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Biophysical Characteristics of Swelling-Activated Cl^- Channels in Human Tracheal 9HTEo-Cells

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Abstract. The question of whether a single molecule can account for every observed swelling-activated Cl^- current deserves to be addressed and biophysical description seems to be an adequate criterion to classify these channels. We studied the biophysical properties of swelling-activated Cl^- currents in 9HTEo-cells using whole-cell and outside-out patch clamp recordings. Hypotonic shock activated outwardly rectifying currents that inactivated at potentials higher than 20 mV. The decay phase of the current was well fitted by two exponential functions and both time constants were voltage-dependent. Two voltage-dependent time constants were also necessary to describe reactivation. The midpoint of current inactivation was 54 mV. The voltage dependence of kinetics did not significantly change by modifying the extracellular NaCl concentration while the inactivation midpoint slightly shifted. In conclusion, our results indicate that the voltage-dependent properties of the swelling-activated Cl^- currents in 9HTEo- cells are largely independent from the extracellular ionic strength and the extracellular Cl^- concentration.

Excised patches from cells exposed to hypotonic shock showed single channel currents that inactivated at positive membrane potentials and displayed chord conductance of ~60 pS at 100 mV and of ~20 pS at −80 mV. The permeability sequence for the single channel was $I^- > Br^- > Cl^-$ > gluconate and currents were blocked by Reactive blue 2. These properties indicate that intermediate conductance outwardly rectifying channels are responsible for the macroscopic swelling-activated current.

Key words: Cl^- channels — Patch clamp — Swelling-activated Cl^- currents — Single channels — Human tracheal cells

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Introduction

The role of anion channels in cell volume regulation has received particular attention. Cells maintain their volume within a narrow range even when exposed to osmolarity variations. The cell swelling caused by extracellular osmolarity decrease is followed by activation of independent Cl^- and K^+ pathways (Hoffmann, Simonsen & Lambert, 1984). The resulting KCl efflux with obligated water restores the initial cell volume. Osmotic loss of water is driven not only by these ions but also by the efflux of small organic osmolytes which leave the cell through a volume-sensitive Cl^- channel (Jackson & Strange, 1993; Ballatori et al., 1995; Boese, Wehner & Kinne, 1996; Galietta et al., 1997). The ionic current flowing through these channels is characterized by outward rectification of the current-voltage relationship, by a permeability sequence of $I^- > Br^- > Cl^-$ and by current inactivation at very positive membrane potentials (Ackerman et al., 1994; Basavappa et al., 1995; Gosling, Smith & Poyner 1995; Jackson & Strange, 1995*a*,*b;* Levitan & Garber, 1995; Meyer & Korbmacher, 1996). However, other studies pointed to swelling-activated Cl^- channels exhibiting characteristics different from those previously mentioned. For example, swelling-activated Cl^- currents that do not inactivate have been reported (Doroshenko & Neher, 1992; Lewis, Ross & Cahalan, 1993; Arreola, Melvin & Begenisich, 1995; Best, Sheader & Brown, 1996; Szücs et al., 1996*b*) and different permeability sequences have been described (Best et al., 1996; Winpenny et al., 1996). The literature also provides contradictory data at the single channel level. Probably because of the difficulties of recording single channel events, the properties of the channel underlying swelling-activated Cl^- currents are still not clearly identified. Indeed, channels of low (Doroshenko & Neher, 1992; Ho, Duszyk & French, 1994; Lewis et al., 1993; Nilius et al., 1994) and intermediate (*see* for review

Hoffmann & Dunham, 1995; Strange & Jackson, 1995) conductance have been described. Furthermore, the transduction mechanism leading from cell volume change to channel activation is as yet undetermined. Second messengers may be involved in the activation and regulation of the swelling-activated Cl^- channel. Nevertheless, the role of intermediates seems to vary from one preparation to another. Contradictory data on the sensitivity to intracellular calcium (Basavappa et al., 1995; Gosling et al., 1995; Galietta, et al., 1997), on the role of the cytoskeleton (Foskett & Spring, 1985; Oike et al., 1994; Schwiebert, Mills & Stanton, 1994; Levitan et al., 1995; Nilius, Eggermont & Voets, 1996) and on the requirement of intracellular ATP to maintain the Cl^- conductance (Lewis et al., 1993; Ballatori, Simmons & Boyer, 1994; Rasola et al., 1994; Jackson, Morrison & Strange, 1994; Gosling et al., 1995; Verdon et al., 1995; Best et al., 1996; Meyer & Korbmacher, 1996; Szücs et al., 1996*a*) have been reported. All this variability suggests the existence of different types of swelling-activated Cl^- channels and of cell type specific regulatory pathways. Nevertheless, the variability could be ascribed, at least in part, to several variables of experimental conditions, including the osmotic gradient, the ionic strength or the permeant anion concentration. Therefore, detailed descriptions of the biophysical properties of these channels and accurate control of experimental conditions would probably help to clarify the problem.

Human tracheal 9HTEo- cells respond to hypotonic challenge by activating a Cl^- current, $ICl_{(vol)}$ (Rasola, et al., 1992; Rasola et al., 1994; Galietta et al., 1997), which has a permeability sequence of $I^- > Br > Cl^-$ and it is also permeable to the small amino acid taurine (Galietta et al., 1997). Here, we present the kinetic parameters of macroscopic $ICl_{(vol)}$ and the single channel conductance and selectivity. We found that the properties of voltage-dependent inactivation and reactivation are largely independent from the extracellular ionic strength and Cl^- concentration of the extracellular solution. Single channel recordings suggest that an outwardly rectifying Cl^- channel of intermediate conductance (~60 pS at 100 mV), with a permeability sequence of $I^- > Br^- > Cl^-$ and blocked by Reactive blue 2 is responsible for the macroscopic $ICl_{(vol)}$.

Materials and Methods

Cell Culture

9HTEo- cells were obtained from human tracheal epithelium by transformation with the SV40 large T antigen (Gruenert et al., 1988). Cells were grown in 35 mm plastic Petri dishes in Dulbecco's modified Eagle's medium and Ham's F12 (Hyclone, Utah) 1:1 with 10% serum (Fetal Clone II, Hyclone), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in 5% CO_2. Cells were seeded at a density of 25,000 cells/dish and used in the first to third day after plating, when cells were smaller and isolated, to allow good voltage control. Cells had a capacitance of 21.6 ± 0.6 pF (mean ± SEM, n = 60).

Electrophysiology

Macroscopic and single channel currents were recorded in the whole-cell and outside-out configurations of the patch-clamp technique, respectively. Borosilicate glass pipettes were pulled and fire polished to obtain resistances in the range 1–3 MΩ, as measured in the working solution. For single channel experiments the pipettes were coated with Sylgard (Dow Corning, Wiesbaden, Germany). Cells were used only when access resistance was less than 6 MΩ. Currents were measured with a standard patch-clamp amplifier (EPC-7, List, Darmstadt, Germany). Stimulation and data acquisition were carried out with a 16-bit AD/DA converter (ITC-16, Instrutech, New York) controlled by a personal computer (Atari, Mega/STE, CA). For whole-cell the signal was low-pass filtered with a 4-pole Bessel filter (4302, Ithaca, New York) set at a cutoff frequency of 1 kHz and sampled at 2 kHz. For single channel the signal was filtered at a cutoff frequency of 2.5 kHz and sampled at 5 kHz. To minimize liquid junction potentials, the reference electrode was connected to the bath through a 0.5 M KCl filled agar bridge. Junction potentials (about zero with halides and 4 mV with gluconate) were measured and used to correct for the reversal potential of membrane currents. Solutions were changed by a gravity-based perfusion system with the tip of the inflow pipette near the patched cell. To ensure that other mechanosensitive currents were not activated during perfusion opening, all experiments were done under continuous perfusion. All experiments were performed at room temperature (21–24°C).

Statistics

Group data are presented as mean ± SEM and significances were evaluated by Student t test for unpaired data.

Solutions

Pipettes were filled with the following solution (in mM): 60 CsCl, 45 Cs_2SO_4, 1 $MgCl_2$, 0.5 EGTA, 10 Hepes-CsOH, 45 Mannitol, at pH 7.3 (277 mosm/kg). The extracellular solution contained (in mM): 130 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes-NaOH, 10 Glucose, 30 Mannitol, at pH 7.3 (298 mosm/kg). Standard hypotonic extracellular solution contained (in mM): 117 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes-NaOH, 10 Glucose, 13 Mannitol, at pH 7.3 (265 mosm/kg).

The hypotonic solution described above was obtained by a reduction of mannitol and of NaCl concentration with respect to the isotonic solution. To study the effect of the ionic strength on voltage-dependent parameters we used two hypotonic solutions with different ionic strength. A high-ionic-strength hypotonic solution was prepared as the standard isotonic solution but omitting mannitol (pH 7.3, 263 mosm/kg). A low-ionic-strength hypotonic solution was prepared by reducing the NaCl concentration while increasing mannitol to maintain the osmolality. This solution contained (in mM): 50 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes-NaOH, 10 Glucose and 135 Mannitol (pH 7.3, 266 mosm/kg).

To study the relative permeability of single channels we used the high-ionic-strength hypotonic solution, where 130 NaCl was equimolarly replaced by NaI, NaBr or Na-gluconate. All chemicals were purchased from Sigma (St. Louis, MO).

Results

Current-Voltage Relationship

The reduction of bath osmolality from 295 to 265 mosm/kg by replacing the isotonic with the standard hypotonic solution activated currents ($ICl_{(vol)}$ that reached a stable value in about 10 min. The holding potential was kept at −5 mV. Membrane currents were recorded in response to 2-sec pulses to voltages in the range −100 to 120 mV. Pulses were preceded by a 2-sec pulse to −100 mV to allow full recovery of the channels from voltage-dependent inactivation. The currents peaked instantaneously and, at membrane potentials higher than 20 mV, inactivated to a non-zero steady-state level (Fig. 1*A*). The current-voltage relationship of the peak current (I_p) and of the current measured at the end of the 2-sec long pulse (I_s) is depicted in Fig. 1*B*. The I_p-voltage relationship was outwardly rectifying. The reversal potential −10.9 ± 0.4 mV ($n = 21$) approximates the theoretical value of −13 mV, estimated considering the relative sulfate permeability. $P_{SO4}/P_{Cl} = 0.12$ (Rasola et al., 1992).

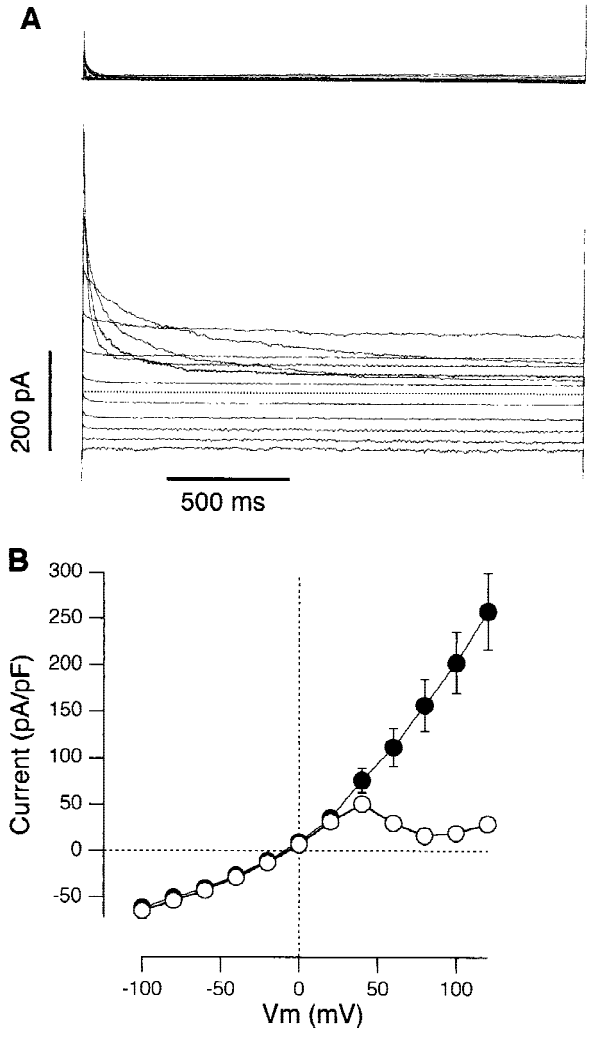

Fig. 1. Current-to-voltage relationship. (*A*) Current traces from one representative experiment before (upper trace) and after (lower trace) the reduction of bath osmolality from 295 to 265 mosm/kg. Holding potential was kept at −5 mV. Currents were recorded in response to 2-sec voltage pulses in the range −100 to 120 mV. Test pulses were preceded by a 2-sec prepulse to −100 mV to allow recovery from inactivation. The dotted line indicates the zero-current level. (*B*) Current to voltage relationship of $ICl_{(vol)}$. Each point represents the mean of 10 experiments and vertical bars are standard errors of the mean (SEM). I_p (closed symbols) and I_S (open symbols) were measured at 7 and 1995 msec of the voltage pulse respectively and corrected by membrane capacitance.

Steady-State Inactivation

The amplitude of the currents decreased when positive prepulses were used. The voltage dependence of the steady-state inactivation was estimated from measurements of I_p in response to a 2-sec long test pulse to 120 mV following conditioning prepulses (V_{pp}) of 2 sec to potentials between −100 and 120 mV (Fig. 2*A* and *B*). As the duration of the test pulse is about 12 times longer than the slowest inactivation time constant at 120 mV (*see* next section), it can be considered that the current reached steady state. Thus, the current measured at the end of the pulse, I_S, represents the non-inactivating current. At 120 mV, where steady-state inactivation was evaluated, I_S was constant and represents 10.4 ± 0.6% of I_{pmax}. The inactivable fraction of the current, I_{in}, defined as the difference between the peak and steady-state current, $I_{in} = I_p - I_S$, was fitted to a Boltzmann function:

$$\frac{I_{in}}{(I_{in})_{max}} = \frac{1}{1 + \exp\left(\frac{V_{pp} - V_{hi}}{a}\right)} \quad (1)$$

where $(I_{in})_{max}$ is the maximum value of I_{in}, V_{hi} is the half-inactivation potential and a is the steepness of the voltage-dependence of inactivation. The steady-state inactivation parameters in 16 different experiments were $V_{hi} = 54.2 \pm 1.4$ mV, and $a = 8.9 \pm 0.4$ mV.

Inactivation Kinetics of the Currents

The inactivation kinetics of $ICl_{(vol)}$ was evaluated on pulses of 2.5 sec at test potentials equal or higher than 40 mV. The decay phase of the current was well fitted with a double exponential function (*see* Fig. 3*A*):

$$I(t) = I_{fast}e^{(-t/\tau_{fast})} + I_{slow}e^{(-t/\tau_{slow})} + I_n \quad (2)$$

where I_{fast} and I_{slow} are respectively the current contribution that inactivated with the fast and slow time constants, τ_{fast} and τ_{slow} respectively, and I_n is the non-inactivating current at a given potential. In Fig. 3*B* are

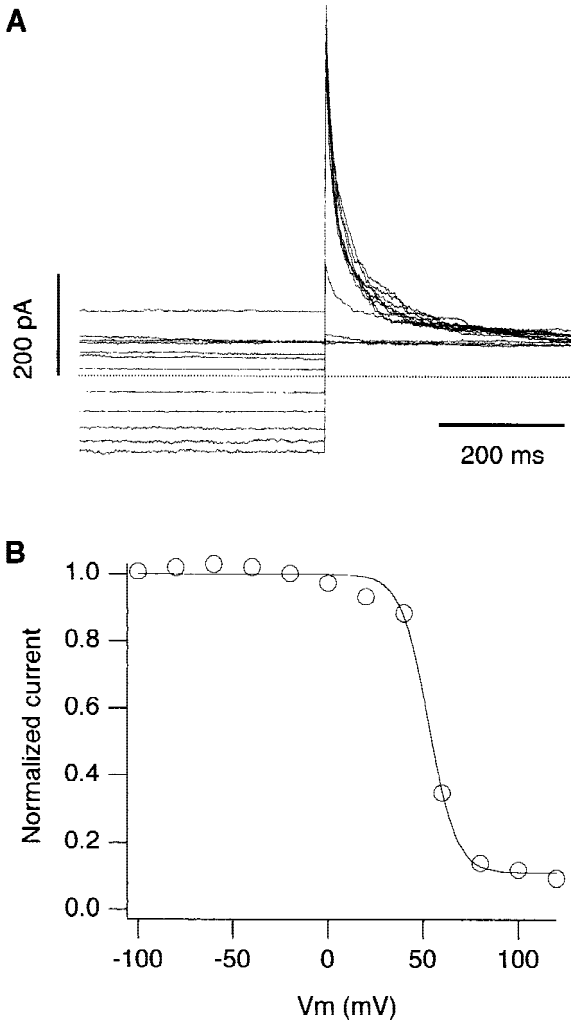

Fig. 2. Steady-state inactivation. Steady-state inactivation was evaluated with a double pulse protocol. Instantaneous currents were measured at 120 mV following prepulses of 2 sec to various membrane potentials from −100 to 120 mV. (*A*) Traces showing the instantaneous current at 120 mV. (*B*) The normalized peak current, I_p/I_{pmax} at 120 mV was plotted *vs.* the potential of the conditioning prepulses and fitted to Eq. 1. This experiment yields V_{hi} = 52.9 mV and the slope parameter a = 7.3 mV. The fraction of non-inactivating current is 0.1.

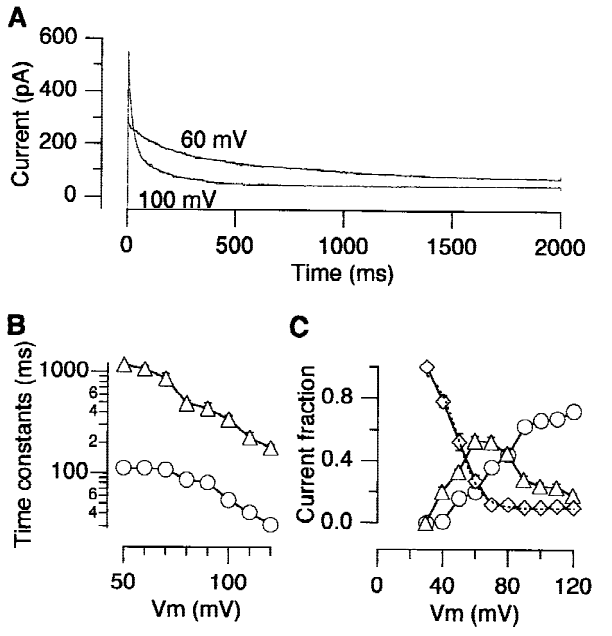

Fig. 3. Inactivation kinetics of $I\mathrm{Cl}_{(vol)}$. (*A*) Inactivation kinetics of $I\mathrm{Cl}_{(vol)}$ current evoked from a holding potential of −100 mV at test pulses of 100 and 60 mV. The decay phase is well fitted by two exponentials. (*B*) Voltage dependence of the fast (circles) and the slow (triangles) time constants. (*C*) Contribution of fast (circles), slow (triangles) and non-inactivating (diamonds) current fractions to total current at different membrane potentials. Each point represents mean and SEM of 8 to 10 different cells. The dashed line represent the fit of non-inactivating current fraction to a Boltzmann function.

represented the fast and slow time constants measured in 8 different experiments, as a function of the membrane potential. Both time constants were voltage-dependent and decreased with increasing potentials. The time constant of the fast component, τ_{fast}, changed from 30 msec at 120 mV to 120 msec at 50 mV and that of the slow component, τ_{slow}, between 200 msec at 120 mV to 1200 msec at 50 mV. The contribution of each component changed with the membrane potential. Figure 3*C* presents the fractions of the total current that inactivated with fast and slow time constants and the non-inactivating current fraction as a function of the test potential. The fast component increased monotonically from zero at 40 mV to 0.7 at 120 mV. The slow component increased from zero at 30 mV, reached a top value of about 0.5 between 60 and 70 mV and then decreased as the membrane potential further increased. The non-inactivating component decreased steeply above 30 mV reaching a minimal value of 0.1 that remained constant from 70 to 120 mV. As expected, the voltage-dependence of non-inactivating fraction mimics the voltage-dependence of the steady-state inactivation. Indeed, the fit of the non-inactivating component with a Boltzmann function yielded a V_{hi} = 49.8 mV, and a = 6.9 mV.

Recovery from Inactivation

To study the reactivation kinetics of $I\mathrm{Cl}_{(vol)}$ currents we used a double pulse protocol. Channels were inactivated with a prepulse of 1 sec to 100 mV. The prepulse was followed by test pulses of 2.5 sec to different voltages between −100 and −20 mV. Membrane potential hyperpolarization resulted in reactivation of $I\mathrm{Cl}_{(vol)}$. The time course of current reactivation was fitted with a double exponential function. The voltage-dependence of recovery from inactivation is shown in Fig. 4. Both time con-

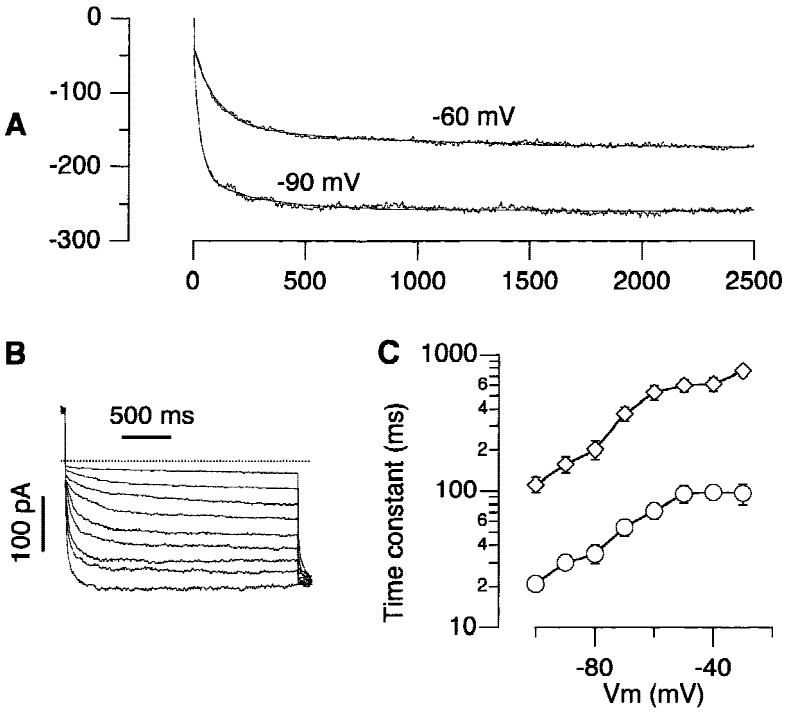

Fig. 4. Recovery from inactivation of $I\mathrm{Cl}_{(vol)}$. Channels were inactivated with a 1-sec prepulse to 100 mV. The prepulse was followed by test pulses of 2.5 sec to different voltages between −100 and −30 mV. In (*A*) two representative traces, at −60 and −90 mV, with the corresponding fits are shown. Reactivation is well described by the sum of two exponential functions. (*B*) Family of reactivating currents of the same experiment. (*C*) Mean and SEM of reactivation fast (circles) and slow (diamonds) time constants of 12 different experiments is shown as a function of membrane potential.

stants increased with increasing potentials. The fast component τ_{fast} changed from near 20 msec at −100 mV to about 100 msec at −30 mV, and τ_{slow} between 100 msec at −100 mV to 800 msec at −30 mV.

Sensitivity of Voltage-Dependent Parameters to Extracellular Ionic Strength

We repeated the study of voltage-dependent parameters using hypotonic solutions with the same osmolarity but with different ionic strength (*see* Materials and Methods). The steady-state inactivation parameters in 8 experiments using the high-ionic-strength hypotonic solution ($[\mathrm{Cl}]_o$ = 138 mM) were V_{hi} = 58.2 ± 2.1 mV, a = 8.5 ± 1.2 mV and the non-inactivating current was 11.3 ± 0.78% of I_{pmax}. As expected for an anion-permeable channel, reducing the extracellular Cl^- concentration by using the low-ionic-strength hypotonic solution ($[\mathrm{Cl}]_o$ = 58 mM) resulted in a reduction of the outward current and a rightward shift of the reversal potential. With this solution V_{hi} was 49.8 ± 3.5 mV, a was 8.2 ± 0.9 mV and the non-inactivating current was 10.9 ± 0.8% of I_{pmax} (n = 8). While the steepness factor a and the fraction of non-inactivating current remained unmodified with the different solutions, current measured in low-strength/low-Cl^- solution tended to inactivate at less depolarizing potential. However, the differences were not statistically significant ($P > 0.05$).

Figure 5 shows the time constants of inactivation (Fig. 5*A*) and recovery from inactivation (Fig. 5*B*) obtained in experiments done using solutions with different ionic strength. The voltage dependence of kinetic parameters was not substantially modified by the composition of the extracellular solution. Figure 5 also shows the fast (*C*), slow (*D*) and non-inactivating (*E*) current fractions as a function of the test potential. Increasing the ionic strength and the Cl^- concentration slightly shifted all the curves to the right. The fit of the non-inactivating fraction to a Boltzmann function (Fig. 5*E*) yielded half inactivation potential values similar to those of steady-state inactivation. Indeed, with 58 and 138 mM extracellular Cl^- V_h was 47 mV and 56 mV, respectively. The non-inactivating current fraction and a remained unmodified.

Single-Channel Properties

For single channel studies, the $I\mathrm{Cl}_{(vol)}$ current was activated with a hypotonic shock in whole-cell configuration and then the membrane was excised obtaining a membrane patch in the outside-out configuration. Patches excised in this way always contained many channels (n = 28). Conversely, patches excised from cells that were not previously exposed to hypotonic shock did not show any channel activity (n = 7). Single channel currents were studied in response to test pulses to various potentials from a holding potential of −5 or −100 mV. The number of open channels slowly diminished with time and usually after 10–15 min channel activity completely disappeared. Interestingly, single channels inactivated at

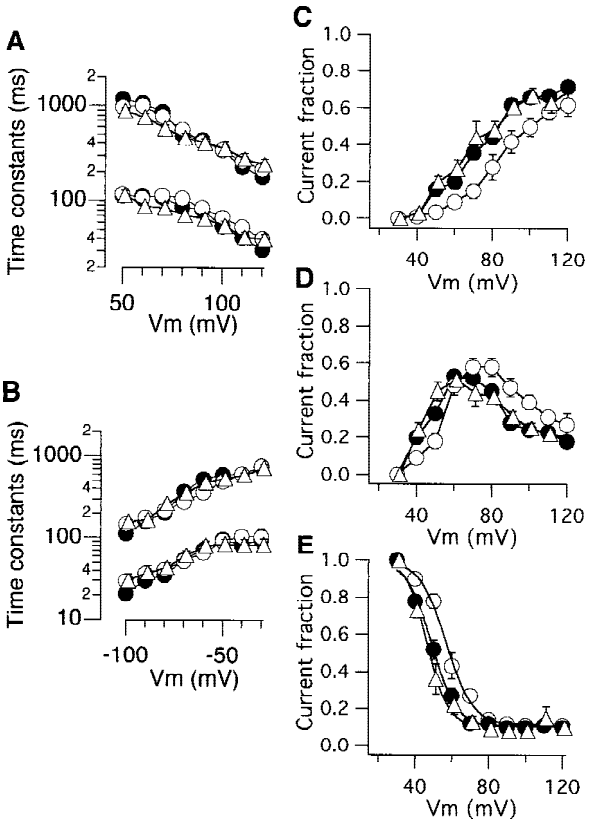

Fig. 5. Sensitivity of voltage-dependent parameters to extracellular ionic strength/Cl^- concentration. Inactivation (*A*) and reactivation (*B*) time constants of $ICl_{(vol)}$ elicited with hyposmotic solutions of different ionic strength. Time constants obtained using solutions with high ($[Cl]_o$ = 138 mM, open circles, n = 8) and with low ionic strength ($[Cl]_o$ = 58 mM, open triangles, n = 7) are compared with time constants obtained using the standard hypotonic solution ($[Cl]_o$ = 125 mM, closed circles) that are replotted from Figs. 3 and 4. Contribution of fast (*C*), slow (*D*) and non-inactivating (*E*) current fractions to total current at different membrane potentials and in the presence of different ionic strength. The non-inactivating current fractions were fitted to Boltzmann functions and the values of V_{hi} were 46.6, 49 and 55.9 mV for solutions with low, intermediate and high ionic strength. The values of the steepness factor a were 5.9, 6.9 and 7.5 mV, respectively.

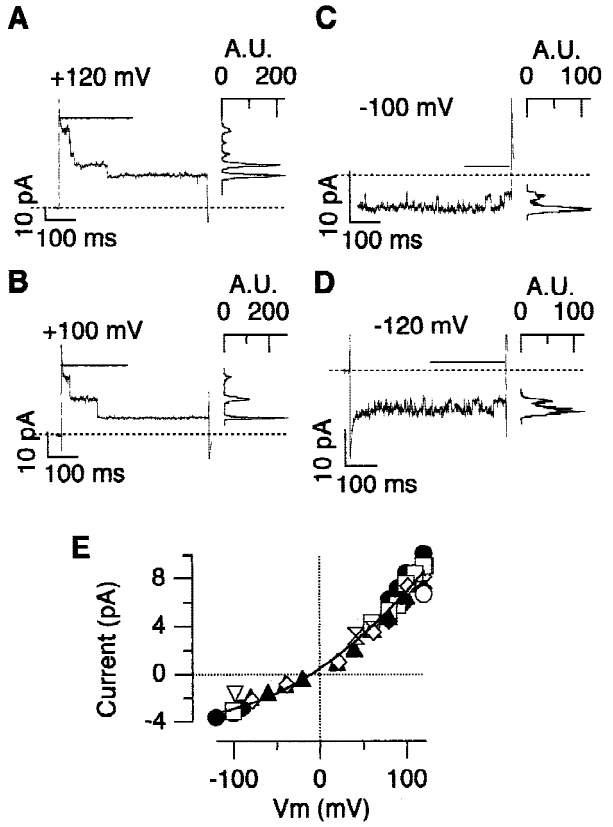

Fig. 6. Single channel current-to-voltage relationship. Single channel events from one representative experiment at different membrane potentials (*A,B,C* and *D*). Currents were recorded from an outside-out patch pulled from a cell previously stimulated by a hypotonic shock. Each of these traces was recorded in response to a 600-msec voltage pulse to the indicated potentials from a holding potential of −5 or −100 mV. Upward deflections represent outward current. Straight lines over the traces indicate the part of the current over which the amplitude histogram was done. On the right of each trace the histogram is given in arbitrary units (A.U.) *vs. i* in pA. Histograms were fitted with Gaussian functions and the value of *i* was obtained from the difference of mean values and was 9, 8.3, −3.5 and −3.7 pA at 120, 100, −100 and −120 mV, respectively. (*E*) The single channel current-voltage relationship was obtained from 11 different cells. Each symbol type represents a single experiment. The continuous line represents the fit of the mean current at each membrane potential with a polynomial function. The current reversal potential is −12 mV.

positive membrane potentials as the macroscopic $ICl_{(vol)}$ current while no inactivation was observed at negative potentials (*see* Fig. 6). Histograms of current amplitude (Fig. 6*A–D*) were fitted with Gaussian functions, and the difference between mean values was taken as the elementary current flowing through the channel at a given potential. Single channel events from one representative experiment and the current-voltage relationship obtained from 11 different experiments are shown in Fig. 6*E*. The reversal potential was −12 mV which is a value similar to that obtained in whole cell. The single channel conductance changed with potential, resulting on a rectification similar to that of the macroscopic currents. Indeed, the single channel chord conductance was 21 ± 3.7 pS between −80 and −100 mV (n = 4) and 63.8 ± 3.8 pS between 100 and 120 mV (n = 10).

We studied the permeability of single channels using hypotonic extracellular solutions containing different anions. We estimated the reversal potential interpolating the unitary current measured at various membrane potentials (Table 1). The relative permeability of these anions with respect to Cl^-, calculated from the Goldman-Hodgkin-Katz zero-current expression, was 1.23, 1.03 and 0.19 for I^-, Br^- and gluconate, respectively. These values are similar to those described for the macroscopic

Table 1. Relative anion permeability of single swelling-activated Cl^- channels

Anion	$V_{r(mV)}$	P_x/P_{Cl}	n
I^-	−16.5 ± 0.65	1.23 ± 0.04	4
Br^-	−13.8 ± 1.06	1.03 ± 0.07	4
Cl^-	−11.9 ± 1.25	1.00	11
Gluconate	+20 ± 0.95	0.19 ± 0.03	4

For each anion, reversal potential (V_r) was used to calculate P_x/P_{Cl}. Values are mean ± SEM.

swelling-activated Cl^- current in this cell line (Rasola et al., 1992).

We recently described the effect of reactive blue 2 (RB) as a voltage-dependent blocker (Galietta et al., 1997) of macroscopic $ICl_{(vol)}$. A further evidence that the single channel events seen in outside-out patches underlie the macroscopic current activated after hypotonic shock was obtained using this blocker. The addition of 100 μM RB reduced the open time without affecting the single channel conductance (Fig. 7*A*). Washing the drug increased the open time but the number of channels never returned to the values seen before blocker application. This partial recovery was probably due to the channel activity rundown described above. Although an accurate determination of open and close time constants was precluded by the impossibility of obtaining one-channel patches and because of the current rundown, we took advantage of the rundown and measured the mean open time of successive traces in the presence of RB and after washing, when the apparent number of channels was equal to one. A mean open time of 2.6 msec at 120 mV (over 1733 events), in the presence of 100 μM RB, was significantly lower than that measured after washing, of 38.1 msec (over 243 events). The average of several single channel traces before and after RB addition (Fig. 7*B*) resembled the macroscopic current seen in whole cell configuration (Fig. 7*C*), strongly suggesting that the single channel events indeed underlie the macroscopic $ICl_{(vol)}$.

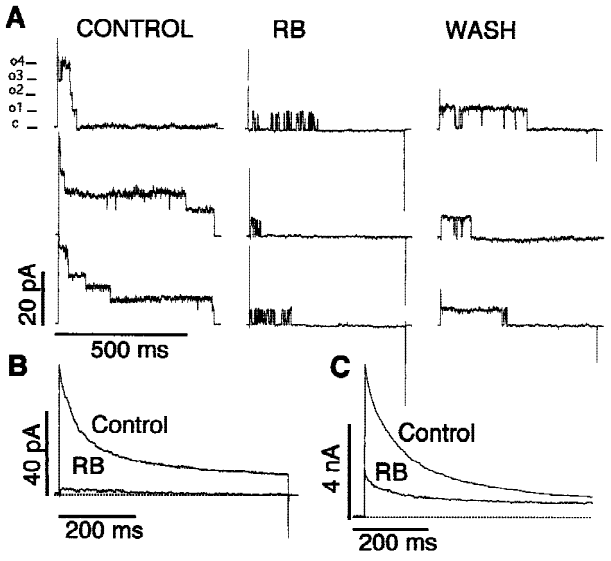

Fig. 7. Blocking effect of RB on single channels and comparison with the macroscopic current. (*A*) Single channel events recorded at 120 mV in outside-out patches. Currents were recorded in response to a 600 msec pulse from a holding of −5 mV in control condition, in the presence of 100 μM RB and after washing. RB reduced the mean open time without affecting the single channel conductance. (*B*) Average of several successive single channel traces at 120 mV before and after 100 μM RB addition. (*C*) Macroscopic current recorded in whole cell configuration at 120 mV following the hypotonic shock and blocked with 100 μM RB.

Discussion

Swelling-activated Cl^- channels are involved in different physiological and pathological processes (*see* for review McManus et al., 1995 and Wright & Rees, 1997). We have characterized the voltage-dependence of the swelling-activated Cl^- channel in human tracheal 9HTEo- cells and identified the single channel conductance underlying the whole-cell currents. Reduction of the extracellular osmolarity activates Cl^- currents ($ICl_{(vol)}$) reaching plateau levels in a few minutes. The $ICl_{(vol)}$ is characterized by an outward rectification of the current-voltage relationship and by voltage-dependent current inactivation and reactivation. Swelling-activated Cl^- channels with biophysical characteristics similar to those reported here have been previously described in rat C6 glioma cells (Jackson & Strange, 1995*a*,*b*), in rat osteoblast-like cells (Gosling et al., 1995), in T84 and B-cell myeloma cells (Levitan & Garber, 1995), in M-1 mouse cortical collecting duct cells (Meyer & Korbmacher, 1996) and in BC_3H1 myoblasts (Voets, Droogmans & Nilius, 1997). Indeed, in these cell types, voltage-dependent parameters are qualitatively similar to those described here. The number of exponential terms to describe inactivation varies from one (Jackson & Strange, 1995*a*) to two (this work, Levitan & Garber, 1995; Meyer & Korbmacher, 1996; Voets et al., 1997). Recovery from inactivation was fitted with one (Levitan & Garber, 1995) or two exponential terms (this work, Jackson & Strange, 1995*a;* Voets et al., 1997). The different number of exponentials considered sufficient to describe kinetics could depend on the different duration of test pulses used to study these parameters, that varies from 0.5 sec (Levitan & Garber, 1995) to 4 sec (Jackson & Strange, 1995*a*). Indeed, with short pulses that do not reach steady-state, the fast component is easily determined while the duration of the slow time constant may be underestimated. On the contrary, the use of long pulses allow a careful evaluation of the slow component,

while the fast component (lasting few tens of milliseconds) could be underestimated.

The main difference between $ICl_{(vol)}$ from different cell types seems to be the set-point of the voltage sensor. Indeed, the half inactivation potential of 9HTEo- cells is about 54 mV, which is similar to that of rat osteoblast-like cells (Gosling et al., 1995). Conversely, this value is about 15 mV more positive in rat C6 glioma cells (Jackson & Strange, 1995*a*), in M-1 mouse cells (Meyer & Korbmacher, 1996), in T84 cells (Levitan & Garber, 1995) and in BC_3H1 myoblasts (Voets et al., 1997), and 50 mV more positive in B-cell myeloma cells (Levitan & Garber, 1995). This variability could be ascribed to differences in the experimental conditions, including the osmotic gradient, the ionic strength or the permeant anion concentration. Our results demonstrate that voltage-dependent parameters of $ICl_{(vol)}$ are not significantly influenced by the ionic strength or by the Cl^- concentration of the extracellular solution. Indeed, the strong reduction of extracellular Cl^- from 138 to 58 mM shifted only by 8 mV to negative the half inactivation potential. The small effect of external Cl^- is at variance with two recent reports. Voets et al. (1997) described that decreasing the Cl^- concentration of the extracellular solution from 110 to 42 mM shifted V_h by 22 mV to negative and hastened the inactivation process by decreasing the slow inactivation time constant and by increasing the contribution of the fast inactivating component. On the second report Stutzin et al. (1997) described that the degree of current deactivation was also dependent on external Cl^-, and an important leftward shift of the half inactivation potential and an increase of the non-inactivating current fraction was found with low Cl^- concentrations. We have not an explanation for such a difference, but it is feasible that a basic common channel associates with a cell-specific subunit that regulates the Cl^- sensitivity. Alternatively, it is conceivable that the sensitivity of voltage-dependent parameters to extracellular Cl^- might be modulated by other more indirect factors.

Another factor that could modulate the voltage sensitivity of the channel is the osmotic gradient. The degree of hyposmolarity used in the different studies ranged from 11% in the present study to 32% (Meyer & Korbmacher, 1996), but seems unlikely that this difference alone could account for the different voltage sensitivity reported by various authors since Levitan and Garber (1995) studying under the same conditions B-cell myeloma and T84 cells found very different half inactivation potentials (73 mV *vs.* 105 mV, respectively).

In some cell types, intracellular ATP seems to be important for maintaining the swelling-activated Cl^- current (Jackson & Strange, 1995*a;* Levitan & Garber, 1995; Meyer & Korbmacher, 1996). The ATP requirement seems to be cell type-dependent, since 9HTEo- cells do not need it to maintain the current while, with exactly the same solutions and experimental protocols, NIH 3T3 cells evidenced transient currents if ATP was not added to the pipette solution (O. Zegarra-Moran, *unpublished results*). However, there is not a correlation between the ATP concentration used in different studies and the voltage dependence, i.e., a higher ATP concentration is not related to a more depolarizing V_{hi}. This observation makes unlikely a role of ATP in the voltage dependence of the channel.

The intracellular ionic strength/Cl^- concentration might also be responsible for the difference in voltage-dependent parameters. Indeed, Emma et al. (1997) reported that intracellular electrolytes regulate the volume set-point of the swelling-activated Cl^- channel in C6 glioma cells. It is possible that also the voltage-dependent channel activity is regulated by the intracellular ionic composition. Taken together, we cannot completely exclude that experimental conditions play a role in the voltage set-point of the channel. Nevertheless, it is more likely that the differences in the voltage sensitivity could be explained by postulating cell type specific regulatory mechanisms or heterogeneity of swelling-activated Cl^- channels.

In contrast with the voltage dependence of the channel, other characteristics such as the anionic selectivity seem to be less variable among $ICl_{(vol)}$ in different cells. Indeed, the permeability sequence $I^- > Br^- > Cl^-$ > gluconate is shared by the $ICl_{(vol)}$ from 9HTEo- (Rasola et al., 1992), rat C6 glioma (Jackson & Strange, 1995*a*), T84, B-cell myeloma (Levitan & Garber, 1995), rat osteoblast-like (Gosling et al., 1995), mouse M-1 cells (Meyer and Korbmacher, 1996) and BC_3Hl (Voets et al., 1997). Moreover, in 9HTEo- cells $ICl_{(vol)}$ is also permeable to the small amino acid taurine (Galietta et al., 1997). The relative taurine permeability $P_{tau}/P_{Cl} = 0.3$ is of the same order of magnitude of that observed in rat C6 cells (0.2; Jackson & Strange, 1993), in skate hepatocytes (0.17; Ballatori et al., 1995), and in rat inner medular collecting duct (0.15; Boese et al., 1996).

Outside-out patch experiments revealed the presence of outwardly rectifying channels only from cells that were stimulated by hypotonic shock. This finding suggests that these channels are responsible for the macroscopic $ICl_{(vol)}$. This interpretation is supported by the following evidences: (i) the permeability sequence $I^- > Br^- > Cl^-$ > gluconate is identical to that determined for macroscopic $ICl_{(vol)}$ in 9HTEo- cells (Rasola et al., 1992); (ii) channels inactivate at positive membrane potentials and the average of single channel traces resembles the time course of the macroscopic $ICl_{(vol)}$; (iii) single channel conductance change with membrane potential and the outward rectification is similar to that of macroscopic currents; (iv) single channel currents are blocked by RB, an inhibitor of the macroscopic $ICl_{(vol)}$ (Galietta et al., 1997). The single channel chord conduc-

tances, about 60 pS at 100 mV and 20 pS at −80 mV, are consistent with those described in C6 glioma (Jackson & Strange, 1995*b*), T84 (Worrell et al., 1989) and mouse M-1 cells (Meyer & Korbmacher, 1996). Swelling activated Cl^- channels with conductances lower than 10 pS (Doroshenko & Neher, 1992; Lewis et al., 1993; Ho et al., 1994; Nilius et al., 1994) could represent a different channel type or, as suggested by Jackson and Strange (1995*b*) an underestimation of the stationary noise analysis. Indeed, these authors compared the single channel conductance of $ICl_{(vol)}$ in C6 glioma cells using stationary noise analysis, non-stationary noise analysis and single channel records. They concluded that stationary noise analysis underestimates the unitary conductance because it incorrectly assumes that the swelling-induced increase in the macroscopic current is due to a graded increase in the open probability of a constant number of channels. These authors proposed instead that channel activation probably involves an abrupt switch of the channels from a state with zero open probability to a state with very high open probability, clearly at potentials were current do not inactivate.

In conclusion, the $ICl_{(vol)}$ of 9HTEo- cells seems to be similar if not identical to swelling activated Cl^- currents described in C6 glioma, T84, myeloma, osteoblast-like, BC_3H1 and M-1 cells. The corresponding channel could be termed VSOAC (Volume-Senstive Organic osmolyte/Anion Channel) as suggested by Jackson et al. (1994). On the other hand, this current seems to be different from volume-sensitive Cl^- currents described in bovine chromaffin cells (Doroshenko & Neher, 1992), in T-lymphocytes (Lewis et al., 1993), in endothelial cells (Szücs et al., 1996*b*), in parotid acinar cells (Arreola et al., 1995), in HIT-T15 and RIN m5F insulinoma cells (Best et al., 1996) which exhibit different kinetics and a weak voltage dependence. In addition, the permeability sequence of the currents in the last 3 cell types is different from VSOAC. $ICl_{(vol)}$ from 9HTEo- is also different from another swelling and hyperpolarization activated Cl^- current termed ClC-2 (Gründer et al., 1992) whose biophysical characteristics, i.e., the inwardly rectifying *I/V* and the relative selectivity to halides ($Br^- \sim Cl^- > I^-$) do not match those of $ICl_{(vol)}$. Hence, hypotonic shock seems to activate at least three types of Cl^- channels: VSOAC, ClC-2 and a third type with biophysical features different from the previous two and that could represent an heterogeneous group. Only the molecular identity of ClC-2 has been clearly established (Thiemann et al., 1992). For the other channels few molecular candidates are under study as the multidrug-resistance gene product P-glycoprotein (Gill et al., 1992) and the small peptide I_{Cln} (Paulmichl et al., 1992). Although the question is not yet settled, the P-glycoprotein and the I_{Cln} should be probably regarded as modulators of the channel (Krapivinsky et al., 1994; Hardy, 1995) more than as channels themselves. It has been recently proposed that ClC-3, another member of the the voltage-dependent ClC family, could be the molecule responsible for a volume-activated Cl^- channel (Duan et al., 1997). The authors reported that functional expression of ClC-3 in NIH-3T3 cells resulted in basally active Cl^- conductance with properties identical to those of $ICl_{(vol)}$ in native cells and that one amino acid mutation abolishes the outward rectification and changes the anion selectivity. These results need further corroboration since the experience with other proteins (i.e., *I*Cln, phospholeman, ClC-6) demonstrates that functional expression in systems having native swelling-activated Cl^- channels can upregulate the endogenous current. Also mutagenesis results should be treated cautiously since the mutated protein could activate a different type of endogenous conductance (Buyse et al., 1997). Until the molecular nature of the proteins underlying $ICl_{(vol)}$ is determined, detailed descriptions of current characteristics will help to get a better insight of the problem. In this regard, the biophysical characteristics of volume-sensitive Cl^- currents seem to be a better criterion to classify these channels than pharmacological tools, in large amount not specific.

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