardiac and noncardiac $Cl^-$ currents (1, 4, 25). Therefore, we assessed the effect of diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS), a member of this class, on whole-cell current activity in mouse cardiac myocytes. As shown in Fig. 2B, the outward currents were strongly suppressed about 90.5 ± 2.1% ($P < 0.01$) at +100 mV in a voltage-dependent manner by addition of 500 μM DIDS to the bath solution.

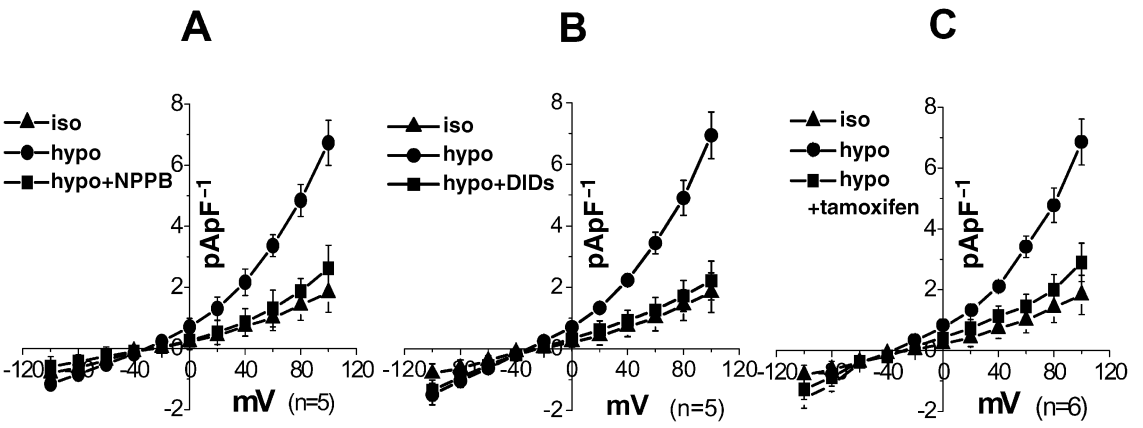


Fig. 2. Sensitivity of swelling-activated $Cl^-$ currents to $Cl^-$ channel blockers. I-V relationships of instantaneous currents recorded before and during exposure to hypotonic solution in the absence and presence of NPPB (A), DIDS (B) and tamoxifen (C). Each symbol represents the mean ± SEM (vertical bar) of 5 or 6 experiments. Hypotonic control data points (circles points) at given voltages between 0 and +100 mV are significantly different from isotonic data points (triangles) and from hypotonic plus blocker data points (squares).

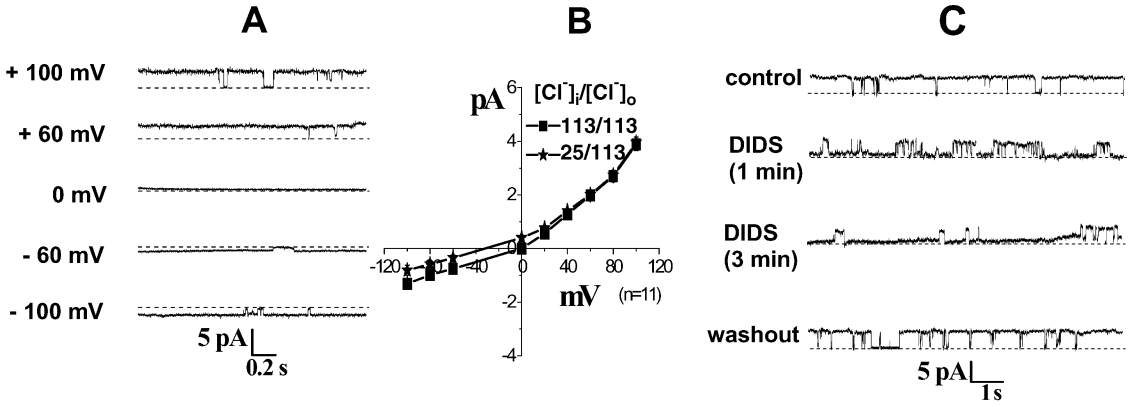


Fig. 3. Properties of single channel currents activated by hypotonic solution in an inside-out patch clamp recording. A: Tracings of channel currents obtained at various membrane potentials in the presence of a symmetrical $Cl^-$ gradient. B: An average I-V relationships of 11 patches studied at different value of $[Cl^-]$ gradients. Each symbol represents the mean ± SEM (vertical bar) of 11 experiments. C: Blockade effect of 500 μM DIDS on hypotonic activated $Cl^-$ single channel currents. The data was a single patch recorded at +100 mV under symmetrical $[Cl^-]$ condition.

Recent studies have shown that tamoxifen (an antiestrogen) is a selective and potent inhibitor of swelling-activated $Cl^-$ channel in cardiac myocytes (21). In this experiment as shown in Fig. 2C, 50 μM tamoxifen inhibited the current significantly (73.1% ± 1.2% at + 100 mV).

*Single Channel Properties of Swelling-Activated $Cl^-$ Channel in Mouse Cardiac Ventricular Myocytes*

Under isotonic condition (305 mOsm/kg $H_2O$), most patches shows no single-channel activity in cell-attached mode. Superfusion with hypotonic bath solution (220 mOsm/kg $H_2O$), about 15-30 min after perfusion, cells swelled obviously. Then cell-attached mode was made and some patches showed single channel open events. The onset of the channel opening was about 18±9 min after perfusion of hypotonic solution. Once a channel activated by swelling was recorded, the patches were excised and inside-out recordings were established.

In inside-out patch recordings, the channel opening was observed at membrane potentials from -100 to +100 mV and was stable for 3-6 min. Fig. 3A showed an example of a patch with single-channel activity in inside-out recording at various transmembrane potentials at symmetrical condition. The absolute ratio value of the mean current was 3.81 ± 0.25 pA at +100 mV and -1.32 ± 0.16 pA at -100 mV (n = 11). The I-V relation was shown in Fig. 3B. The reversal potential turned

out to be more negative when the intracellular $Cl^-$ concentration was decreased from 113 mM to 25 mM.

As in the whole-cell recording, we tested the effect of DIDS on the single channel activity. The addition of 500 μM DIDS to the bath solution (intracellular side of the patch) rapidly inhibited channel opening by increasing closed time and causing increased flicking during the open state (Fig. 3C). At +100 mV, the Po was reduced to 0.23 ± 0.07 from 0.76 ± 0.08 ($P < 0.01$, n = 6). This blockade effect is reversible after washout.

## Discussion

The activation of $I_{Cl.swell}$ is believed to provide one of the initial triggers linking cell swelling to the subsequent loss of osmolytes and water resulting in RVD (5). It appears to be ubiquitously expressed and has been observed in many cardiac cell types including canine cardiac myocytes, rabbit atrial (17, 20), sinoatrial myocytes (7), cultured chick myocytes (15), guinea pig cardiac myocytes (21) and feline ventricular myocytes (6). The macroscopic currents activated by cell swelling in these studies exhibited similar properties, including sensitivity of reversal potential to changes in the $Cl^-$ gradient and pronounced outward rectification. In rabbit atrial and sinoatrial cells, $I_{Cl.swell}$ was further characterized by an anion permeability sequence of $SCN > I > NO_3 > Br^- > Cl^- > F^-$ (7). In guinea pig cardiac myocytes, anion permeability sequence was $I^- > NO_3^- > Br^- > Cl^- > Asp^-$. Consistently, the swelling-activated $Cl^-$ channel currents in mouse cardiac ventricular myocytes in this experiment also have the same high $Cl^-$ selectivity, outward rectification, and anion permeability sequence of $I^- > Br^- > Cl^-$. These results are in good agreement with that in other cardiac myocytes. Nevertheless, the time and voltage-dependent inactivation are not as apparent as that in guinea pig myocytes (21) and that in epithelial cells (12). Because of the limited number of studies that have attempted to measure unitary currents responsible for $I_{Cl.swell}$ in the heart, it is difficult to predict whether or not $I_{Cl.swell}$ is uniformly expressed with similar properties in all types of mammalian cardiac cells. In this experiment, even though we have recorded and observed the properties of single channel activated by cell swelling in inside-out recording, it might be better for us to use on-cell patch clamp recordings to investigate this channel because it is difficult for us to explain why excised patch still have active channel. In this experiments, under isotonic solution, most patches have no active channel. However, under hypotonic condition, we can record the active channel on cell-attached mode and it was still open after excised for several min. There is a concept that swelling-activated chloride channel activation is induced by unfolding of the membrane folds. Maybe this can explain our results.

$I_{Cl.swell}$ in cardiac myocytes, like in many other cell types (11,19), is blocked by millimolar concentration of stilbene derivatives SITS, DIDS (18, 20, 21). The blockade effect by these compounds is usually voltage-dependent, with outward currents more effectively being inhibited, compared with inward currents. The most extensive characterization of sensitivity of $I_{Cl.swell}$ in heart to inhibitors was made by Sorota (18) in canine atrial myocytes. NPPB (10-40 μM) produced a complete blockade of $I_{Cl.swell}$, and DIDS (100 μM)-blocked outward currents more effectively than inward currents. One of the most potent inhibitors of $I_{Cl.swell}$ is the antiestrogen compound tamoxifen, which at 10 μM has been shown to nearly completely block $I_{Cl.swell}$ in guinea pig atrial and ventricular myocytes. Consistently, in mouse cardiac ventricular myocytes, the swelling-activated $Cl^-$ channel was found also showing sensitivity to NPPB, DIDS and tamoxifen, but it needs a relatively higher concentration.

The activities of swelling-activated $Cl^-$ channels imply that not only in RVD but also in many other physiological cell activities, the $Cl^-$ channels are associated with changes in cell volume or shape. Its pathological implications have been pointed with respect to electrical instability of the heart. Because the $E_{Cl^-}$ is near -50 mV and $I_{Cl.swell}$ exhibits marked outward rectification, activation of $I_{Cl.swell}$ during cell swelling would apparently increase outward, repolarizing current during the action potential plateau and a small increase in inward, depolarizing current near the cell's resting potential. Both the action potential shortening and membrane depolarization resulting from activation of $I_{Cl.swell}$ can accelerate the development of reentry arrhythmias (8). During ischemia-reperfusion, cell swelling does occur presumably as a result of increases in tissue osmolarity which will change the activity of $I_{Cl.swell}$ (9, 24). The changes in electrical activity would contribute to the development of cardiac arrhythmias in this condition. Nevertheless, $I_{Cl.swell}$-induced action potential shortening may be antiarrhythmic during cardiac hypertrophy (3). Thus more intensive investigation of functional properties and molecular biological identity of swelling-activated $Cl^-$ channel as well as its pharmacological characteristics has physiological and pathological significance.

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