Cation regulation of anion current activated by cell swelling in two types of human epithelial cancer cells

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1. In epithelial cells, hyposmotic stress induces visible cell swelling and large Cl^- currents, which deactivate on return to isotonic solutions and are abolished by 0·1–0·5 mM DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid). During depolarizing voltage clamp pulses, the currents activate rapidly and show time-dependent relaxation with associated tail currents on return to negative potentials.

2. We used whole-cell and outside-out patch recording to study volume activation of Cl^- currents in the epithelial cancer cell lines H69AR and HeLa S5. In a 210 or 160 mosmol l^{-1} hyposmotic bathing solution containing 90 mM NaCl, 1 mM Ca^{2+} and 1 mM Mg^{2+}, current relaxation was rapid, occurred positive to the Cl^- reversal potential and reduced current to $<30\%$ of its peak level at +100 mV.

3. Replacement of most bath inorganic cations by *N*-methyl-D-glucamine (NMDG) at constant Cl^- concentration and osmolarity eliminated most of the current relaxation and caused an increase in steady-state current levels. Steady-state current was $85 \pm 6\%$ of peak current at +100 mV in NMDG-Cl bath solution. This ratio fell to $55 \pm 2\%$ ($n = 5$) when 1 mM Mg^{2+} was re-added to the bath.

4. Re-addition of Mg^{2+} or other Group II metals (Ca^{2+}, Sr^{2+}, Ba^{2+}) induced immediate changes in current relaxation in a dose- and species-dependent manner. Concentrations of Mg^{2+} as low as 0·1 mM were effective in causing Cl^- current relaxation. The IC_{50} for steady-state current block by external Mg^{2+} was 1·75 mM. The relative efficacy of block at 20 mM concentrations by different cations was $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} \gg Na^+$.

5. Outside-out patch recordings from single cell-swelling-activated Cl^- channels revealed that high bath Mg^{2+} concentrations reduced mean channel open time and burst channel open probability (P_o), giving a flickering appearance to channel openings.

6. These findings suggest that physiological concentrations of Ca^{2+} and Mg^{2+} modulate volume-regulated Cl^-currents in epithelial tissues.

Cell-swelling-regulated Cl^- channels are present in widely diverse tissues from neurones (Oliet & Bourque, 1993) to heart (Tseng, 1992) and epithelia (McCann, Li & Welsh, 1989; Worrell, Butt, Cliff & Frizzell, 1989), and are intimately connected with regulatory volume changes that follow cell swelling or shrinkage and form part of the restorative mechanisms that involve the loss of osmotically active particles (Hoffmann & Simonsen, 1989). These Cl^- channels have been the most studied of the cell-swelling-regulated ion channel systems (McCann *et al.* 1989; Worrell *et al.* 1989; Kubo & Okada, 1992). Single channels show a variety of conductances around 50 pS (Shoemaker, Frizzell, Dwyer & Farley, 1986; Worrell *et al.* 1989; Solc & Wine, 1991; Krouse, Haws, Xia, Fang & Wine, 1994) and activation is independent of intracellular Ca^{2+} levels (Kubo & Okada, 1992). These cell-swelling-regulated Cl^- currents show prominent outward rectification of whole-cell currents and relax at pulse potentials more positive than +50 mV (Worrell *et al.* 1989; Kubo & Okada, 1992). Although various studies have suggested that voltage-dependent inactivation is the mechanism responsible for the current decay (McCann *et al.* 1989; Solc & Wine, 1991), the inactivation rate is often variable (Solc & Wine, 1991) and some single channel studies show that, at positive potentials, the mean open time and open probability is relatively stable (Solc & Wine, 1991) or does not change (Duan & Nattel, 1994). At very large depolarizations (> +100 mV)

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channels can enter a long-lived closed state (Solc & Wine, 1991). In other systems, other mechanisms for current decay include cation block and ion-dependent inactivation. Zinc ions have long been known to block Cl^- channels in skeletal muscle (Stanfield, 1970; Spalding, Taber, Swift & Horowicz, 1990), and the trivalent cations La^{3+} and Gd^{3+} also block cell-swelling-activated Cl^- channels (Ackerman, Wickman & Clapham, 1994). Mg^{2+} ions are well known to block a wide variety of cation channels. NMDA receptors are gated by external Mg^{2+} (Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984). Potassium channels are often blocked by cations, especially Mg^{2+} from the inside (Vandenberg, 1987), which confers rectification properties (Hille, 1991). Here we suggest that external cations (primarily divalent), at physiological concentrations, can modulate the Cl^- current magnitude and its decay positive to the Cl^- reversal potential. Most current regulation occurs at low concentrations of the divalent cations Mg^{2+} and Ca^{2+}.

METHODS

Cell lines

The human small-cell lung cancer (H69AR) and cervical carcinoma (HeLa S5; from ATCC, Rockville, MD, USA) cell lines used in this study were kindly provided by Dr S. P. C. Cole (Mirski, Gerlach & Cole, 1987). Cells were maintained in RPMI 1640 medium (Gibco Laboratories, Burlington, Ontario, Canada) supplemented with 5% heat-inactivated, supplemented bovine calf serum (HyClone Laboratories, Logan, UT, USA) and 4 mM L-glutamine. The H69AR cell line was grown in sterile glass bottles at 37 °C in a humidified atmosphere with 5% CO_2. To prepare cells for electrophysiological recordings, H69AR cells were syringed using a 20-gauge needle and seeded onto poly-L-lysine (0·01% w/v)-coated glass coverslips. HeLa cells were grown in plastic flasks (Corning Co., Corning, NY, USA) and maintained as above. Prior to plating, HeLa cells were treated with 0·05% trypsin and 0·5 mM EDTA (Gibco) for 10 min at 37 °C, then washed with fivefold excess normal growth medium and centrifuged for 5 min at 800 r.p.m. The cell fraction was resuspended in RPMI medium before seeding onto poly-L-lysine (0·01% w/v)-coated glass coverslips. All cells were incubated on coverslips in 35 mm Petri dishes for 12–24 h prior to use.

Electrophysiology

Coverslips were removed from the incubator prior to experiments and placed in 35 mm Petri dishes containing isotonic bath solution and were maintained at room temperature. Electrodes of 1–5 MΩ resistance were pulled from TW150 glass (World Precision Instruments, MA, USA) on a horizontal micropipette puller (Model P-87, Sutter Intrument Co.), fire-polished and filled with an internal solution that contained (mM): CsCl, 130; ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 10; $MgCl_2$, 1; *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid (Hepes), 5; Na_2ATP, 4; GTP, 0·1; pH 7·2 with CsOH. The standard isosmotic (290 mosmol l^{-1}) bath solution contained (mM): NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; $BaCl_2$, 1; Hepes, 10; sodium acetate, 2·8. $BaCl_2$ was added to block inwardly directed K^+ currents. The osmolarity of internal and external solutions was measured using freezing point depression (Advanced Instruments, Model 3L). Hypotonic bath solution (210 mosmol l^{-1}) was made by reducing the NaCl to 90 mM. This resulted in a +8 mV shift in the reversal potential for Cl^- ions (E_{Cl}). *N*-methyl-D-glucamine (NMDG; 100 mM) was used as a substitute for NaCl in a hyposmotic solution termed 210-NMDG solution with 20 mM sucrose to maintain osmolarity (pH 7·4 with HCl). In this situation the ideal objective was to omit all cations (except H^+) from the bath solution. However, a general property of divalent cations is their ability to screen fixed negative charges on the inner and outer aspects of the lipid bilayer, loss of which results in a perturbation of the electric field surrounding the voltage sensors of ion channels (Hille, 1991). Since the efficacy of screening is relatively non-selective and requires large changes in concentrations (i.e. a 10-fold change in Mg^{2+} produces a 10–15 mV shift in voltage-dependent properties), it is unlikely that changes in surface charge screening affect our results. However, wherever possible, we kept 1 mM Ba^{2+} present in the external solutions to provide a source of external cations since we found (Fig. 3) that Ba^{2+} had a lesser effect on the anion channels than the lower atomic number divalent cations. In the solutions of increased divalent cation concentration (up to 20 mM Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}), the NaCl or NMDG-Cl concentration was reduced proportionally, down to 50 mM, in order to keep the external [Cl^-] constant. Sucrose was added as required to prevent small deviations in osmolarity from 210 mosmol l^{-1}. Experiments were carried out at 22–23 °C, and in all cases the measured osmolarities of solutions were rounded to the nearest 10 mosmol l^{-1}. Axopatch 1D or Axopatch 200A amplifiers (Axon Instruments, Foster City, CA, USA) were used for voltage clamp measurements and data were filtered at 5 kHz prior to digitization via a Labmaster DMA interface. For single channel recording, data were filtered at 1–2 kHz and sampled at 2–5 kHz. The pCLAMP suite of programs was used for data acquisition and analysis. Analogue capacity compensation and 80–90% series resistance (R_s) compensation were used during whole-cell measurements. Prior to compensation the mean series resistance was 8·5 ± 3·7 MΩ (mean ± S.D., $n = 10$) and capacitance (C_m) was 29·6 ± 8·6 pF for H69AR cells and R_s and C_m were 9·4 ± 2·8 MΩ and 29·9 ± 9·0 pF, respectively, for HeLa cells. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St Louis, MO, USA). Rapid solution change was obtained using 12 V solenoids (Lee Valve Co., Essex, UK) which switched a series of individual pipes leading solution directly into the floor of the experimental chamber.

RESULTS

We have previously shown that osmotically induced cell swelling results in the activation of Cl^- channels in H69AR cell lines (Jirsch, Loe, Cole, Deeley & Fedida, 1994). Repetitive exposure to hyposmotic solutions resulted in large reversible increases in membrane current. An example of membrane currents in an H69AR cell, in response to a range of voltage clamp step pulses during exposure to 210 mosmol l^{-1} solution is shown in Fig. 1*A*. Most of the current could be deactivated by re-exposure to control 290 mosmol l^{-1} solution (Fig. 1*B*), and much larger cell-swelling-activated current could be induced by exposing the cell to 160 mosmol l^{-1} bath solution (Fig. 1*C*). 4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) selectively blocked cell-swelling-

activated Cl^- channels at 0·2 mM in both cell types (Jirsch *et al.* 1994). The very large currents which activated in hyposmotic bath solutions showed some degree of outward rectification (Fig. 1*A*, *C* and *D*), but the most striking characteristic of the current traces at depolarized potentials was a marked current decay which results in cross-over of current tracings after 50–200 ms (see also Kubo & Okada, 1992). Tails were apparent on repolarization and these reflect reversal of the decay process that occurred during prior depolarizations (McCann *et al.* 1989; Solc & Wine, 1991). Mean *I–V* curves for peak and steady currents in 290 and 210 mosmol l^{-1} bath solution are shown in Fig. 1*D*. The marked current relaxation apparent at depolarized potentials in 210 mosmol l^{-1} solution can be seen, such that the *I–V* relation of end-of-pulse currents shows a region of negative slope positive to +40 mV. The mean difference current (between peak and steady-state current levels) for current traces in 210 and 290 mosmol l^{-1} bath is shown in Fig. 1*E*. In the hyposmotic situation, large difference

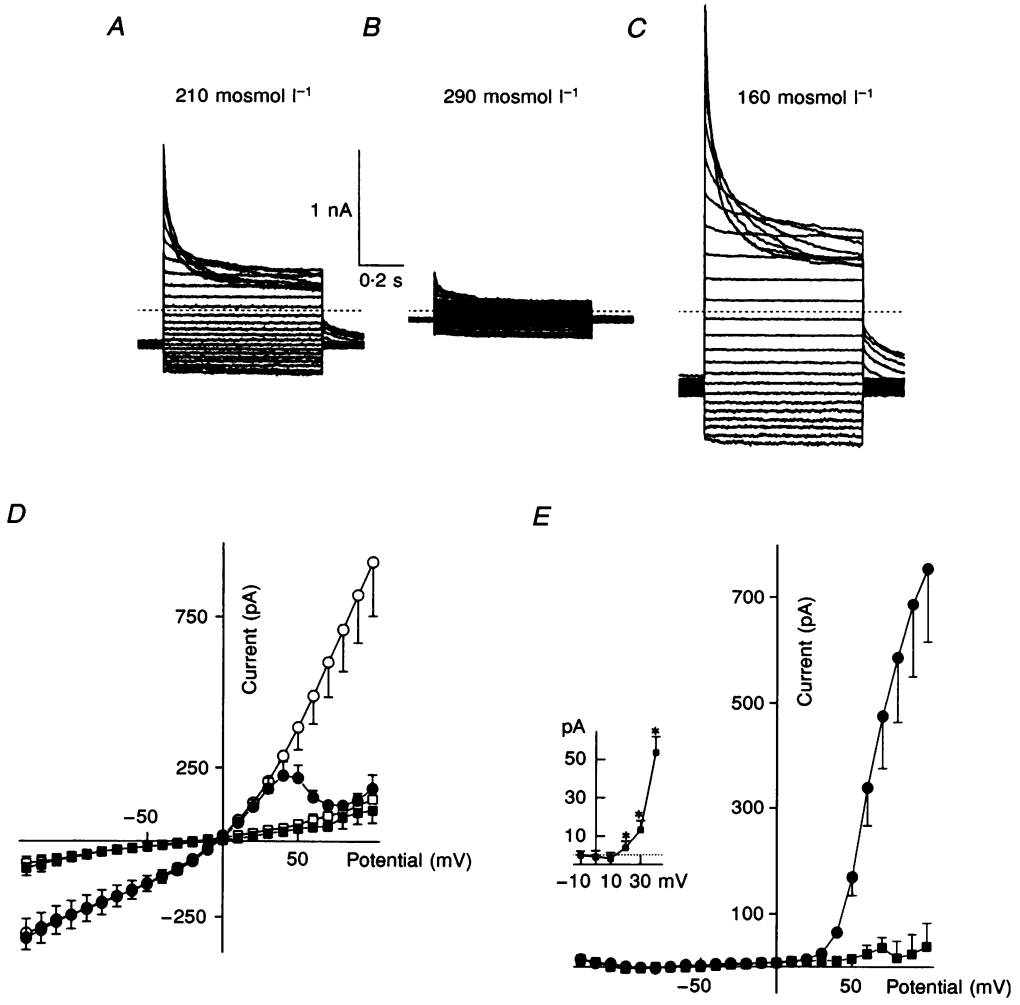

Figure 1. Voltage dependence of swelling-activated Cl^- channels

Pipette contained Cs^+-filling solution (Methods). Isosmotic (290 mosmol l^{-1}) bath contained 135 mM NaCl and hyposmotic solutions (210 and 160 mosmol l^{-1}) contained decreased NaCl (90 and 65 mM). Currents recorded during 750 ms pulses from −60 mV to between −130 and +100 mV. Currents generated by an H69AR cell in hypotonic, isotonic and very hypotonic environments shown in *A*, *B* and *C*, respectively, as indicated by osmolarities above. The graph in panel *D* is the mean (bars indicate ± S.E.M.) *I–V* relation for H69AR cells ($n = 10$) in isosmotic (squares) and hyposmotic (circles) environments. In each case, the peak (open symbols) and steady-state (filled symbols) currents are shown. No data normalization has been applied. Panel *E* depicts the mean difference current (peak − steady-state currents) for isosmotic (■) and hyposmotic 210 mosmol l^{-1} (●) conditions. The inset in panel *E* shows the mean difference current between the two conditions in the voltage range between −10 and +40 mV. Asterisks indicate points significantly different from zero by Wilcoxon's paired-sample *t* test ($P < 0·05$).

currents are apparent by +40 or +50 mV. The hypotonically activated difference current (obtained by subtraction of the difference currents in 210 and 290 mosmol l^{-1} baths) is shown in the inset to Fig. 1*E*. A significant difference from zero current is only present for voltage pulses positive to +10 mV, that is, positive to the reversal potential for current through the Cl^- channel. This significant difference current appeared positive to the Cl^- reversal potential, even when the reversal potential was made negative by substitution of varying amounts of caesium aspartate for CsCl in the filling solution (data not shown). This Cl^- reversal potential dependence of current decay suggested to us that something other than a purely voltage-dependent process

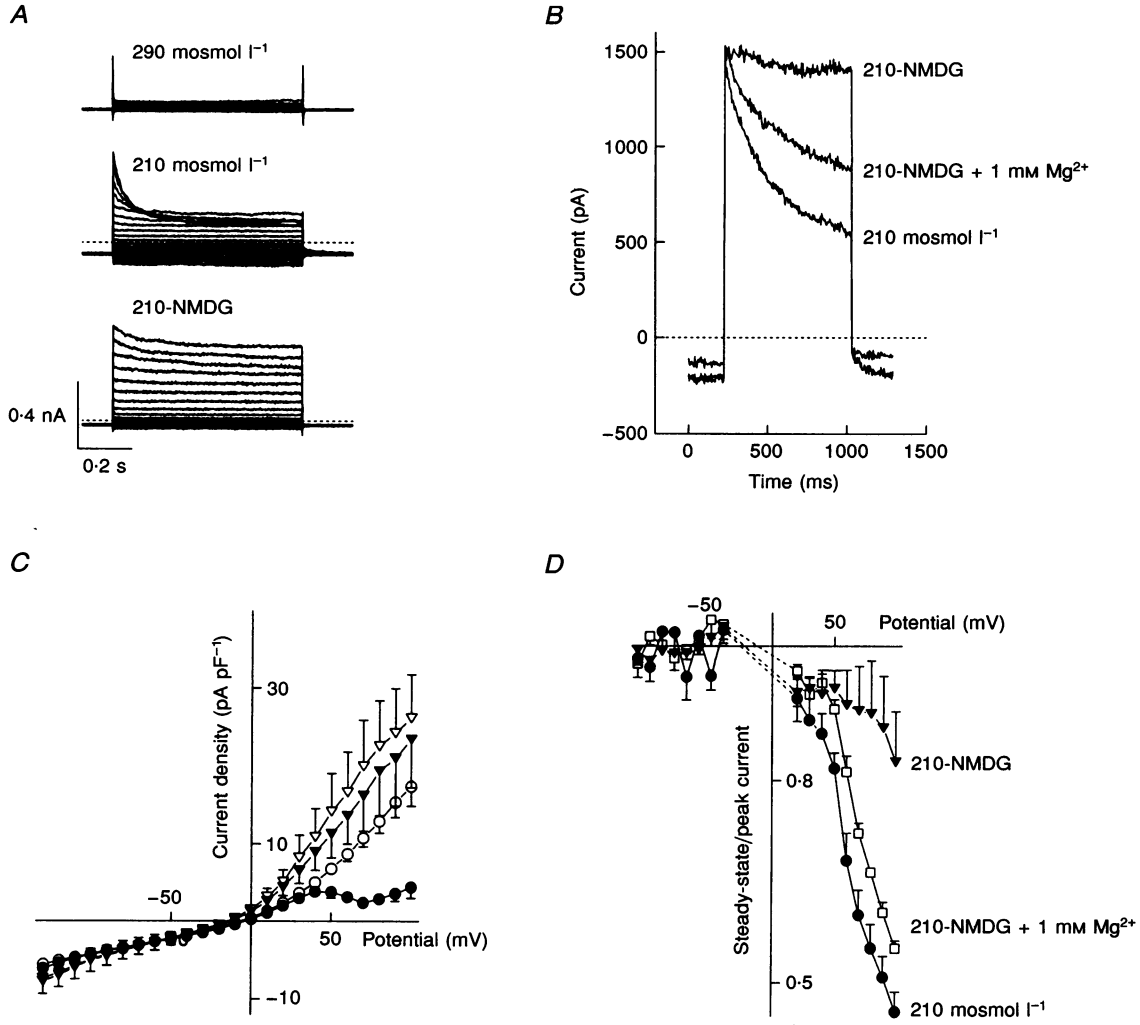

Figure 2. Inorganic cation reduction modulates time-dependent cell-swelling-induced Cl^- current

Solutions as in Fig. 1 except for 210-NMDG which contained (mM): NMDG, 100; Hepes, 10; $BaCl_2$, 1; and sucrose to 210 mosmol l^{-1}. Currents generated by an H69AR cell in isotonic (+1 mM Mg^{2+}) and hypotonic (+1 mM Mg^{2+}) and 210-NMDG (0 mM Mg^{2+}) environments shown in panel *A*. In *B*, no inorganic cations except H^+ were present in the 210-NMDG bath solution. Addition of 1 mM Mg^{2+} to 210-NMDG solution restores time-dependent relaxation. This HeLa cell was pulsed every 10 s from −60 to +80 mV for 750 ms while the bath solution was changed from the standard hypotonic (90 mM NaCl) to 210-NMDG and then to 210-NMDG + 1 mM Mg^{2+}. Current traces from all three solutions have been superimposed. *C*, mean *I–V* relation for 5 H69AR cells in normal 210 mosmol l^{-1} bath (circles) and 210-NMDG (triangles) solutions. Both peak (open symbols) and steady-state (filled symbols) shown. Data have been normalized to cell capacitance obtained from integration of uncorrected capacity transients. *D*, plot of the ratio of steady-state current to peak current ($n = 4$ for each condition) for 210 (●), 210-NMDG (▲) and 210-NMDG + 1 mM Mg^{2+} (□) at each potential during *I–V* curve generation in HeLa cells (data from six cells).

might be modulating the current relaxation. In support of this idea, we were able to remove much of the time dependence of the osmotically activated current by replacement of bathing cations with the positively charged organic molecule NMDG. Representative *I–V* curves obtained in standard (290 mosmol l^{-1}), hyposmotic (210 mosmol l^{-1}) and 210-NMDG solutions are shown in Fig. 2*A*. Exposure to 210 mosmol l^{-1} solution caused the typical cell-swelling-activated current with prominent relaxation of current tracings at positive potentials. Subsequent replacement of all cations (except $BaCl_2$, 1 mM) with NMDG (bottom tracings) led to an immediate slowing of current relaxation. The *I–V* curve was obtained after 1 min exposure to 210-NMDG bath solution. Mean *I–V* data are shown in Fig. 2*C*. Here, data obtained in 210 mosmol l^{-1} bath (circles) show the decrease between peak (○) and steady-current (●) at positive potentials. By contrast, data obtained in the presence of 210-NMDG show somewhat larger peak currents with only a relatively small current decay over the 1 s pulse. No change in current reversal potential occurred in the presence of NMDG, and even at very positive membrane potentials there is no evidence for marked current relaxation from peak to steady-state current levels.

These data confirmed first that the current is an anion current which lacks significant small cation permeability, and second that use of 1 mM $BaCl_2$ to maintain membrane surface charge screening is effective in preventing alteration of the potential dependence of channel gating. The failure of currents to decay rapidly in the presence of 210-NMDG was corrected rapidly by the addition of 1 mM $MgCl_2$ to the bath. This experiment is illustrated in Fig. 2*B*. Here, another cell was equilibrated in 210 mosmol l^{-1} hypotonic solution and then exposed to cation-free NMDG solution. Currents immediately failed to relax (but note that typically, in the absence of all divalent cations, current records became noisier) and after 30 s exposure, inclusion of $MgCl_2$ in the bath caused a return of current decay. These changes in time dependence were immediate upon changing solutions, which was accomplished within 1 s using the rapid solution changer (see Methods), so it is unlikely that Mg^{2+} ions were able to enter cells and mediate current relaxation from the inside. Moreover, changes in pipette Mg^{2+} concentration did not affect the time dependence of the current (data not shown). The mean amount of current decay was quantified by measuring the ratio of the final current to peak level at different potentials in

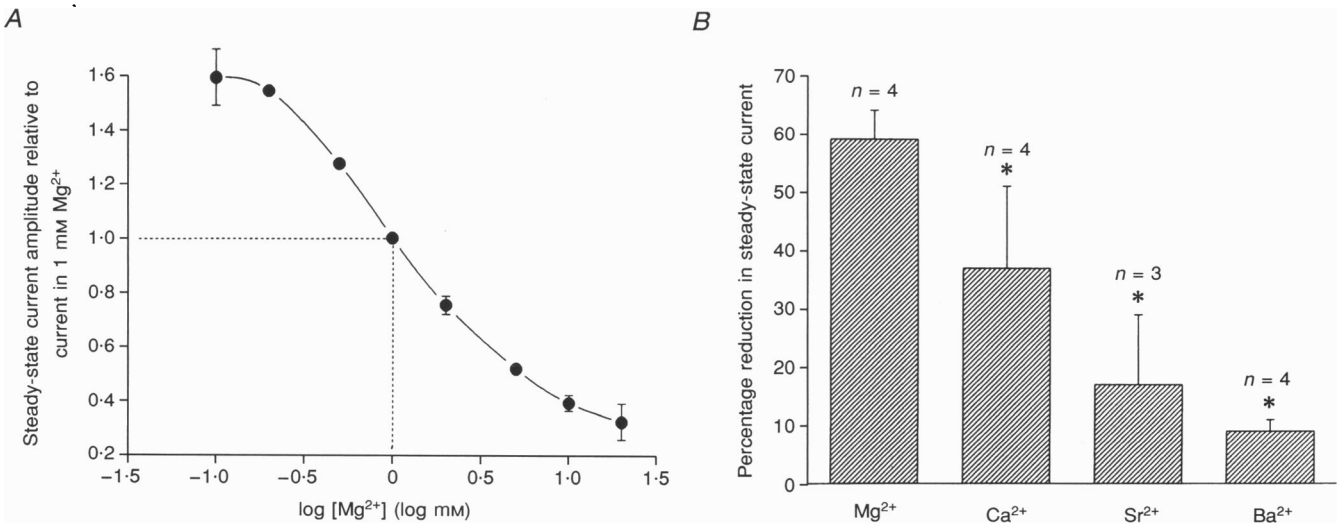

Figure 3. Concentration dependence of extracellular divalent cation decrease of cell-swelling-activated Cl^- current

A, HeLa cells ($n = 6$) and H69AR cells ($n = 4$) were initially swollen in 210-NMDG + 1 mM Mg^{2+} to activate the Cl^- current and then pulsed to +80 mV as described in Fig. 2. Bath Mg^{2+} concentration was then varied from 100 μM to 20 mM, without variation in bath Cl^- concentration (Methods). A dose–response curve for current block by Mg^{2+} was constructed by measuring steady-state current levels and then normalizing these to steady-state current level in 1 mM Mg^{2+}. Data points are mean ± S.D. in each case and joined by a line through data. The dotted lines denote the current at standard 1 mM bath Mg^{2+}. *B*, other divalent cations affect the current in a manner consistent with cation block. Data from HeLa cells; *n* indicates number of cells studied. Fractional reductions of steady-state currents at +80 mV were determined by comparing currents in standard 210 mosmol l^{-1} hypotonic solution to those in 20 mM solutions of other Group II divalent cations. Data bars show ± S.D. Protocols were as described for panel *A*. Asterisks indicate significant differences ($P < 0.05$) between Mg^{2+} and other cation data.

these three external solutions (Fig. 2*D*). For pulse potentials up to +10 mV, no current relaxation was observed. At more positive potentials, current relaxations in hyposmotic baths became more prominent. The graph shows that replacement of Groups I and II cations with 210-NMDG removed most of the current decay (but not all). For cells re-exposed to 210-NMDG + 1 mM Mg^{2+}, the mean current decay was significantly increased although not to the level seen in 210 mosmol l^{-1} hypotonic solution alone. These data suggest that although Mg^{2+} is a potent inhibitor of the current and can effect current relaxation, Ca^{2+} and the monovalent cations Na^+ and K^+ present in the standard hyposmotic bath solution also account for part of the effects of cation removal.

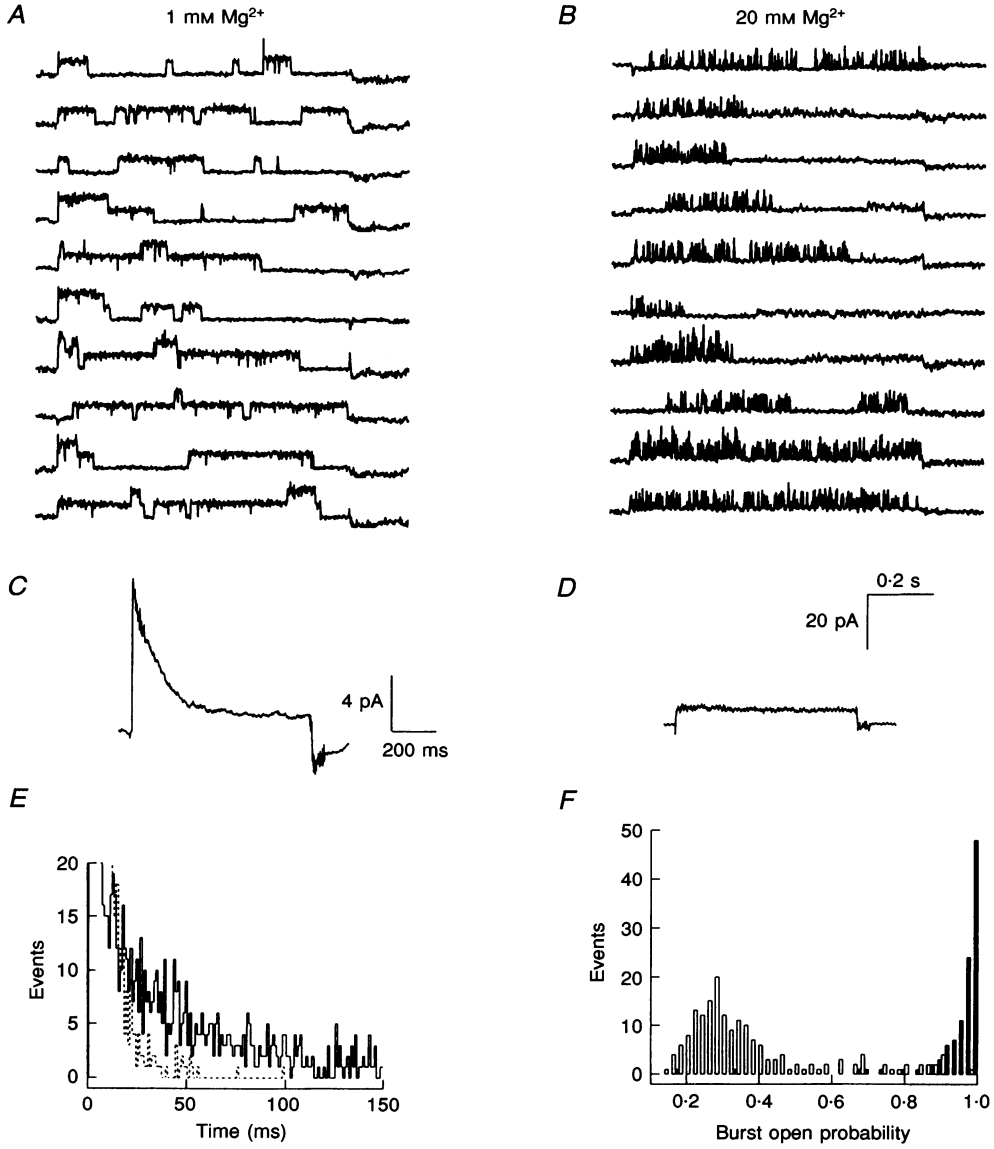

Figure 4. Regulation of cell-swelling-activated Cl^- channels in excised outside-out membrane patches from H69AR cells

A, patch containing two channels, repetitive sweeps of channel openings in response to 800 ms steps to +100 mV from a holding potential of −60 mV. Depolarizations were applied at 0·5 Hz. Linear leak and capacity currents have been subtracted using occasional blank sweeps. *B*, patch exposed to a bath solution containing 20 mM Mg^{2+} (see Methods). Same voltage protocols as in *A*. *C* and *D*, ensemble average currents for 190 traces in *A* and 90 sweeps in *B*. *E*, analysis of open times during depolarization to +100 mV for patches in 1 mM Mg^{2+} (continuous line) and 20 mM Mg^{2+} (dashed line). It can be seen that only brief channel openings occur in the presence of 20 mM Mg^{2+}. *F*, channel openings analysed in a burst mode. The open probability during bursts is shown for patches in 1 mM Mg^{2+} (filled bars) and 20 mM Mg^{2+} (open bars). The burst termination was determined using a closed time duration of 20 ms.

The osmotically activated Cl^- current of H69AR and HeLa cells was reversibly modulated by Mg^{2+} in a dose-dependent manner (Fig. 3). A dose–response curve was constructed for H69AR cells using bath solutions where Mg^{2+} was the only inorganic cation present (see Methods). Here, current levels were measured at the end of 1 s voltage clamp pulses and normalized to the current levels in a 210-NMDG + 1 mM Mg^{2+} bathing solution. We did this as seals and recording were always established in a normal isotonic bath solution (see Methods), and cell-swelling currents were produced by initial exposure to the standard 210 mosmol l^{-1} bath solution which contained 1 mM Mg^{2+}. Currents in 1 mM Mg^{2+} were 37% smaller than in the absence of cations and 20 mM Mg^{2+} reduced the current by 68% ($n = 10$). The IC_{50} for this effect was 1·75 mM Mg^{2+}, well within its physiological range (0·5–2·0 mM). To determine whether this modulatory action of Mg^{2+} was specific or a more generalized effect shared by a variety of divalent cations, we compared the response of HeLa cells to 20 mM concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} (Fig. 3*B*). Currents were compared between the standard hyposmotic solution and those containing 20 mM concentrations of the various inorganic cations. The effects of these cations varied in an atomic size-dependent manner such that 20 mM Mg^{2+} reduced steady current by 59 ± 5% but 20 mM Ba^{2+} only reduced current by 9 ± 2%. The reduction in 20 mM Mg^{2+} was significantly greater than for any other cation ($P < 0·05$). One-way ANOVA was used to test for significant differences and Student–Newman–Keuls test used for multiple comparisons of pairs of cations. The effects of Ca^{2+}, Sr^{2+} and Ba^{2+} were all significantly less than that of Mg^{2+} ($P < 0·05$, for each cation compared with Mg^{2+}). Other pairs of cations were also significantly different from each other except Ba^{2+} *vs.* Sr^{2+}.

It was possible to examine the action of Mg^{2+} on single cell-swelling activated Cl^- channels. In the absence of cell swelling, outside-out patches from these cells did not contain active channels ($n = 8$) and we observed Cl^- channels by exposing cells to hypotonic bath solutions after establishing the whole-cell recording mode and then excising patches into the outside-out mode (Jirsch *et al.* 1994). After exposure to 210 mosmol l^{-1} bath, we observed patches containing a variable number of channels. A typical example of a patch that contained only two channels is illustrated in Fig. 4*A*. Here long bursts of openings of the Cl^- channel are observed. The channel was able to reopen repeatedly during prolonged depolarizations, which suggests that the channel can enter and exit a non-absorbing, but quite long-lived closed state at depolarized potentials. Single channel openings showed prominent outward rectification and ensemble averages in 1 mM $MgCl_2$ (Fig. 4*C*) at +100 mV were similar to whole-cell currents with marked current relaxation over the duration of the voltage clamp pulse (Fig. 1). The ensemble current shows rapid decay over the 1 s duration of the pulse and reaches an apparent steady-level after about half of the duration of the depolarization. The cause of the current decay apparent from the records in Fig. 4*A*, is a decrease in channel open probability (P_o) during the maintained depolarization, but this does not leave channels in an absorbing closed state that they are unable to leave at depolarized potentials. In the presence of 20 mM Mg^{2+} bursts of channel openings were also long, but the channel open probability during bursts was reduced markedly (Fig. 4*F*, the burst P_o decreased from >0·9 in control to a mode of 0·25 in 20 mM Mg^{2+}). In seven cells exposed to 1 mM Mg^{2+} the mean burst open probability was 0·88 ± 0·06 (mean ± S.E.M.) and 0·36 ± 0·17 (mean ± S.D.) for three cells exposed to 20 mM bath Mg^{2+}. Long-duration openings were prevented in the presence of high concentrations of Mg^{2+} (Fig. 4*E*) and the overall effect on the ensemble average was to reduce current greatly (Fig. 4*D*). The flickering block observed with very high concentrations of Mg^{2+} is very reminiscent of block of cation channels by divalent cations (Hille, 1991) or indeed stilbene block of cell-swelling-activated Cl^- channels (Solc & Wine, 1991).

DISCUSSION

This report provides evidence for the novel observation that divalent cations modulate cell-swelling-activated Cl^- channels from the external side by impeding the influx of Cl^- ions. The large, outwardly rectifying Cl^- channel has been described previously in epithelial cells from various tissues (Worrell *et al.* 1989; Solc & Wine, 1991; Kubo & Okada, 1992; Jirsch *et al.* 1994) and we identified it on the basis of its *I–V* relation, lack of dependence on intracellular Ca^{2+}, reversal potential shifts with alterations of the transmembrane Cl^- gradient, block by stilbene derivatives and large single channel chord conductance (at +100 mV) of 53 pS in symmetrical Cl^- concentrations.

The time-dependent relaxation of cell-swelling-activated Cl^- channels is a common (Worrell *et al.* 1989; Solc & Wine, 1991; Kubo & Okada, 1992) but not universal (Tseng, 1992; Stoddard, Steinbach & Simchowitz, 1993; Duan & Nattel, 1994) property of these currents and during strong depolarizations is usually ascribed to a voltage-dependent gating process (McCann *et al.* 1989; Worrell *et al.* 1989; Solc & Wine, 1991; Ackerman *et al.* 1994; Krouse *et al.* 1994). The entry of single Cl^- channels into an absorbing closed state seems restricted to very positive membrane potentials (Solc & Wine, 1991), and at less positive potentials a variable decrease in open probability with time is the major mechanism of current decay (inactivation).

Cations can block membrane Cl^- channels. Zn^{2+} has long been known to inhibit Cl^- efflux from skeletal muscle (Stanfield, 1970; Spalding *et al.* 1990), as do H^+ ions

(Spalding *et al.* 1990; Ackerman *et al.* 1994), but other divalent cations, specifically Mg^{2+}, seem to be without extracellular action (Spalding *et al.* 1990). Trivalent cations have recently been shown to be potent blockers of cell-swelling-activated Cl^- channels in *Xenopus* oocytes (Ackerman *et al.* 1994), where micromolar concentrations of La^{3+} and Gd^{3+} are effective. Mg^{2+} does regulate various Cl^- transporters: cytosolic Mg^{2+} inhibits a Cl^--dependent K^+ efflux in red blood cells (Lauf, Erdmann & Adragna, 1994). Intracellular Mg^{2+} also regulated the appearance of cell-swelling-activated Cl^- channels in neutrophils (Stoddard *et al.* 1993).

We have found that low concentrations of divalent cations, including Mg^{2+}, are able to reversibly modulate both steady current levels and current relaxations. Ackerman *et al.* (1994) have shown that decreases in H^+ in the external bath modulate cell-swelling-activated Cl^- channels in *Xenopus* oocytes by decreasing current relaxation at positive potentials. We did not change bath H^+ concentration, but we observed that when other external monovalent and divalent cations were replaced by NMDG, the current relaxation was mostly removed within a few pulses (Fig. 2). The ability of cations to cause anion current relaxation decreased as the atomic number increased for cations in the series $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} \gg Na^+$ (Fig. 3). The remaining current relaxation seen in the NMDG bath solution could be caused by H^+ ion effects on the channel (Ackerman *et al.* 1994) and/or blockade by the 1 mM $BaCl_2$ maintained in the bath.

Time-dependent relaxation of the Cl^- current appeared at potentials positive to the reversal potential, and became very marked around +50 mV. We suggest that Cl^- influx through channels was required to 'drag' extracellular cations into a blocking site within the pore of the channel. This effect became more marked towards +50 mV such that a negative slope region appeared in the *I–V* relation. At very large depolarizations, the mean steady-state *I–V* relations (Fig. 1) turned upward again. This leads to the speculation that at very large depolarizations and physiological concentrations of Mg^{2+}, Cl^- ions are able to force the blocking ions through the Cl^- channels and into the cells, as has been suggested for block of delayed rectifier K^+ channels by Cs^+ (French & Wells, 1977).

Our single channel data in normal hypotonic bath solution (1 mM Mg^{2+}, Fig. 4) suggested that up to +100 mV, where prominent relaxation of single channel ensemble averages was seen, the underlying single channel mechanism was a decrease in open probability with time of depolarization. In the presence of high Mg^{2+} concentrations, we observed a flickering block of the Cl^- channel. The channel open probability during bursts was markedly reduced and channel open time was less. No systematic alteration in the latency to first opening was observed.

Our conclusion from these experiments is that cations strongly modulate the amplitude and relaxation of cell-swelling-activated Cl^- channels. Further experiments are required on single channels and whole-cell currents in the absence of cations to establish the quantitative contribution of voltage-dependent inactivation and voltage-dependent cation block as mechanisms for Cl^- channel current relaxation at depolarized potentials.

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