Synergetic activation of outwardly rectifying Cl^- currents by hypotonic stress and external Ca^{2+} in murine osteoclasts

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1. An outwardly rectifying Cl^- (OR_{Cl}) current of murine osteoclasts was activated by hypotonic stimulation. The current was characterized by rapid activation, little inactivation, strong outward rectification, blockage by DIDS and permeability to organic acids (pyruvate and glutamate).

2. The hypotonically activated OR_{Cl} current was inhibited by intracellular dialysis with an ATP-free pipette solution, but not by replacement of ATP with a poorly hydrolysable ATP analogue adenosine 5′-*O*-(3-thiotriphosphate). The current amplitude was reduced when intracellular alkalinity increased over the pH range 6·6–8·0.

3. Intracellular application of cytochalasin D occasionally activated the OR_{Cl} current without hypotonic stress, but inhibited activation of the OR_{Cl} current by hypotonic stimulation. The hypotonically activated OR_{Cl} current was unaffected by a non-actin-depolymerizing cytochalasin, chaetoglobosin C, but partially inhibited by deoxyribonuclease I.

4. Removal of extracellular Ca^{2+} inhibited activation of the OR_{Cl} current by hypotonic shock, but did not reduce the current once activated. The hypotonically activated OR_{Cl} current was partially decreased by intracellular dialysis with 20 mM EGTA.

5. With 10 mM Ca^{2+} in the extracellular medium, the OR_{Cl} current was activated in response to more minor decreases in osmolarity than with 1 mM Ca^{2+}. The increased sensitivity to hypotonicity was mimicked by increasing the intracellular Ca^{2+} level (pCa 6·5).

6. These results suggest that hypotonic stimulation and a rise in the extracellular Ca^{2+} level synergistically activate the OR_{Cl} channel of murine osteoclasts, and that the activating process is modified by multiple intracellular factors (pH, ATP and actin cytoskeletal organization).

Osteoclasts secrete protons and various enzymes to degrade bone matrix, and play a crucial role in bodily Ca^{2+} homeostasis. Both organic and inorganic bone degradation products are transcytosed through osteoclasts and liberated into the extracellular space (Nesbitt & Horton, 1997; Salo *et al.* 1997). During the bone resorption cycle, osteoclasts face dynamic changes in diverse extracellular/intracellular environmental factors, such as osmolarity, Ca^{2+} levels, pH, metabolic activities and organization of cytoskeletons (Zaidi *et al.* 1993; Lakkakorpi & Väänänen, 1996). The ruffled membrane proton secretion via a vacuolar-type H^+-ATPase requires anion efflux through the Cl^- channel. Resultant accumulation of HCO_3^- and reduction of intracellular Cl^- are compensated for via the HCO_3^-–Cl^- exchanger in the basal membrane (Schlesinger *et al.* 1994). Transmembrane Cl^- flux is thus closely related to the functional state of osteoclasts. Chloride channels have been demonstrated in rat (Sims *et al.* 1991), avian (Blair & Schlesinger, 1990), rabbit (Kelly *et al.* 1994) and murine osteoclasts (Shibata *et al.* 1997), but still little is known about their role in osteoclast functions.

So far, the only known activating extracellular stimulus for Cl^- channels of osteoclasts is either a rise in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) (Fujita *et al.* 1996; Shibata *et al.* 1997) or hypotonic stress (Kelly *et al.* 1994). Resorbing activities are inhibited by a rise in $[Ca^{2+}]_o$ possibly via a low-affinity receptor-like $[Ca^{2+}]_o$-sensing molecule (Zaidi *et al.* 1995). Cell swelling induced by hypotonic shock may also lead to changes in various cellular events, such as ion transport through the plasma membrane, organization of the cytoskeleton and metabolic activities (Lang *et al.* 1998). Osteoclasts would be exposed to both a rise in $[Ca^{2+}]_o$ and an osmotic imbalance during the bone resorption. We have shown that a rise in $[Ca^{2+}]_o$ activates an outwardly rectifying Cl^- (OR_{Cl}) channel in *in vitro*-generated murine osteoclasts (Shibata *et al.* 1997) which have the characteristics of mature osteoclasts (Akatsu *et al.* 1992; Shioi *et al.* 1994), but the hypotonic effects on their Cl^- channels remain to be solved.

This study aimed at characterizing hypotonically activated Cl^- channels in murine osteoclasts and studying the co-operative action of the two stimuli, a rise in $[Ca^{2+}]_o$ and osmotic perturbation. We show that the hypotonically activated Cl^- current shares common electrophysiological features with the OR_{Cl} current activated by a rise in $[Ca^{2+}]_o$, and that a rise in $[Ca^{2+}]_o$ shifts the osmolarity set-point for the current activation to respond to a minor change in osmolarity. In addition, we provide evidence that the hypotonically activated OR_{Cl} current requires extracellular Ca^{2+} and intracellular ATP and is inhibited by intracellular alkalization and perturbation of the actin cytoskeleton. A preliminary report has been published (Sakai *et al.* 1997).

METHODS

Cell culture

Osteoclasts were generated from a co-culture of bone marrow cells of male, 5- to 8-week-old mice (C3H/HeN) with a marrow-derived stromal cell line (ST2; Riken Cell Bank, Tsukuba, Japan). The mice were killed by cervical dislocation. Bone marrow cells obtained from the femurs and tibias were centrifuged at 300 *g* for 7 min at 4 °C, and incubated in α-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS), streptomycin (0·1 mg ml^{-1}) and penicillin (100 u ml^{-1}) at 37 °C in a 95% air–5% CO_2 atmosphere overnight. Non-adherent cells were collected by centrifugation at 300 *g* at 4 °C for 7 min and incubated in a phosphate-buffered saline solution containing 0·02% pronase and 1·5 mM EDTA for 15 min at 37 °C. The pronase reaction was stopped by heat-inactivated horse serum (0·2 ml per 10 ml pronase solution), and the cell suspension was layered on ice-cold horse serum. After 15 min of sedimentation at unit gravity in the ice-cold horse serum, the uppermost part of the layers was collected, transferred into cold horse serum and then centrifuged at 800 *g* at 4 °C for 10 min. The bone marrow cell pellet was suspended in fresh α-MEM supplemented with 10% FCS at 1×10^6 cells ml^{-1} and co-cultured with ST2 cells (1×10^5 cells ml^{-1}) in the presence of 1α,25$(OH)_2D_3$ (10^{-8} M) and dexamethasone (10^{-7} M) at 37 °C in a 95% air–5% CO_2 atmosphere. The total medium was changed twice a week. ST2 cells were removed by incubation with 0·1% bacterial collagenase (collagenase S-1)–0·1% bovine serum albumin (BSA) in α-MEM for 10–30 min at 37 °C before recordings were made.

Identification of cells

After culturing for 5–7 days, osteoclasts were identified as multinucleated cells with a unique morphology (a flattened cell body, developed lamellipodia and retraction fibres) and tartrate-resistant acid phosphatase (TRAP) activity (Shioi *et al.* 1994). Actin rings and podosomes containing F-actin were identified by staining with rhodamine-conjugated phalloidin.

Solutions

The standard external solution contained (mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0·1% BSA and 10 Hepes-NaOH (pH 7·3) (299 ± 2 mosmol l^{-1}, $n = 5$ solutions). The osmolarity of solutions was measured using a freezing-point depression osmometer (OS osmometer, Fiske, MA, USA). Hypotonic solutions were made by reducing NaCl concentration. In the high Ca^{2+} (10–40 mM) or K^+-free solutions, the concentration of NaCl was changed to adjust the osmolarity to the required value. The Cl^- concentration of the medium containing 10 mM Ca^{2+} was 3 mM higher than that containing 1 mM Ca^{2+}. The low Cl^- hypotonic solutions were made by replacing NaCl with sodium isethionate, sodium pyruvate or sodium glutamate. The standard pipette solution contained (mM): 150 potassium glutamate, 3 $MgCl_2$, 1 EGTA, 1 Na_2ATP and 10 Hepes-KOH (pH 7·3). To examine the effects of intracellular pH on the currents, BAPTA (1 mM) was substituted for EGTA to stabilize the free Ca^{2+} concentration at different pH values. EGTA (20 mM) was added to keep the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) low in some experiments. To investigate the effects of the elevated $[Ca^{2+}]_i$ on current activities, pipette solutions containing high Ca^{2+} (pCa 6·5) were prepared with the appropriate amounts of EGTA and $CaCl_2$, which were estimated using the CHELATOR program (Schoenmakers *et al.* 1992). Potassium glutamate was replaced by caesium methanesulphonate to block K^+ currents. Alkaline pipette solutions (pH 7·8–8·0) were made by adjusting the pH with KOH, and the acidic pipette solution (pH 6·6) was buffered with 120 mM Mes. In the solutions containing high Mes or EGTA, potassium glutamate was reduced to compensate for the osmolarity. The osmolarity of these pipette solutions was maintained at between 280 and 290 mosmol l^{-1}.

Electrophysiological recordings

Recordings were made from osteoclasts cultured for 5–14 days. Culture dishes were placed on the stage of an inverted microscope (Diaphot-TMD, Nikon, Japan), and cells were monitored with a CCD camera (KY-F55MD, Olympus, Japan). The borosilicate glass pipettes had a resistance of 5–8 MΩ. Series resistance compensation (70–90%) was conducted to reduce the voltage error. The reference electrode was a Ag–AgCl wire connected to the bath solution through a Ringer–agar bridge. The zero current potential before formation of the gigaseal was taken as 0 mV.

Whole-cell currents were recorded by an amplifier (Axopatch 200A, Axon Instruments) at room temperature (20–24 °C). Current signals were digitized at 2 kHz with an analog-to-digital converter (Maclab/4S, ADInstruments, New South Wales, Australia), stored and analysed by a personal computer. Voltage ramps (0·24 mV ms^{-1} from −120 to +120 mV) were applied at a holding potential of −60 mV every 10 s. Leak current was determined from the linear portion of the current–voltage (I–V) relationship when either inward or outward current was absent or when the currents were eliminated by the blockers. The outward conductance was obtained from the I–V relationship between +80 and +100 mV, after subtraction of the leak current. Within this voltage range, the conductance estimated from the voltage ramp method was almost identical to that obtained by measuring the peak current amplitude evoked by voltage steps. The hypotonically activated conductance was obtained by subtracting the data before hypotonic stimulation from the maximum conductance evoked by the hypotonic stimulation. Data are expressed as means ± S.E.M., and were tested using Student's unpaired t test (unless otherwise stated). Values of $P < 0·05$ were considered to be significant. The P values between 0·05 and 0·2 are described in the figure legends.

Chemicals

Mes and BAPTA were purchased from Dojindo Laboratories (Kumamoto, Japan), collagenase S-1 from Nitta Gelatin Co. (Osaka, Japan), and 1α,25$(OH)_2D_3$ from Solway Duphar (Weesp, The Netherlands). All other chemicals were obtained from Sigma Chemical Co. A concentrated stock solution of Na_2ATP (500 mM) was prepared in 1 M Tris-Cl, stored in a freezer, and added to the internal medium before use. Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and deoxyribonuclease I (DNase I) were dissolved in distilled water. Cytochalasin D, chaetoglobosin C and DIDS were dissolved in DMSO. The final DMSO concentration was less than 0·1%, which itself did not affect the membrane currents.

RESULTS

Hypotonic stimulation activates an outwardly rectifying Cl^- current

The resting membrane potential of osteoclasts was -58 ± 2 mV ($n = 72$ osteoclasts) and whole-cell capacitance was 85 ± 3 pF ($n = 145$). In the standard solution, an inwardly rectifying K^+ (IR_K) current was exhibited in most *in vitro*-generated murine osteoclasts, but outward currents were generally small or negligible (Shibata *et al.* 1996, 1997). We previously reported that a rise in $[Ca^{2+}]_o$ activated an outwardly rectifying Cl^- (OR_{Cl}) current (Shibata *et al.* 1997). A similar OR current was activated by hypotonic stimulation. Figure 1*A* shows whole-cell currents evoked by voltage steps applied at a holding potential of -60 mV. When the osmolarity of the external medium was reduced to 60% of the control, a rapidly activating and little-inactivating current was evoked by depolarization (Fig. 1*A*, middle). The reduction of the peak current during a 500 ms-long depolarization (+80 or +100 mV) was $6{\cdot}5 \pm 2{\cdot}0$% ($n = 10$). A Cl^- channel blocker, DIDS, blocked the outward current (Fig. 1*A*, right). The percentage inhibition of the hypotonically activated OR current by 50 and 100 μM DIDS was 97 ± 3% (minimum–maximum, 84–100%; $n = 6$) and 98 ± 1% (92–100%; $n = 9$). Swelling of cells during hypotonic challenges was confirmed under the microscope, although this was not quantified. Figure 1*B* (left) shows a time course of changes in the outward conductance when the cell was perfused with the hypotonic solution (70% of control osmolarity) at about 0·1 ml s^{-1} (volume of the recording chamber, 2 ml). To eliminate the IR_K current, K^+ was omitted from the extracellular medium. Exposure to the hyposmotic solution reversibly activated the outward conductance. The current–voltage (*I–V*) relationships obtained by voltage ramps before (*a*), during (*b*) and after (*c*) exposure to the hypotonic solution are superimposed in Fig. 1*B*, right. The OR conductance was decreased by 30–100% of the maximum within 5–10 min after washout of the hypotonic solution. Immediately after switching from the hypotonic to the normotonic solution, OR currents transiently increased in 10 of 37 cells.

Figure 1*C* shows the *I–V* relationships of the hypotonically activated currents recorded in the K^+-free extracellular medium. The OR current was decreased in amplitude and activated at more positive potentials by lowering the extracellular Cl^- concentration of the hypotonic solution. A least-squares fit of a semilogarithmic plot of the reversal potential had a slope of 55 mV per tenfold change in $[Cl^-]_o$ (Fig. 1*D*). The hypotonically activated OR current was also observed when intracellular K^+ was totally replaced by Cs^+ (data not shown). These results suggest that the hypotonically activated OR current is carried mainly by Cl^- and shares common features with the OR_{Cl} current activated by a rise in $[Ca^{2+}]_o$ (Shibata *et al.* 1997). The permeability ratio between monovalent cations and Cl^- (P_{cation}/P_{Cl}), calculated from the reversal potentials using the Goldman–Hodgkin–Katz equation, was $0{\cdot}09 \pm 0{\cdot}02$ ($n = 9$). When the organic ions pyruvate$^-$ and glutamate$^-$ were substituted for the Cl^- of the hypotonic solution (70%, 109 mM anions), the reversal potential was shifted in a positive direction by $28{\cdot}7 \pm 1{\cdot}6$ mV ($n = 3$) and $50{\cdot}9 \pm 1{\cdot}9$ mV ($n = 4$), respectively, giving a permeability ratio $P_{pyruvate}/P_{Cl}$ of $0{\cdot}32 \pm 0{\cdot}02$ ($n = 3$) and $P_{glutamate}/P_{Cl}$ of $0{\cdot}13 \pm 0{\cdot}01$ ($n = 4$). The hypotonically activated OR conductance, calculated from the *I–V* relationship between +80 and +100 mV positive to the reversal potential, was reduced to $39{\cdot}4 \pm 8{\cdot}7$% ($n = 3$) of the Cl^- conductance with pyruvate$^-$ and $8{\cdot}5 \pm 3{\cdot}8$% ($n = 4$) with glutamate$^-$.

The relationship between the extracellular osmolarity and the OR conductance is summarized in Fig. 2. The OR conductance, normalized by the cell capacitance, was obtained from seven cells which were first suspended in the standard solution and then subsequently challenged with various hypotonic media. The OR conductance recorded at 100, 90, 80 and 70% of control osmolarity was $5{\cdot}4 \pm 0{\cdot}8$, $4{\cdot}9 \pm 1{\cdot}4$, $12{\cdot}5 \pm 5{\cdot}3$ and $121{\cdot}3 \pm 30{\cdot}8$ pS pF^{-1} ($n = 7$), respectively. In later experiments, the hypotonically activated OR_{Cl} current was examined by exposure to the solution at 70% of control osmolarity for about 5 min unless described otherwise.

Hypotonic activation of the OR_{Cl} current depends on intracellular ATP and pH

The rate of OR_{Cl} current activation varied among cells. In some cells, the hypotonically activated OR_{Cl} current spontaneously decreased despite continued hypotonic stimulation. On average, the OR_{Cl} current developed gradually for at least 3–5 min with the standard pipette solution containing 1 mM ATP (Fig. 3*A*, ○; $n = 22$). The current activation was inhibited by dialysis with an ATP-free solution (□, $n = 6$), but not by replacing ATP with 1 mM ATPγS, a poorly hydrolysable ATP analogue (△, $n = 6$). Figure 3*B* summarizes the maximum hypotonically activated OR conductance during exposure to the hypotonic solution, obtained by subtracting the conductance before stimulation. The conductance with 1 mM ATP was $79{\cdot}5 \pm 11{\cdot}4$ pS pF^{-1} ($n = 37$) (leftmost column in Fig. 3*B*). Inhibitory effects of depletion of ATP on the activation were not evident when the hypotonic shock was applied 3 min after formation of the whole-cell configuration ($50{\cdot}2 \pm 23{\cdot}8$ pS pF^{-1}, $n = 6$), but were significant when the stimulus was applied at 10 min ($15{\cdot}3 \pm 6{\cdot}8$ pS pF^{-1}, $n = 6$). Pretreatment with 5 mM sodium azide for 20–80 min followed by dialysis with the ATP-free solution for 10 min did not further inhibit the OR current ($22{\cdot}2 \pm 17{\cdot}8$ pS pF^{-1}, $n = 6$) (Fig. 3*B*). Most ($84{\cdot}8 \pm 6{\cdot}7$%, $n = 5$) of this small current was blocked by 100 μM DIDS, but the remaining DIDS-insensitive current, characterized by slow activation kinetics on depolarization, seemed to differ from the OR_{Cl} current. The second conductance, unidentified at this moment, was occasionally seen before hypotonic stimulation, but the amplitude was small even at potentials greater than +100 mV and was not increased by hypotonic stimulation. The hypotonically activated OR_{Cl} current with 1 mM

ATPγS was $89{\cdot}8 \pm 34{\cdot}2$ pS pF^{-1} ($n = 4$), significantly greater than that with the ATP-free solution ($P < 0{\cdot}05$), so that the major portion of the activated OR_{Cl} current seems to depend on non-hydrolytic ATP binding.

The hypotonic activation of the OR_{Cl} current was also inhibited by increasing the pH of the pipette solution (Fig. 4*A*). To minimize the difference in the Ca^{2+}-buffering action at a different pH, 1 mM BAPTA was substituted for 1 mM EGTA in the pipette solutions (Schoenmakers *et al.* 1992). The maximum OR_{Cl} conductance activated during exposure to hypotonic stimulation was $182{\cdot}2 \pm 31{\cdot}9$ pS pF^{-1} ($n = 7$) at pH_i 6·6, $112{\cdot}2 \pm 30{\cdot}8$ pS pF^{-1} ($n = 13$) at pH_i 7·3,

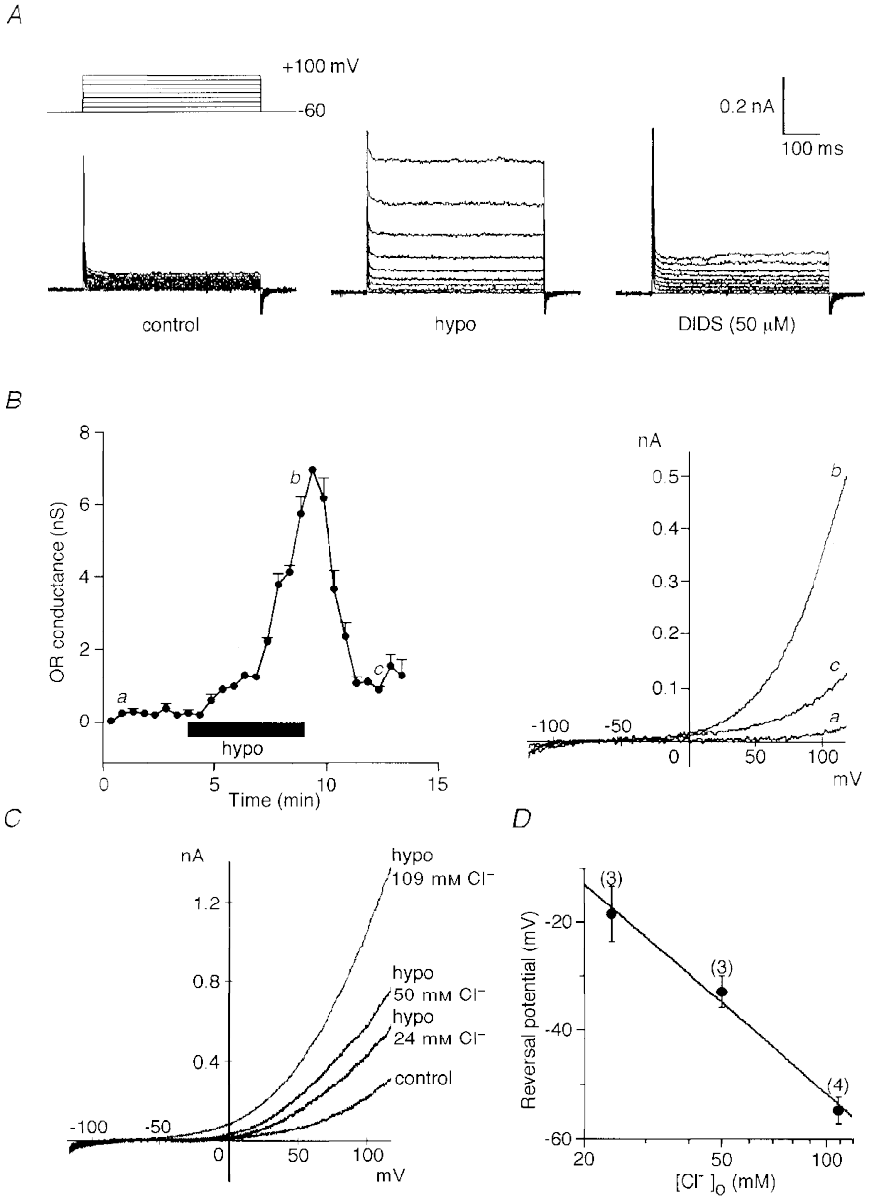

Figure 1. Hypotonically activated OR_{Cl} current of murine osteoclast

A, whole-cell currents evoked by a series of 500 ms voltage steps in control (left), during exposure to hypotonic solution (middle) and after addition of DIDS (right). *B*, time course of outward conductance in a cell exposed to hypotonic shock (left). The conductance was calculated from *I–V* relationships obtained by voltage ramps. Each point represents the mean of three consecutive responses at an interval of 10 s. Some error bars are smaller than the actual symbols. The *I–V* relationships at times indicated by *a*, *b* and *c* are superimposed (right). *C*, *I–V* relationship of the hypotonically activated currents in a cell recorded with different $[Cl^-]_o$. *D*, semilogarithmic plot of the reversal potential (means ± S.E.M.) of the hypotonically activated current against $[Cl^-]_o$. The number of cells examined is given in parentheses. The line indicates a least-squares fit of the data. The osmolarity of the hypotonic solution was 180 mosmol l^{-1} (60% of control osmolarity) in *A* and 210 mosmol l^{-1} (70%) in *B–D*. The extracellular medium was K^+ free in *B* and *C*. Leak currents were subtracted in *B* (right) and *C*, but not in *A*.

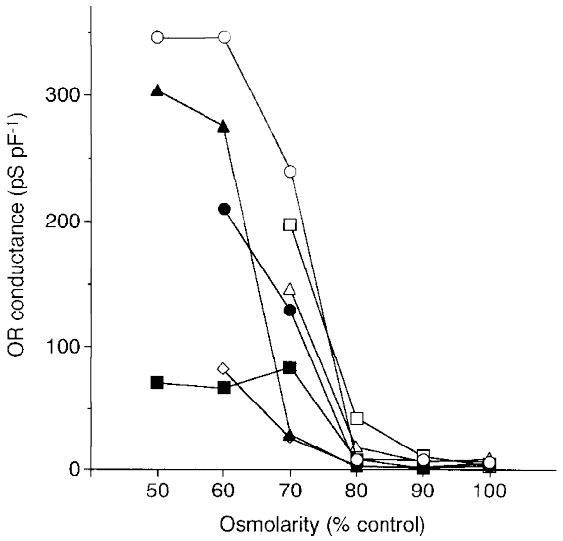

Figure 2. Osmolarity–conductance relationship for hypotonically activated OR current

The hypotonically activated OR conductance in seven cells is plotted against the osmolarity of the extracellular medium. The conductance was normalized by the cell capacitance. The osmolarity is expressed as a percentage of the control (100%, 299 mosmol l^{-1}). The osmolarity of the extracellular medium was subsequently decreased from 100% and data were obtained at about 5 min following perfusion of each hypotonic solution.

and $43{\cdot}2 \pm 9{\cdot}6$ pS pF^{-1} ($n = 12$) at pH_i 7·8–8·0 (Fig. 4*B*). The conductance with the alkaline solution was significantly larger than that at pH_i 7·3 ($P < 0{\cdot}05$) and pH_i 6·6 ($P < 0{\cdot}005$), suggesting that pH_i is crucial for the hypotonic activation of the OR_{Cl} current. The effects of pH_i were evident even when the cell was exposed to the hypotonic solution within 5 min following the rupture of the patch membrane.

Cytochalasin D inhibits the hypotonically activated OR_{Cl} current

Intracellular dialysis with cytochalasin D (1 μM) gradually activated the OR_{Cl} current without hypotonic stimulation in 7 out of 11 cells, often in association with bleb formation (Fig. 5*A* and *B*). The cytochalasin D-activated current shared common electrophysiological features with the hypotonically activated OR_{Cl} current. With pipette solutions containing

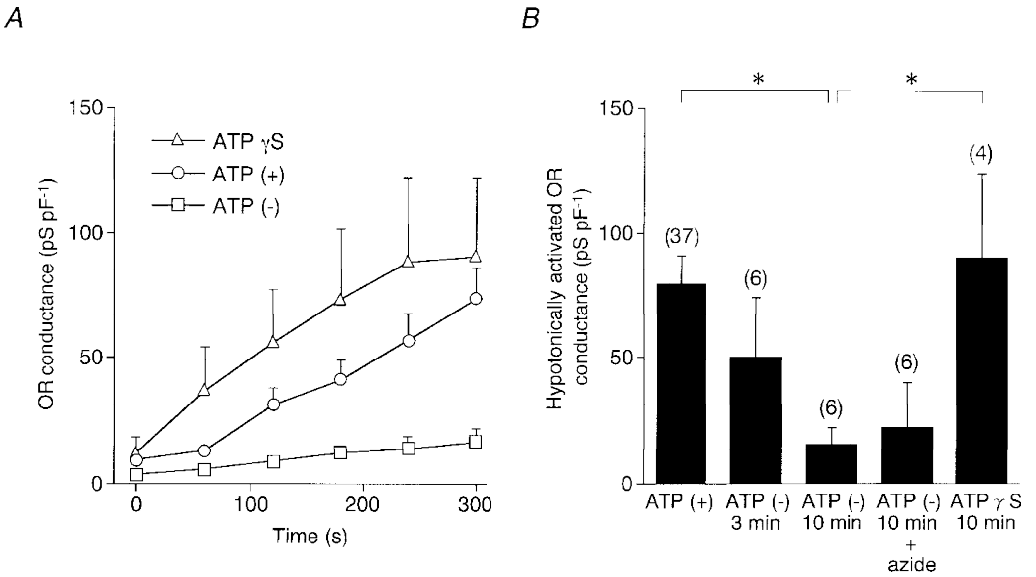

Figure 3. ATP dependence of hypotonically activated OR current

A, time courses of the OR conductance following hypotonic stimulation with ATP-containing (○, $n = 22$), ATPγS (1 mM)-containing (△, $n = 4$) and ATP-free (□, $n = 6$) pipette solutions. Cells were dialysed for 10 min with ATP-free or ATPγS-containing pipette solutions and then exposed to the hypotonic solution (70% of control osmolarity for 5 min) at time zero. *B*, the hypotonically activated OR conductance. Pretreatment with 5 mM sodium azide for 20–80 min was followed by dialysis with ATP-free solution for 10 min. Data are means and S.E.M. Number of cells examined is given in parentheses. * $P < 0{\cdot}05$ for ATP (+) and ATPγS *versus* ATP (−) for 10 min. The P value for ATP (−) plus sodium azide *versus* ATP (+) was 0·06.

0·1, 0·3 and 1 μM cytochalasin D, the OR conductance recorded at 3–4 min was 6·5 ± 1·7 ($n = 5$), 18·1 ± 2·1 ($n = 4$) and 41·7 ± 13·4 pS pF^{-1} ($n = 11$), respectively (Fig. 5*C*). The hypotonically activated OR_{Cl} current was significantly inhibited by dialysing cells for 3–4 min with cytochalasin D at both 0·1 μM (15·6 ± 5·2 pS pF^{-1}, $n = 5$) and 1 μM (9·9 ± 3·0 pS pF^{-1}, $n = 4$), where the current activation was negligible without hypotonic stimulation (Fig. 5*D*). Chaetoglobosin C (1 μM), a cytochalasin without depolymerizing action, did not affect the hypotonically activated OR_{Cl} current (79·4 ± 25·4 pS pF^{-1}, $n = 5$). Dialysis for 10 min with 10 μM DNase I, which inhibits actin polymerization by binding to globular actin, did not affect the hypotonically activated OR_{Cl} conductance (64·9 ± 21·5 pS pF^{-1}, $n = 8$). With 30 μM DNase I, however, the OR_{Cl} conductance was smaller than 18 pS pF^{-1} ($n = 4$) except for one cell (101·2 pS pF^{-1}) (29·3 ± 18·1 pS pF^{-1}; $n = 5$). Neither chaetoglobosin C nor DNase I (10–30 μM) activated the OR current without hypotonic stimulation.

Extracellular Ca^{2+} is required for the hypotonically activated OR_{Cl} current

Figure 6*A* shows the OR_{Cl} conductance of a cell which was successively exposed to nominally Ca^{2+}-free, Ca^{2+}-free hypotonic, and Ca^{2+} (1 mM)-containing hypotonic solutions. The OR_{Cl} current was not activated by hypotonic stimulation in the absence of Ca^{2+}. Subsequent perfusion with the Ca^{2+}-containing hypotonic solution reversibly activated the OR_{Cl} current. Figure 6*B* summarizes the results of similar experiments in seven cells. The hypotonically activated OR_{Cl} current in the absence of Ca^{2+} was significantly smaller (11·2 ± 4·7 pS pF^{-1}, $n = 7$) than that in the presence of Ca^{2+} (55·8 ± 13·3 pS pF^{-1}, $n = 7$) ($P < 0·01$ with Student's paired t test). Removal of the extracellular Ca^{2+}, however, did not reduce, but rather increased the current once activated ($n = 7$, data not shown).

When cells were exposed to hypotonic shock 10 min after dialysis with the pipette solution containing 20 mM EGTA ($n = 7$), the hypotonically activated OR_{Cl} conductance was generally small (24·1 ± 5·4 pS pF^{-1}, $n = 6$) except for one cell (111·9 pS pF^{-1}). Thus a low basal $[Ca^{2+}]_i$ seems to be required for the hypotonic activation of the OR_{Cl} current. It was noted that the OR_{Cl} current was not decreased by washout of the hypotonic solution, possibly because of profound cellular disturbances due to chelating intracellular Ca^{2+}.

Synergetic activation of the OR_{Cl} current by hypotonic stimulation and a rise in $[Ca^{2+}]_o$

A rise in the $[Ca^{2+}]_o$ activates the OR_{Cl} current (Shibata *et al.* 1997). The amplitude of the OR_{Cl} current in the presence of 10 and 40 mM Ca^{2+} was 20·6 ± 5·3 pS pF^{-1} ($n = 18$) and 116·9 ± 28·5 pS pF^{-1} ($n = 18$) (Fig. 7*A*). Figure 7*B* represents a typical experiment in which hypotonic stimulation was applied in the presence of 10 mM Ca^{2+}. Even with 90% of the control osmolarity, exposure to the solution activated the OR_{Cl} current. The hypotonically activated OR_{Cl} conductance at 90, 80 and 70% of control osmolarity was 23·7 ± 11·2, 64·2 ± 24·3 and 102·9 ±

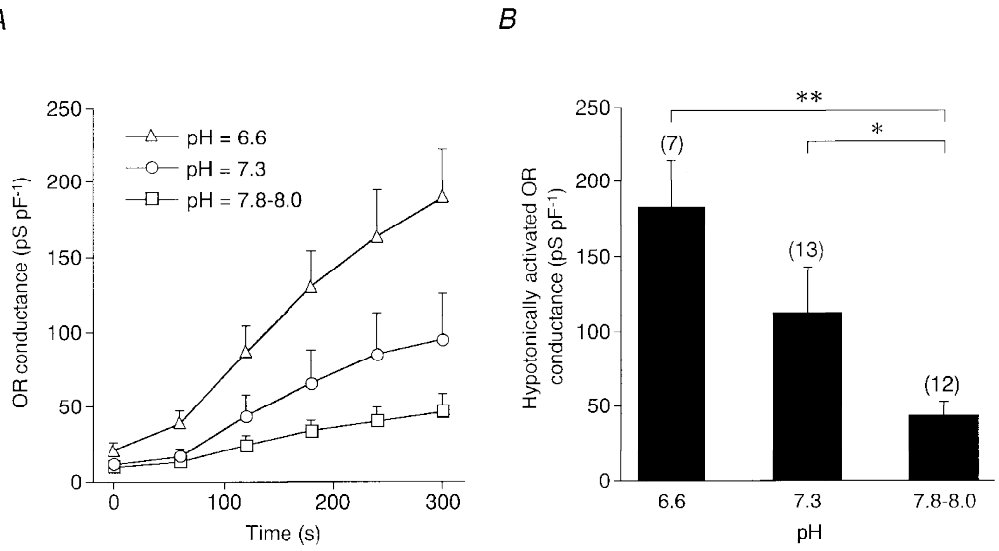

Figure 4. pH dependence of hypotonically activated OR current

A, time courses of the OR conductance following hypotonic stimulation at different intracellular pH values. Cells were dialysed with pipette solutions of variable pH for 10 min, and then exposed to the hypotonic solution (70% of control osmolarity for 5 min) at time zero. The pH of the standard pipette solution was 7·3. The pipette solutions contained 1 mM BAPTA, as a substitute for EGTA, to minimize the difference in free Ca^{2+} concentration at different pH values. *B*, the hypotonically activated OR conductances at different pH values. Data are means and S.E.M. Number of cells examined is given in parentheses. * $P < 0·05$. ** $P < 0·005$. The P value for pH 6·6 *versus* pH 7·3 was 0·16.

30·7 pS pF^{-1} ($n = 7$) in the 10 mM Ca^{2+}-containing external medium (Fig. 8, △), and 1·3 ± 1·1, 7·6 ± 5·3 and 115·8 ± 30·8 pS pF^{-1} ($n = 7$) in the standard medium containing 1 mM Ca^{2+} (○). Values at 90–80% were significantly larger with 10 mM Ca^{2+} although there was no difference in those at 70%, suggesting that a rise in $[Ca^{2+}]_o$ is likely to sensitize the channel to respond to more minor decreases in osmolarity.

A rise in $[Ca^{2+}]_o$ leads to elevation of $[Ca^{2+}]_i$ in osteoclasts (Miyauchi *et al.* 1990; Zaidi *et al.* 1993). To examine the role of a rise of $[Ca^{2+}]_i$ in increasing sensitivity to osmolarity, hypotonic stimulation was applied to cells dialysed for 10 min with a pipette solution containing high Ca^{2+} (pCa 6·5, 316 nM). The extracellular medium contained 1 mM Ca^{2+}. Intracellular dialysis with the high Ca^{2+} pipette solution itself did not activate the OR current (6·5 ± 0·8 pS pF^{-1}, $n = 11$). The hypotonically activated OR_{Cl} conductance at 90, 80 and 70% of control osmolarity was 11·8 ± 6·0 ($n = 8$), 54·1 ± 22·4 ($n = 7$) and 113·2 ± 36·1 pS pF^{-1} ($n = 9$) (Fig. 8, □). The values at 80% were significantly larger than the control, while there was no significant difference in those at 90 and 70%. Thus an increase in $[Ca^{2+}]_i$ did not activate the OR_{Cl} current by itself, but might shift the osmolarity set-point for the activation to a more minor hypotonicity.

DISCUSSION

Hypotonically activated OR_{Cl} current in murine osteoclasts

In murine osteoclasts, a strong elevation of $[Ca^{2+}]_o$ (⩾ 20 mM) activates a sizable OR_{Cl} current (Shibata *et al.* 1997). The present study provides evidence that hypotonic stress also activates an OR_{Cl} current. The hypotonically activated Cl^- current and the high $[Ca^{2+}]_o$-activated Cl^- current share the same kinetics (rapid activation and little inactivation on depolarization), strong outward rectification and sensitivity to DIDS, suggesting that the same class of OR_{Cl} channel underlies the two currents. In rabbit osteoclasts an OR_{Cl} current characterized by similar kinetics

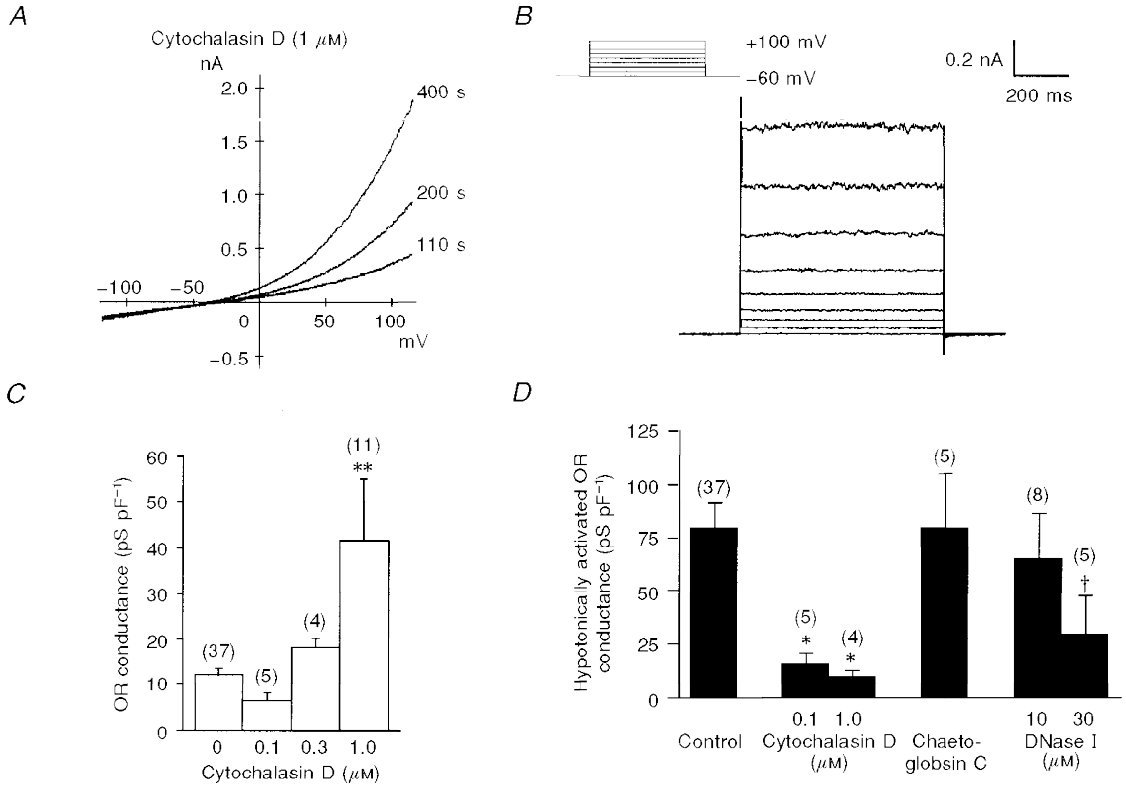

Figure 5. Effects of cytochalasins and DNase I on hypotonically activated OR current

A, *I–V* relationship recorded at 110, 200 and 400 s following intracellular dialysis with a pipette solution containing 1 μM cytochalasin D. *B*, a family of the cytochalasin D-induced currents evoked by voltage steps at around 300 s. *C*, the OR conductances in cells dialysed with cytochalasin D (0·1–1·0 μM for 3–4 min) without hypotonic stimulation. ** $P < 0·005$ compared with the control (0 μM cytochalasin D). The P value for 0 *versus* 0·1 μM cytochalasin D was 0·18. *D*, the OR conductance activated by hypotonic stimulation (70% of control osmolarity for 5 min) in cells dialysed with cytochalasin D (0·1 and 1·0 μM for 3–10 min), chaetoglobosin C (1 μM, 10 min) and DNase I (10 and 30 μM, 10 min). Data are means and S.E.M. Number of cells examined is given in parentheses. With 30 μM DNase I, hypotonic stimulation failed to activate the current in 4 of 5 cells. * $P < 0·05$ and † $P = 0·12$ compared with control.

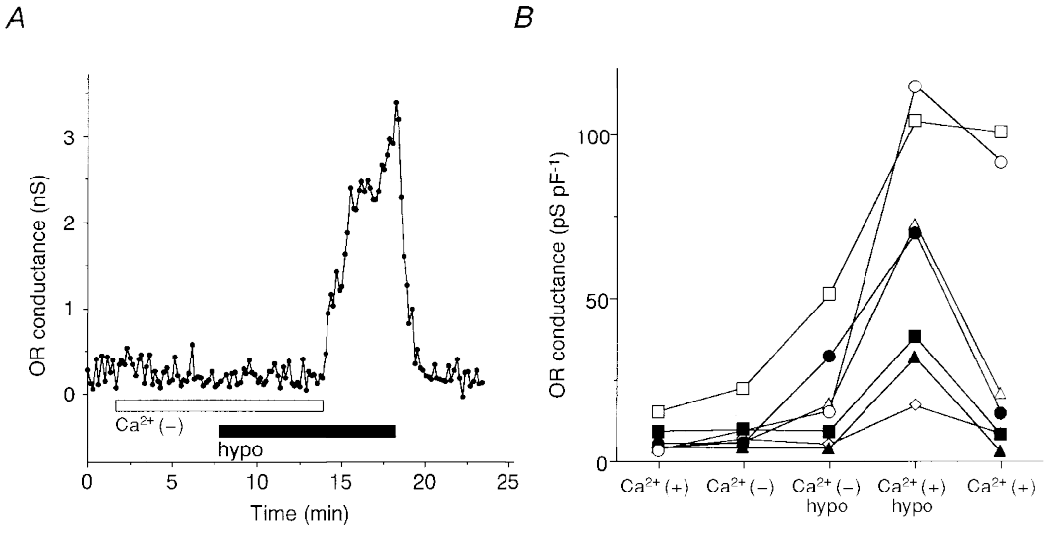

Figure 6. Extracellular Ca^{2+} dependence of hypotonically activated OR current

A, representative time course of the OR conductance when a cell was successively exposed to nominally Ca^{2+}-free, Ca^{2+}-free hypotonic or Ca^{2+} (1 mM)-containing hypotonic solutions. The osmolarity of the hypotonic solutions was 70% of control. *B*, the OR_{Cl} conductances induced by similar experiments in seven cells. Data were obtained at about 5 min following perfusion of each solution (sequentially, from left to right).

and pharmacology is activated by hypotonic stimulation (Kelly *et al.* 1994). The properties of hypotonically activated Cl^- currents of osteoclasts differ from those of either the ClC-2-type Cl^- channels or the maxi-Cl^- channels (Strange *et al.* 1996).

ATP dependence of the hypotonically activated OR_{Cl} current

A deficiency of intracellular ATP inhibited hypotonic activation of the OR_{Cl} current of murine osteoclasts. The inhibition was not apparent when the hypotonic challenge

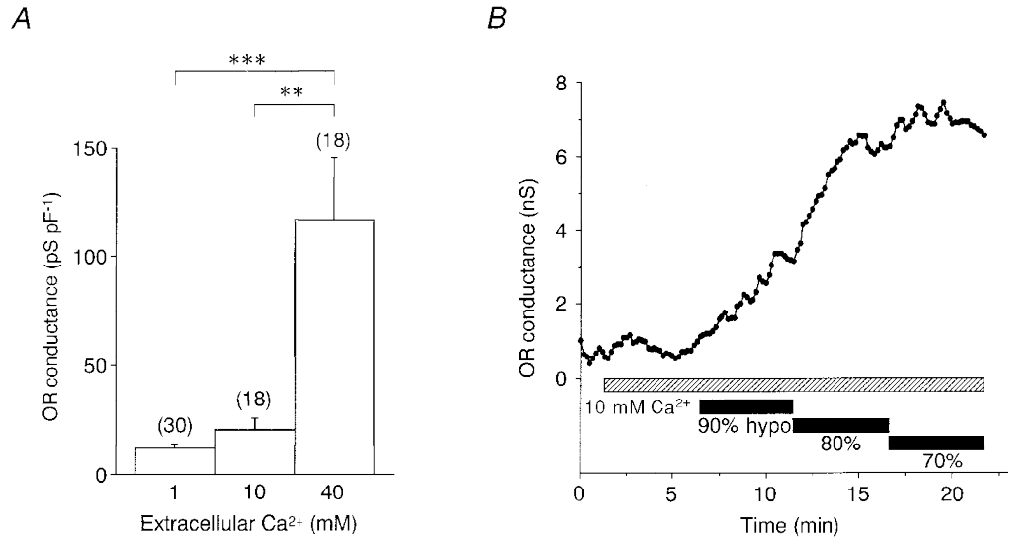

Figure 7. Effects of high extracellular $[Ca^{2+}]$ on hypotonically activated OR current

A, OR conductance (means and S.E.M.) produced by a rise in $[Ca^{2+}]_o$ in the normotonic solutions. The standard extracellular medium contained 1 mM Ca^{2+}. ** $P < 0{\cdot}005$. *** $P < 0{\cdot}0005$. The P value for 1 mM Ca^{2+} *versus* 10 mM Ca^{2+} was 0·09. *B*, time course of the OR conductance when a cell was exposed to a series of hypotonic solutions (90–70% of control osmolarity) in the presence of 10 mM Ca^{2+}.

was applied at 3 min following formation of the whole-cell configuration. Considering that the inhibitory effects of dialysis with either alkaline or cytochalasin D-containing pipette solutions were evident after a few minutes, ATP deficiency is likely to need a certain period to be effective. In rabbit osteoclasts, the hypotonically activated OR_{Cl} current was not inhibited by ATP depletion, although the period for the dialysis was not stated (Kelly *et al.* 1994). As substitution of ATPγS for ATP did not affect the hypotonically activated OR_{Cl} current, non-hydrolytic ATP binding seems to be responsible for the activation, although it is not known whether ATP binds to the channel or to unidentified intracellular regulatory molecules. In the present study, even when cells were dialysed with an ATP-free pipette solution for 10 min after pretreatment with 5 mM sodium azide for 20–80 min, a small but significant OR_{Cl} current was activated by hypotonic shock. Thus we cannot exclude the possibility that ATP-insensitive pathways are partially involved in the activating process, although the major portion of the hypotonically activated OR_{Cl} current indeed depends on non-hydrolytic ATP binding. The requirement for non-hydrolysing ATP for the hypotonic activation of Cl^- channels is reported in many mammalian cells (Jackson *et al.* 1994; Oike *et al.* 1994). As organic acids (pyruvate and glutamate) can permeate through the OR_{Cl} channel, this ATP dependency may work as a negative feedback mechanism to prevent loss of metabolic intermediate anions when cellular energy production is low (Jackson *et al.* 1994: Okada, 1997).

Effects of intracellular pH on the hypotonically activated OR_{Cl} current

In many cell types, cell swelling leads to cytosolic acidification (Lang *et al.* 1998). The present study showed that the hypotonically activated OR_{Cl} current was larger at a lower pH_i. During bone resorption, osteoclasts actively secrete protons into the resorbing pit and generate a load of cytoplasmic equivalent, primarily as HCO_3^-. The pH_i is influenced by the culture substrate (Lehenkari *et al.* 1997), extracellular pH (Teti *et al.* 1989; Nordström *et al.* 1997), and activities of diverse pH_i regulatory mechanisms including the HCO_3^-–Cl^- exchanger, the H^+ channel and the Na^+–H^+ exchanger (Schlensinger *et al.* 1994; Nordström *et al.* 1997). It is conceivable that the OR_{Cl} current activity dependent on changes in pH_i may modulate resorbing actions (Teti *et al.* 1991).

Involvement of actin cytoskeleton in the hypotonically activated OR_{Cl} current

Actin filaments are depolymerized or disrupted during osmotic cell swelling in a variety of cells (Lang *et al.* 1998). Activation of volume-sensitive Cl^- currents is often accompanied by reorganization of F-actin cytoskeleton (Strange *et al.* 1996; Okada, 1997). Cytochalasins have a number of actions on actin filaments (Sampath & Pollard, 1991), including inhibition of both polymerization and depolymerization of F-actin and disruption of F-actin, suggesting that the effects of cytochalasin D may arise from multiple combinations of these actions. Intracellular application of 1 μM cytochalasin D activated the OR_{Cl} current in 7 out of 11 cells. Intense reorganization of actin filaments by cytochalasin D may underlie this activation, as the current activation was often accompanied by bleb formation. DNase I, which inhibits polymerization of F-actin by binding to G-actin, did not activate the current by itself. The hypotonically activated OR_{Cl} current was inhibited by even 0·1 μM cytochalasin D, which did not activate the OR_{Cl} current by itself, but not by chaetoglobosin C, a cytochalasin without actin-depolymerizing action. Cytochalasins have been reported to inhibit activation of Cl^- channels by hypotonic stimulation in other cell types (Fatherazi *et al.* 1994; Schwiebert *et al.* 1994). DNase I also inhibited the hypotonically activated OR_{Cl} current partially, but the inhibitory action seems to be moderate compared with that of cytochalasin D, since a rather high concentration (30 μM) of DNase I was needed for the inhibition. Taking

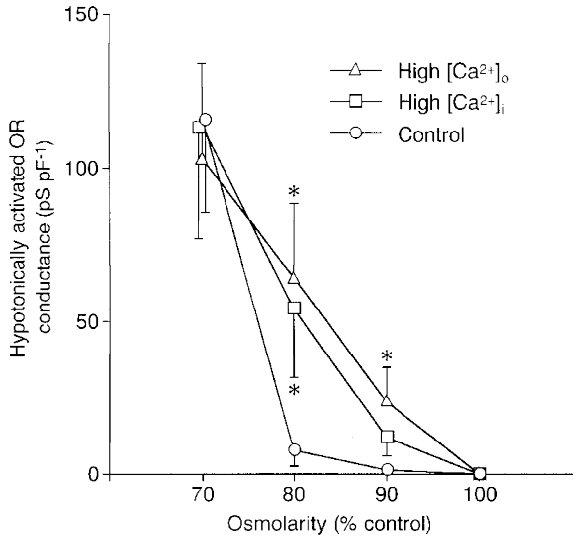

Figure 8. Effects of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ on the osmolarity–conductance relationship

The hypotonically activated OR conductance (means and S.E.M.) in control (○, $n = 7$), with high $[Ca^{2+}]_o$ (10 mM) (△, $n = 7$) and with high $[Ca^{2+}]_i$ (pCa 6·5) (□, $n = 7$–9). The $[Ca^{2+}]_o$ was 1 mM and the $[Ca^{2+}]_i$ was < 10 nM in the control condition. After treatment with these solutions for 10 min, the osmolarity of the extracellular medium was decreased. Data were obtained at about 5 min following the perfusion of each solution. * $P < 0·05$ compared with control.

these findings together, organization of actin filaments would be involved in the current activation machinery. Depletion of intracellular ATP alters actin cytoskeleton (Molitoris *et al.* 1991), and actin polymerization is inhibited as alkalinity increases over the pH range 6·6–8·3 (Sampath & Pollard, 1991). This may partly explain the inhibitory effect of ATP deficiency and intracellular alkalization on the hypotonically activated OR_{Cl} current.

Osteoclasts have organized membrane domains and change their shape and polarity according to functional states (Teti *et al.* 1991; Aubin, 1992). Osteoclasts form F-actin-containing podosomes or actin rings even on the culture dishes and disruption of actin rings suppresses the pit-forming activity (Zhang *et al.* 1995; Lakkakorpi & Vänäänen, 1996). A scattered punctate pattern of podosomes in the lamellipodia was often decreased by hypotonic stimulation in association with cell swelling (authors' unpublished observation), so hypotonic shock could inhibit bone resorption via reorganization of the actin cytoskeleton.

Effects of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ on the hypotonically activated OR_{Cl} current

In some cell types, regulatory volume decrease or activation of Cl^- channels by hypotonic shock depends on $[Ca^{2+}]_o$ (McCarty & O'Neil, 1992; Strange *et al.* 1996). The hypotonically activated OR_{Cl} current of murine osteoclasts was inhibited in the nominally Ca^{2+}-free solution, contrary to rabbit osteoclasts (Kelly *et al.* 1994). Removal of extracellulsr Ca^{2+}, however, did not reduce the current once activated, as reported in pancreatic duct cells (Verdon *et al.* 1995). Thus extracellular Ca^{2+} seems to be required for activation of the current, but not for sustained activity. Cell swelling leads to a rise in $[Ca^{2+}]_i$ in a variety of cells and, in some cell types, the rise in $[Ca^{2+}]_i$ due to Ca^{2+} influx is suggested to activate Cl^- channels (McCarty & O'Neil, 1992; Lang *et al.* 1998). Addition of high concentrations of EGTA to the pipette solutions partially inhibited the hypotonically activated OR_{Cl} current in murine osteoclasts, as in bovine vascular endothelial cells (Szücs *et al.* 1996) and rat carotid body cells (Carpenter & Peers, 1997). However, neither the introduction of 0·3–10 μM Ca^{2+} into the pipette solution nor the addition of ionomycin into the extracellular medium activated the OR_{Cl} current (Shibata *et al.* 1997). As reported in bovine vascular endothelial cells in which the hypotonically activated Cl^- current is unaffected by $[Ca^{2+}]_i > 50$ nM (Szücs *et al.* 1996), it is likely that a rise in $[Ca^{2+}]_i$ does not activate the OR_{Cl} channel directly but that a low basal $[Ca^{2+}]_i$ is needed for the activation.

During bone resorption, $[Ca^{2+}]_o$ in the resorptive pit is estimated to increase up to 40 mM (Silver *et al.* 1988), which results in suppression of the resorptive activity of osteoclasts by retraction of lamellipodia, reduction of podosome expression, de-adhesion of osteoclasts and inhibition of release of resorptive enzymes (Miyauchi *et al.* 1990; Zaidi *et al.* 1993). Sensitivity to increased $[Ca^{2+}]_o$ depends on their activation phases (Lakkakorpi *et al.* 1996). In the present study, a rise in $[Ca^{2+}]_o$ changed the osmolarity set-point for the current activation. The increased sensitivity to hypotonicity was mimicked by increasing $[Ca^{2+}]_i$ (pCa 6·5). A rise in $[Ca^{2+}]_o$ increases $[Ca^{2+}]_i$ to $\geqslant$ 200–300 nM by evoking Ca^{2+} release from the internal stores and Ca^{2+} influx through the plasma membrane in osteoclasts (Miyauchi *et al.* 1990; Zaidi *et al.* 1993). Actin filaments depolymerize by binding Ca^{2+} to gelsolin (Lang *et al.* 1998), which could modify the intracellular signalling for the activation of the OR_{Cl} channel. Thus an elevation in $[Ca^{2+}]_i$ would participate in the sensitization of the channel to hypotonicity at least partly, but the entire intracellular machinery underlying the synergetic action of a rise in $[Ca^{2+}]_o$ and hypotonicity on the OR_{Cl} current remains to be proved.

Physiological implications

Several roles of Cl^- channels can be postulated in osteoclasts. First, the membrane potential would be changed by the activity of the channel, although the direction and the magnitude depend on $[Cl^-]_i$. The membrane potential may be crucial in determining the $[Ca^{2+}]_i$ during bone resorption (Shankar *et al.* 1995). Second, the Cl^- channels may be responsible for Cl^- efflux through the ruffled membrane to maintain electroneutral HCl secretion into the resorptive lacuna. The ruffled border Cl^- channel incorporated in planar lipid bilayers has a current with similar properties to the hypotonically activated OR_{Cl} current (sensitivity to stilbene sulfonate and strongly outwardly rectified conductance) (Schlesinger *et al.* 1997). Third, the Cl^- channels may contribute to $[Cl^-]_i$ homeostasis by compensating for changes in $[Cl^-]_i$ due to the massive Cl^- flux during bone resorption. Fourth, the Cl^- channels may work as for organic solute transport, as suggested in swelling-activated organic osmolyte and anion channels (Strange *et al.* 1996). Fifth, the Cl^- channels could be involved in morphological changes during the resorption cycle, since they are one of the most efficient mechanisms of regulating cell volume (Okada, 1997).

In conclusion, we have demonstrated that hypotonicity and a rise in $[Ca^{2+}]_o$ synergistically activate the OR_{Cl} current of murine osteoclasts. Both stimuli could be inhibitory signals for resorbing activity, so that activation of the OR_{Cl} channel would rescue osteoclasts by the above mechanisms. As activation processes are modulated by pH_i, ATP and the organization of F-actin, the channel activities seem to depend greatly on the cellular functional states. The degree of swelling in response to hypotonicity was also varied among cells, although the change in volume was not quantified. The large variability of the current size may be explained partly by diversity in either the regulatory mechanisms or the amount of swelling. We previously suggested that the OR_{Cl} current could be activated only in a severe resorbing phase because efficient activation of the OR_{Cl} current needed a much higher $[Ca^{2+}]_o$ (> 20 mM) than that required for inhibition of the IR_K current (Shibata *et al.* 1997). The present study indicates that sizable OR_{Cl} currents may be activated in response to a minor change in osmolarity and a small increase in $[Ca^{2+}]_o$ if the two stimuli co-exist.

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