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Influence of K-Cl cotransporter activity on activation of volume-sensitive Cl^- channels in human osteoblasts

Margot Bräuer,[1] Eva Frei,[1] Lutz Claes,[2] Stephan Grissmer,[1] and Heike Jäger[1]
[1]*Department of Applied Physiology and* [2]*Institute of Orthopedic Research and Biomechanics, University of Ulm, 89081 Ulm, Germany*

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Bräuer, Margot, Eva Frei, Lutz Claes, Stephan Grissmer, and Heike Jäger. Influence of K-Cl cotransporter activity on activation of volume-sensitive Cl^- channels in human osteoblasts. *Am J Physiol Cell Physiol* 285: C22–C30, 2003. First published March 12, 2003; 10.1152/ajpcell.00289.2002.—The whole cell recording mode of the patch-clamp technique was used to study the effect of hypotonic NaCl or isotonic high-KCl solution on membrane currents in a human osteoblast-like cell line, C1. Both hypotonic NaCl or isotonic high-KCl solution activated Cl^- channels expressed in these cells as described previously. The reversal potential of the induced Cl^- current is more negative when activated through hypotonic NaCl solution (-47 ± 5 mV; $n = 6$) compared with activation through isotonic high-KCl solution (-35 ± 3 mV; $n = 8$). This difference can be explained by an increase in intracellular $[Cl^-]$ through the activity of a K-Cl cotransporter. Potassium aspartate was unable to activate the current, and furosemide or DIOA suppressed the increase in Cl^- current induced by isotonic high-KCl solution. In addition, we used the polymerase chain reaction to demonstrate the presence of KCC1–KCC4 mRNA in the osteoblast-like cell line. From these results, we conclude that human osteoblasts express functional K-Cl cotransporters in their cell membrane that seem to be able to induce the indirect activation of volume-sensitive Cl^- channels by KCl through an increase in the intracellular ion concentration followed by water influx and cell swelling.

potasium-chloride cotransporter; KCC1–KCC4; chloride channels; extracellular potassium concentration buffering

IN HUMAN OSTEOBLASTS a Cl^- channel seemed to get activated by either hypotonic solution or isotonic high-KCl solution (10). The high degree of similarity of the induced currents in respect to their electrophysiological as well as pharmacological properties argued for the activation of one type of Cl^- channel by the two solutions (10). The activation of Cl^- channels by hypotonic solutions has been described in a variety of other cell types (1, 2, 5, 9, 18, 20, 21, 26, 30–32, 34) and might be due to the lower osmolarity of the extracellular solutions causing water to enter the cell, therefore producing cell swelling. This swelling might stretch the cell membrane, and stretch could open mechanosensitive Cl^- channels. This mechanism seemed plausible because suction applied to the patch pipette interior opposed the induction of Cl^- currents, implying that the Cl^- channels might be modulated by a change in the mechanical stress on the membrane (21). In addition, the induced currents could be inhibited by extracellular hypertonicity induced by adding sucrose, an argument showing that the activation of the current did not depend on the ionic strength in the external solution but rather on the transmembrane osmotic pressure (intracellular osmolarity > extracellular osmolarity) (21).

The mechanism by which isotonic high KCl can activate the volume-sensitive Cl^- channel is not clear. Two distinct possibilities could account for the activation of the Cl^- channels by KCl: first, a K^+ binding site at the Cl^- channel directly activating the channel, and second, a more indirect mechanism that would also result in cell swelling. Another idea about how swelling can happen could be through the activity of a cotransporter activated by K^+ and Cl^-, increasing intracellular K^+ ($[K^+]_i$) and Cl^- concentration ($[Cl^-]_i$). This increase in the intracellular ion concentration would cause water to follow, thereby increasing cell volume. The resulting cell swelling could then be the stimulus to open the stretch-activated Cl^- channels.

The goal of this study was to distinguish these two possibilities for a direct or more indirect activation of the Cl^- channel in osteoblasts by KCl. We present evidence that human osteoblasts express functional K-Cl cotransporters (KCCs) in their cell membrane that seem to induce indirect activation of volume-sensitive Cl^- channels by isotonic high-KCl solution through an increase in the intracellular ion concentration followed by water influx and cell swelling. In addition, we could also demonstrate the presence of KCC1–KCC4 mRNA. Some of the results have been reported in preliminary communications (3, 4).

EXPERIMENTAL PROCEDURES

Cell culture. A human osteoblast-like cell line, C1 (immortalized human osteogenic precursor cells), was obtained from Dr. Brian A. Ashton (The Robert Jones & Agnes Hunt Orthopaedic and District Hospital, Oswestry, UK), cultured in DMEM (10% FCS) with 2 mM glutamine, and maintained continuously in a humidified 5% CO_2 incubator at 37°C.

Address for reprint requests and other correspondence: H. Jäger, Dept. of Applied Physiology, Univ. of Ulm, 89081 Ulm, Germany (E-mail: heike.jaeger@medizin.uni-ulm.de).

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Solutions. All experiments were done at room temperature (21–25°C). The cells under investigation were normally bathed in NaCl solutions containing (in mM) 164.5 NaCl, 0 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, adjusted to pH 7.4 with NaOH. The osmolarity of the NaCl solution was adjusted with either sucrose or mannose to 330 mosmol/l. We used this solution lacking K^+ to prevent the activation of the K-Cl cotransporter under these conditions. However, similar results were obtained by using NaCl solutions containing 4.5 mM KCl. In KCl solutions, all the NaCl of the NaCl solution was replaced by an equal amount of KCl. In K-aspartate solution, all the KCl was replaced by K-aspartate. The composition of the internal pipette solution (in mM) was 150 K-aspartate, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 3 Mg_2ATP, and 0.5 GTP, adjusted to pH 7.2 with KOH. The osmolarity was adjusted with either sucrose or mannose to 320 mosmol/l. Hypotonic solutions were prepared by diluting the NaCl or KCl solution appropriately with distilled H_2O. In rare cases, isotonic high-KCl did not induce an induction of Cl^- conductance. In all of those cases, 60% NaCl solution did not induce the Cl^- conductance either, whereas 50% NaCl solution was sufficient to induce the Cl^- conductance. Therefore, the effect of KCl on Cl^- current induction in these cells was investigated by comparing 60% NaCl solution with 60% KCl solution.

Furosemide was purchased from Sigma-Aldrich (Taufkirchen, Germany), and *R*-(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]acetic acid (DIOA) was purchased from RBI (Natick, MA; distributed through BioTrend Chemikalien, Cologne, Germany). Both substances were dissolved in DMSO as stock solutions and diluted appropriately to yield the final concentrations. The final DMSO concentration in our experiments was <0.3%. This concentration had no apparent effect on its own on the property of the Cl^- current as well as the K-Cl cotransporter during our measurements.

Electrophysiology. Experiments were carried out by using the whole cell recording mode of the patch-clamp technique (10–14, 25, 29, 33). Electrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) in two stages, coated with Sylgard (Dow Corning, Seneffe, Belgium), and fire-polished to resistances measured in the bath of 2.5–6 MΩ. Membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software (Pulse/Pulsefit, HEKA Elektronik). Membrane capacitance (C_m), a rough estimate of cell size, was resolved automatically through the EPC-9 software, which fits the exponential relaxation of capacitive currents elicited by voltage steps preceding the voltage ramp protocol used to measure current through the volume-sensitive Cl^- channels. Average C_m was 33 ± 9 pF (mean ± SD, $n = 36$). However, we did not use this procedure to determine changes in cell size during osmotic or KCl-induced swelling because it has been shown, at least in Jurkat T lymphocytes (26), that there is little correlation between cell swelling and membrane capacitance. Analysis was also performed by using IGOR Pro (Wavemetrics, Lake Oswego, OR) software. Some of the displayed ramp current traces were smoothed by the IGOR routine for better visualization. Final figures were created by using CANVAS (Deneba Systems, Miami, FL). Liquid junction potentials that develop at the tip of the pipette if the pipette solution is different from that of the bath were <5 mV and were not corrected for. Each illustrated response was observed at least three times. Values are given as means ± SD.

RT-PCR. Total RNA from the osteoblast cell line C1 cells was extracted by using QIAshredder and the RNeasy mini kit (Qiagen, Hilden, Germany) with RNase-free DNase treatment according to the manufacturer's instructions. For RT-PCR, 4 μg of total RNA were reverse transcribed with the use of oligo(dT) primer and reverse transcriptase (SuperScript first-strand synthesis system for RT-PCR; Invitrogen, Karlsruhe, Germany). cDNA was synthesized for 120 min at 37°C. cDNAs for each KCC isoform were amplified from single-stranded cDNA by PCR with the use of 25 mM $MgCl_2$ and *Taq* PCR DNA polymerase (Qiagen) in the presence of Q-solution. The amplification protocols for individual KCC isoforms were as follows: 35 cycles at 96°C for 30 s, 61°C for 30 s, and 72°C for 1 min, with a final extension time of 15 min. The PCR primers were designed on the basis of the published human cDNA sequences: KCC1 (accession no. XM 043043): sense, 5′-CTACAGGCCCTCCTCATCGTGC-3′, and antisense, 5′-GGCTCGGGGCATCTGCGGAG-3′ (759-bp product); KCC2 (accession no. XM 016773): sense, 5′-CTCAC-CTGCATGGCCACTGTG-3′, and antisense, 5′-CCCACTC-CTCTCCACAATCACG-3′ (405-bp product); KCC3a (accession no. AF211854) and KCC3b (accession no. AF211855): sense, 5′-GACCTGAGTCAGAACTCCATCAC-3′, and antisense, 5′-GATTCCTTGAGTGCGTCATCACTG-3′ (661-bp product, splice form independent); KCC4 (accession no. AF105365): sense, 5′-CCAGCCCGGGAGATGGAAATC-3′, and antisense, 5′-GGCCACGATGAGGAAGGACTCCAG-3′ (358-bp product); and β-actin (accession no. NM_001101): sense, 5′-GTGCGTGCAGGATTTAGG, and antisense, 5′-CCACATCTGCTGGAAGGTGG (551-bp product). Amplification of a 551-bp fragment of β-actin served as control; in case of genomic DNA contamination, a PCR fragment of 815-bp would have been generated. All KCC primer pairs only give products on cDNA. RT-Mastermix (without RNA), C1 RNA only, and PCR-Mastermix (without cDNA) served as controls. The PCR products were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. To verify the sequence of the amplified PCR products, we gel-purified the separated products using the QIAquick gel extraction kit (Qiagen), cloned the pcDNA3.1/V5-HisTOPO TA vector using the TA cloning kit (Invitrogen, Groningen, The Netherlands), and sequenced plasmid DNA from individual clones.

RESULTS

Activation of Cl^- channels by hypotonic NaCl and isotonic high-KCl solution. The basic observation is illustrated in Fig. 1*A*, which shows a series of currents elicited by voltage ramps from −160 to 40 mV. The records from −100 to +20 mV are shown for clarity. In this C1 cell, no outwardly rectifying current was observed unless either the extracellular solution was made hypotonic (Fig. 1*A*) or the NaCl in the isotonic extracellular solution was replaced by KCl (Fig. 1*C*). The induced current reversed at ∼−40 mV for the solution in Fig. 1*A* and at ∼−35 mV for the solution in Fig. 1*C*, indicating in both cases a Cl^- conductance rather than a nonselective leak. For a more detailed description of the reversal potential, see *Changes in* $[Cl^-]_i$. The time course of this induction can best be seen in Fig. 1, *B* and *D*, where the slope of the ramp current, measured at potentials between −60 and −30 mV for the records shown in *A* and *C*, respectively, was plotted against the absolute time after the whole cell

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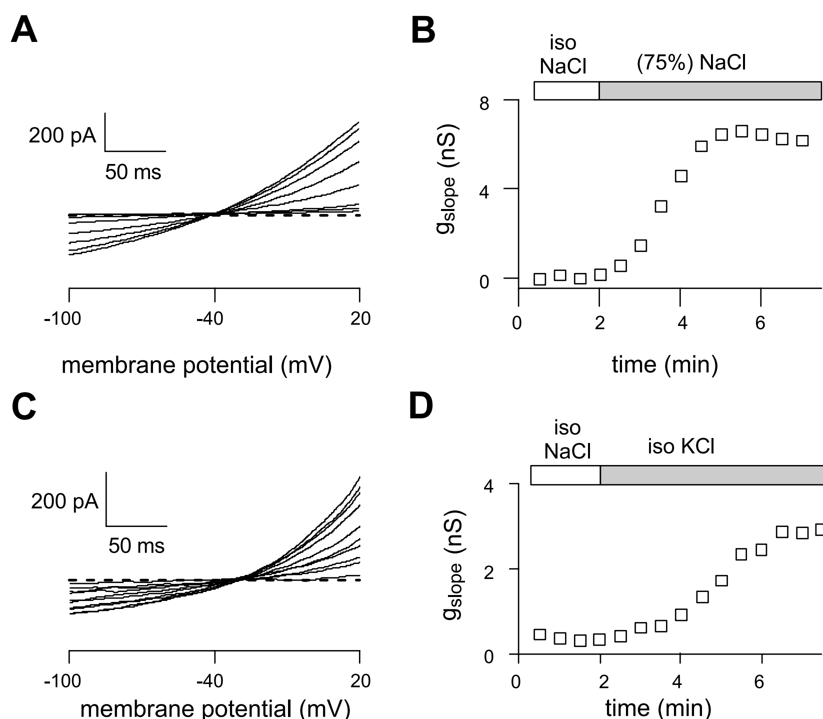

Fig. 1. Cl^- current induction in C1 cells by a hypotonic NaCl solution or an isotonic high-KCl solution. Current was elicited by voltage ramps from −160 to 40 mV within 400 ms at 30-s intervals. Current traces shown from −100 to +20 mV were measured before and after the bath solution was changed from a normal 100% NaCl solution to a hypotonic 75% NaCl solution (*A* and *B*) or to an isotonic high-KCl solution (*C* and *D*). Dotted lines show zero current level. The time course of Cl^- current induction by hypotonic NaCl solution (*B*) or isotonic high-KCl solution (*D*) is shown; conductance obtained from the slope (g_{slope}) of the Cl^- current was determined by fitting a line through the ramp current between −60 and −30 mV from those records shown in *A* and *C*, respectively. The slope of the ramp current was plotted against absolute time after the whole cell recording mode was established. Membrane capacitance was 35 pF in *A* and *B* and 29 pF in *C* and *D*. Iso, isotonic.

recording mode was established. After the solution change, it took about 3 min to fully activate the Cl^- currents through hypotonic solutions, whereas activation by isotonic high KCl seemed a bit slower. In both cases, this rather slow activation time course was not due to a slow exchange of the bath solution, which can be fully exchanged within 20 s, as can be demonstrated by the rapid reduction of slope conductance when the bath solution is exchanged to a solution containing fewer Cl^- that could carry current through the Cl^- channels (data not shown). Figure 1 therefore confirms earlier measurements of the activation of an identical Cl^- conductance by hypotonic NaCl and isotonic high KCl (10) and sets the stage for answering the question about the different mechanism of activation.

No activation of Cl^- channels by isotonic K-aspartate. To find out whether the activation of Cl^- current by an isotonic high-KCl solution depended on K^+ only or on a combination of K^+ and Cl^-, we tried to activate (open) the Cl^- channels in a solution containing isotonic K-aspartate (Fig. 2). This solution, however, did not induce a Cl^- current, as shown in Fig. 2, *A* and *B*. The slope conductance of the ramp current (Fig. 2*A*) obtained when the cell was bathed in K-aspartate was similar to that under control NaCl solution. The inability of the K-aspartate solution to induce the Cl^- current, even though the Cl^- channels might have been activated, could be due to the lack of a current carrier; even when channels would have been activated, no current would be observable. Once the current carrier, i.e., Cl^-, is added to the bath solution, one should expect a sudden increase in Cl^- current. The result of such an experiment can be seen in Fig. 2 where the slope conductance increases in KCl after the cell is bathed in K-aspartate. The scenario mentioned above, however, could not be observed. Instead, after isotonic high-KCl solution was added, it took again about 3–4 min for a full activation of the current, independent of a "pretreatment" with isotonic K-aspartate solution.

This result can be more clearly seen in Fig. 3, where the time course of the activation of the Cl^- current with isotonic high-KCl solution was compared when activated under different conditions. Figure 3*A* shows the time course of Cl^- current activation when the solution was changed from isotonic NaCl to isotonic high KCl. The solid line through the data points was drawn for clarity (Fig. 3*A*), with the time to reach half activation around 2 min. An almost identical activation time course can be seen in Fig. 3*B*, which shows the time course of Cl^- current activation when the solution was changed from isotonic K-aspartate to isotonic high KCl. The solid line from Fig. 3*A* has been drawn in Fig. 3*B*, with a shift along the time axis of about 15 s to the left indicating that the time courses in both cases are almost identical. This finding clearly demonstrates that the activation of the Cl^- current by isotonic high KCl was not a direct effect of K^+ on the channel but, rather, a combined action of K^+ and Cl^-. This in turn suggests that the increase in extracellular K^+ concentration ($[K^+]_o$) in combination with a high $[Cl^-]_o$ could turn on a K-Cl cotransporter, shuttling K^+ and Cl^- into the cell.

Pharmacological evidence for a K-Cl cotransporter. To test this hypothesis of a K-Cl cotransporter, we tried to activate the Cl^- channels in isotonic high-KCl solution in the presence of furosemide or DIOA. Furosemide is known to block the Na-K-2Cl as well as the K-Cl cotransporter, albeit with different sensitivities. Whereas furosemide blocks the Na-K-2Cl cotransporter in the low micromolar range (28; for review see

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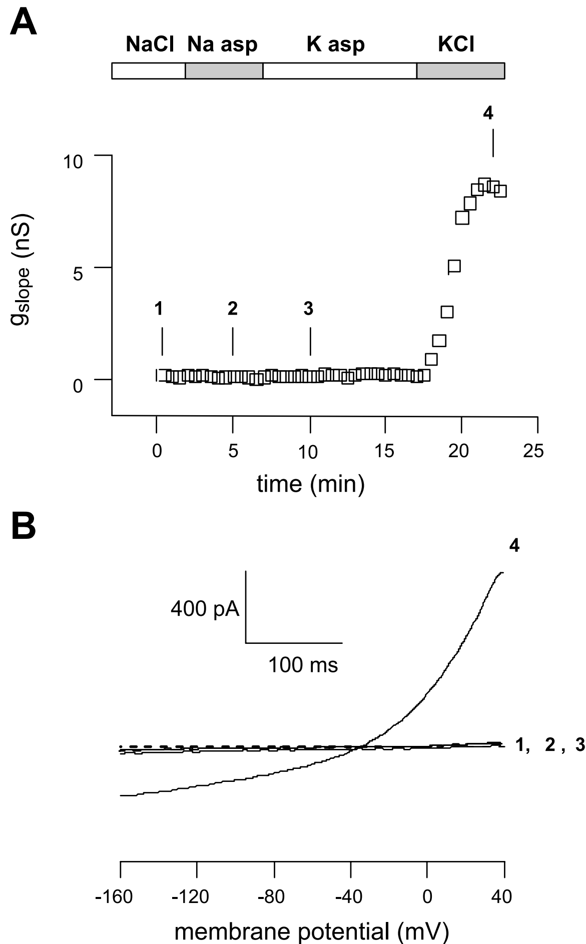

Fig. 2. Cl^- current induction in a C1 cell by isotonic K-aspartate and KCl solution. Ramp current was elicited (*B*) and the time course of the Cl^- current induction (*A*) was measured and shown (as described in Fig. 1) in isotonic K-aspartate (*trace 3*) solution and in isotonic high-KCl solution (*trace 4*). The dotted line in *B* shows zero current level. Membrane capacitance was 37 pF. Na asp, Na-aspartate; K asp, K-aspartate.

Ref. 27), its effect on the K-Cl cotransporter is only observed at much higher concentrations starting around 100 μM (19). Experiments with furosemide showed that only at concentrations higher than 100 μM was a failure to induce the volume-sensitive Cl^- conductance by KCl observed (data not shown). In addition, we assume that our observations are not due to the activity of the Na-K-2Cl cotransporter because all our experiments were performed in the absence of Na^+. DIOA is much more specific for blocking the K-Cl cotransporter rather the Na-K-2Cl cotransporter: whereas 100 μM DIOA blocks the K-Cl cotransporter, the Na-K-2Cl cotransporter is not blocked by this concentration (7, 8). The result of a DIOA experiment is presented in Fig. 4. To begin with, as a control, the volume-sensitive Cl^- conductance was induced in KCl solution compared with NaCl solution (Fig. 4*B*, compare *trace 1* before and *trace 2* after the induction). This induction of the volume-sensitive Cl^- conductance was then reversed by the application of the NaCl solution again. Another challenge for the activation of the Cl^- conductance by KCl failed to induce the current due to the presence of 100 μM DIOA, a concentration that does not inhibit the Na-K-2Cl cotransporter (Fig. 4*B*, *trace 3*). The effect of DIOA was totally reversible, because after washout of DIOA, the volume-sensitive Cl^- conductance could be induced again in KCl solution compared with NaCl solution (Fig. 4*B*, *trace 4*). A better overview of this experiment can be observed in Fig. 4*A*, where the slope of the ramp current is plotted against the time during the experiment. Figure 4 clearly demonstrates that DIOA can prevent the activation of the volume-sensitive Cl^- conductance by KCl, possibly by blocking the K-Cl cotransporter.

Alternatively, DIOA might directly block volume-activated Cl^- channels as has been demonstrated, for example, for other Cl^- channels, like CFTR (16), and the effect of DIOA shown in Fig. 4 could be due to this direct block rather than a blockage of the K-Cl cotransporter. To rule out this possibility, we tested DIOA on the volume-sensitive Cl^- conductance activated by a hypotonic NaCl solution. The result of such an exper-

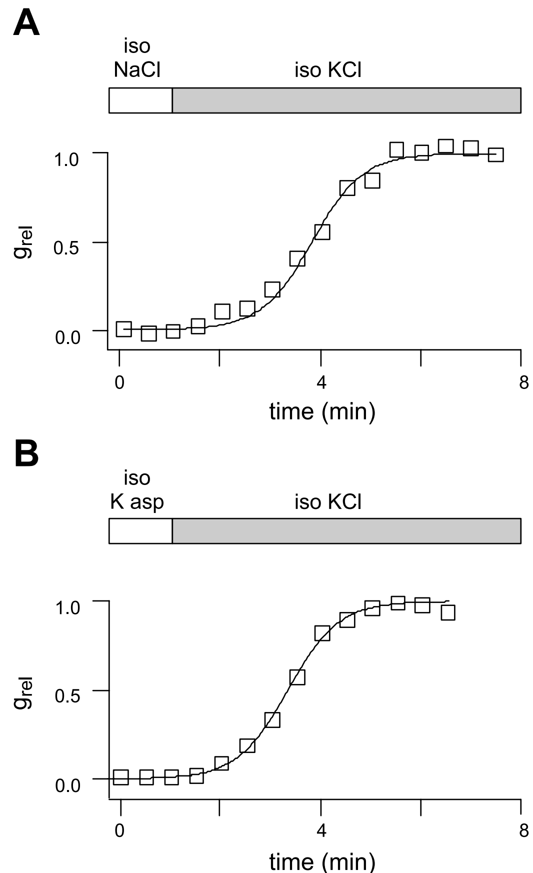

Fig. 3. Comparison of the time course of Cl^- current induction by isotonic high-KCl with NaCl solution (*A*) or K-aspartate solution (*B*) as the preceding solution. Relative slope conductance (g_{rel}) from experiments similar to those shown in Fig. 1 were plotted against time. The time course of Cl^- current induction was measured by changing the solution from isotonic NaCl (*A*) or isotonic K-aspartate (*B*) to isotonic high KCl. The solid line in *A* was drawn by eye and was taken into *B* shifted by about 15 s along the time axis to the left, showing that both time courses of Cl^- current induction are similar with a half-maximal activation after ~2 min.

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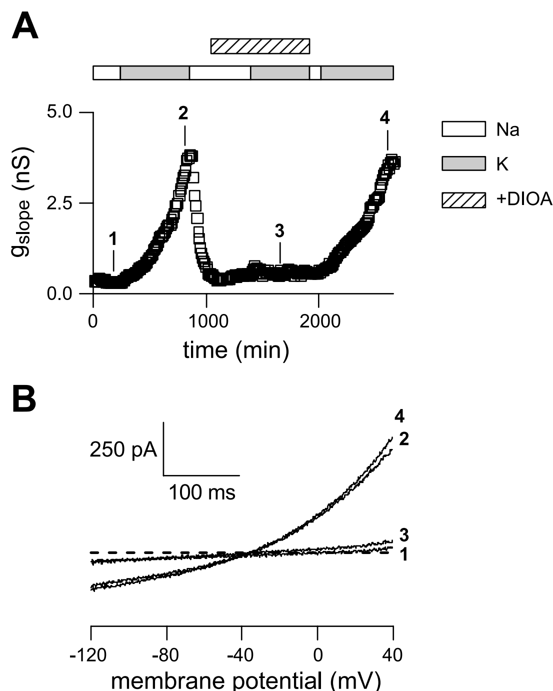

Fig. 4. Inhibition of Cl^- current induction in C1 cells by *R*-(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)-oxy]acetic acid (DIOA). Ramp currents were elicited by voltage ramps from −120 to 40 mV within 400 ms at 10-s intervals similarly to description in Fig. 1 legend. *A*: the time course of the Cl^- current induction was measured and shown as described in legend to Fig. 1 by changing the solution from 60% NaCl (open bars) to 60% KCl (filled bars) before, during, and after the presence of 100 μM DIOA (hatched bar). *B*: ramp currents in 60% NaCl solution (*trace 1*, no Cl^- conductance induced), 60% KCl solution (*trace 2*, after induction of Cl^- conductance), 60% KCl solution in the presence of 100 μM DIOA (*trace 3*, no Cl^- conductance induced), and 60% KCl solution after washout of DIOA (*trace 4*, after induction of Cl^- conductance). The dotted line shows zero current level. Membrane capacitance was 20 pF.

iment is shown in Fig. 5. Ramp current traces are shown before and after the activation of the volume-sensitive Cl^- conductance by 50% NaCl solution. Addition of 100 μM DIOA to this solution reversibly reduced this conductance to about two-thirds of the control conductance in the absence of DIOA. We assume that this partial reduction of the Cl^- conductance by DIOA is not sufficient to explain the failure to induce the activation of the Cl^- conductance by KCl demonstrated in Fig. 4. Therefore, we conclude that it is very likely that DIOA prevented the induction of the Cl^- conductance shown in Fig. 4 mainly through a blockade of the K-Cl cotransporter.

Changes in cell volume. If our hypothesis is correct, that a K-Cl cotransporter is responsible for the indirect activation of volume-sensitive Cl^- channels by KCl through an increase in the intracellular ion concentration, thereby inducing water influx and cell swelling, then we might also be able to verify the changes in cell volume directly. Therefore, we carried out experiments in which we monitored the size of single C1 cells through video imaging before and after bathing the cells in isotonic high-KCl solution. Representative images obtained from such an experiment are shown in Fig. 6. The single C1 cell was bathed in isotonic NaCl solution 5 min before (Fig. 6*A*) and 5 min after (Fig. 6*C*) the bath solution was changed to isotonic high-KCl solution (Fig. 6*B*). For better visualization of cell volume changes, a dotted oval has been drawn around the cell bathed in isotonic high-KCl solution (*B*), and an identical oval has been drawn over the cells shown in *A* and *C*. One can see that the dotted oval is larger than the cells shown in *A* and *C*, suggesting an increase in cell size in isotonic high-KCl solution. We take these imaging experiments as qualitative evidence for a cell volume increase in isotonic high-KCl solution compared with the situation when the cell is bathed in isotonic NaCl solution.

Changes in $[Cl^-]_i$. If our hypothesis is correct, that a K-Cl cotransporter is responsible for the increase in cell volume and, therefore, for the indirect activation of volume-sensitive Cl^- channels in isotonic high-KCl solution, then we should be able to estimate the increase in $[Cl^-]_i$ by comparing the reversal potential of the induced Cl^- current when activated through hypotonic NaCl solution or isotonic high-KCl solution. The Cl^- currents induced through hypotonic (75%) NaCl solution reversed direction around -47 ± 5 mV ($n = 6$). This value is in good agreement with earlier reports (21) on a similar conductance. For a perfectly selective Cl^- conductance, one would expect a reversal potential of −77 mV with $[Cl^-]_i = 6$ mM and $[Cl^-]_o = 127.5$ mM. However, it has been shown (9) that this Cl^- conductance is also slightly permeable to aspartate, the major anion used in our pipette solution. Assuming a permeability ratio for aspartate to Cl^- of about 0.1, as described earlier (21), the calculated reversal potential is $58 \text{ mV} \times \log \{[6 \text{ mM} + (0.1 \times 150 \text{ mM})]/127.5 \text{ mM}\}$, approximately equal to −45 mV, which is in excellent agreement with the observed reversal potential of −47

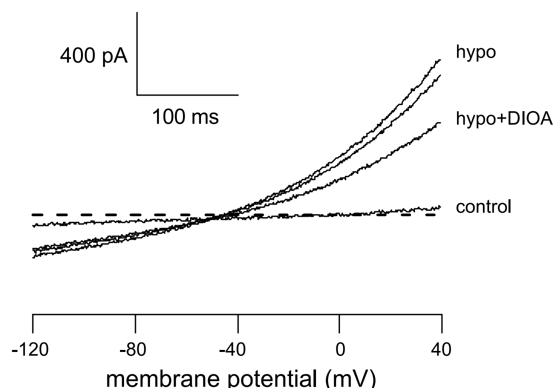

Fig. 5. Direct effect of DIOA on the volume-sensitive Cl^- conductance in C1 cells. Ramp currents were elicited similarly to description in Fig. 4 legend, and the Cl^- conductance was induced by hypotonic (50%) NaCl solution. After full activation, 100 μM DIOA was added to the bath solution. Ramp currents are shown before activation of the Cl^- conductance obtained in isotonic NaCl solution (control), after full activation with 50% NaCl solution before and after (hypo) as well as during (hypo + DIOA) the presence of 100 μM DIOA. The dotted line shows zero current level. Membrane capacitance was 20 pF. Hypo, hypotonic.

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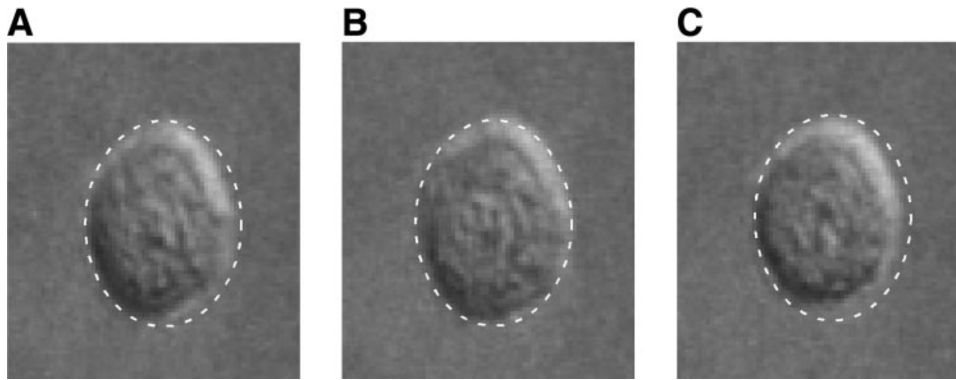

Fig. 6. Effect of isotonic high KCl on cell volume. A single C1 cell is shown bathed in isotonic NaCl solution 5 min before (*A*) and 5 min after (*C*) the bath solution was changed to isotonic high-KCl solution. For better visualization of cell volume changes, a dotted oval has been drawn around the cell bathed in isotonic high-KCl solution (*B*), and the identical oval has been drawn around the same cell shown in *A* and *C*.

mV stated above. The reversal potential of the induced Cl^- current is less negative when activated through isotonic high-KCl solution (-35 ± 3 mV; $n = 8$). This behavior is actually unexpected because $[Cl^-]_o$ in this isotonic high-KCl solution is much higher (~170 mM) than in the hypotonic (75%) NaCl solution (127.5 mM), and the reversal potential should therefore be more negative, or 58 mV × log {[6 mM + (0.1 × 150 mM)]/170 mM}, approximately equal to −53 mV, which is not observed. This discrepancy can be explained by assuming that the difference is due to a difference in $[Cl^-]_i$ induced by the activity of the K-Cl cotransporter. From the difference of the observed (−35 mV) and the calculated (−53 mV) reversal potential, we can estimate $[Cl^-]_i$ for the KCl-induced Cl^- conductance to be about 27 mM. This $[Cl^-]_i$ is ~20 mM higher than under the conditions where we have induced the Cl^- conductance using hypotonic solutions. We assume that the increase in $[Cl^-]_i$ in KCl solution is due to influx through a K-Cl cotransporter. In combination with an identical increase in $[K^+]_i$, this would lead to an increase in osmolarity of the intracellular solution of >10%. Even smaller differences in osmolarity between pipette and bath solution (~6%) have been reported to induce cell swelling and substantial Cl^- current induction (9) and could at least in part explain why extracellularly applied isotonic high-KCl solutions act very similarly to an extracellularly applied hypotonic solution due to the increase in the osmolarity of the intracellular solution by the transporter activity of the K-Cl cotransporter making the intracellular solution hypertonic.

RT-PCR. To characterize the KCC isoforms, RT-PCR was performed with KCC1- to KCC4-specific primers. Total mRNA of exponentially grown C1 cells was used to synthesize cDNA for RT-PCR analysis. As shown in Fig. 7, amplification of KCC1, KCC3, and KCC4 isoforms resulted in PCR products with lengths of 759, 691, and 358 bp as calculated from the database, encoding parts of the KCC1, KCC3, and KCC4 genes. The closely related KCC1 and KCC3 isoforms and the KCC4 isoform that is more homologous to KCC2 were found. A KCC2 transcript was only found at a very low

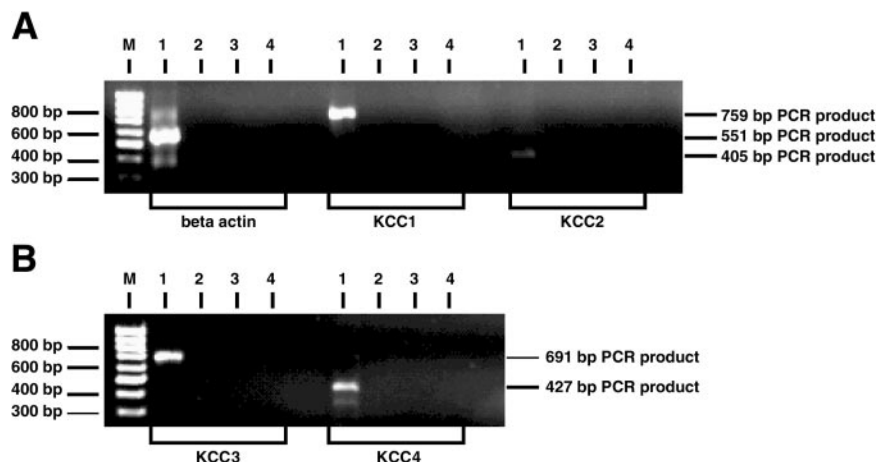

Fig. 7. RT-PCR analysis of K-Cl cotransporter (KCC) isoform in C1 cells. *A*: β-actin, KCC1, and KCC2; *B*: KCC3 and KCC4. Total RNA was prepared from C1 cells and used as template for RT-PCR by using oligonucleotides specific for KCC1, KCC2, KCC3, and KCC4, respectively. Amplification of a 551-bp fragment of β-actin served as control; in case of genomic DNA contamination, a PCR fragment of 815 bp would have been generated. With the KCC1, KCC3, and KCC4 primer pairs, PCR products of the expected length of 759 bp (KCC1), 691 bp (KCC3), and 358 bp (KCC4) were generated by PCR amplification of cDNA. With KCC2 primer pairs, a very low amount of PCR product of 405 bp was generated. *Lane 1*: RT-PCR reaction of individual mRNA samples. As a control for each, the RT-PCR reaction samples without template mRNA (*lane 2*), with RNase-free DNase (*lane 3*), and without cDNA (*lane 4*) were run in parallel. *Lane M*: DNA molecular mass marker. All PCR products were subcloned and verified by sequencing. One representative assay of four individual experiments, using different RT-PCR probes, is shown.

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level in the osteoblast cell line. All PCR products were verified by sequencing.

DISCUSSION

We have characterized a Cl^- conductance in cells from a human osteoblast-like cell line. The Cl^- conductance was activated by either hypotonic NaCl or isotonic high-KCl solution as described earlier on this C1 cell line as well as on other cells, including primary human osteoblasts (10). The activation mechanism by hypotonic NaCl solution was thought to occur via cell swelling through water influx (see Introduction). The activation mechanism by isotonic high-KCl solution could be either through a K^+ binding site at the Cl^- channel directly activating the channel or by a mechanism that somehow result in cell swelling activating the Cl^- channel. The goal of this study was to distinguish these two possibilities, and we present evidence that the induction of the Cl^- current by isotonic high-KCl solution is most likely occurring indirectly via an increase in the intracellular ion concentration through a functional K-Cl cotransporter in the osteoblast cell membrane. This increase in the intracellular ion concentration will result in water influx and, hence, cell swelling as demonstrated in Fig. 6. This swelling in turn will activate the volume-sensitive Cl^- conductance similarly as in hypotonic solutions. Therefore, we present evidence for the functional expression of a K-Cl cotransporter in human osteoblast-like cells.

Physiological role of the K-Cl cotransporter. Volume-sensitive Cl^- channels have been implicated as one limb in the regulatory volume changes that follow cell swelling and form part of the mechanism by which osmotically active solutes leave the cell. The other limb of the regulatory volume decrease (RVD) often involves a K^+ efflux. In addition to these ion channels involved in RVD, K-Cl cotransport has been described to regulate cell volume after swelling by promoting an efflux of K^+ and Cl^-, followed by a loss of water (23). Here we report that a K-Cl cotransporter might be responsible for cell swelling due to a reversed mode of action, transporting K^+ and Cl^- into the cell, especially under conditions where $[K^+]_o$ is elevated.

The activity of the K-Cl cotransporter under isotonic high-KCl solutions will transport K^+ and Cl^- into the cell and thereby increase the osmolarity of the intracellular solution. This change in intracellular osmolarity will draw water into the cell, leading to cell swelling. This in turn will activate the volume-sensitive Cl^- conductance that could lead to Cl^- efflux as a first step in RVD, paralleled by a concomitant K^+ efflux through K^+ channels. The activation of the K-Cl cotransporter in the above-described scenario seems to have a trigger function for ion channel activity. This mechanism might provide a means for the regulation of $[K^+]_o$, especially when K^+ accumulates in the extracellular medium. This increase in K^+ can occur either through K^+ loss from the same cell or when cells in the vicinity die or are destroyed and release their intracellular content, mainly K^+, into the extracellular solution as might be the case for bone fractures. In addition, the K-Cl cotransporter may be important in buffering the extracellularly accumulated K^+, removing it from the extracellular solution as has been suggested for KCC2 in the brain in association with a reversed mode of action of KCC2 under high $[K^+]_o$ (24). Both effects might have physiological consequences for the healing processes after bone fractures or for stabilizing an environment that sustains proper bone function.

Other transport systems and mechanisms. Several different ion transport systems have been described in the literature in a variety of tissues. Three major families of cation Cl^- cotransporter can be distinguished on the basis of the number stoichiometry and type of transported ions as well as their pharmacology: the Na-Cl cotransporters (NCC), the K-Cl cotransporters (KCC), and the Na-K-2Cl cotransporters (NKCC). Several facts argue that the effects we observe are due to the activation of a K-Cl cotransporter and not to the activation of a Na-K-2Cl or Na-Cl cotransporter. First, the observations described in this article are independent of extracellular Na^+, and second, the pharmacological profile of the observation to induce the volume-sensitive Cl^- conductance by KCl pointed also to the activity of a K-Cl cotransporter rather then to a Na-K-2Cl cotransporter because DIOA (at low concentrations) and furosemide (only at higher concentrations) could modify the induction of the volume-sensitive Cl^- conductance by KCl.

Another potential mechanism by which the increase in extracellular KCl may drive KCl into the cell might be due to profound depolarization in high-KCl solution, assuming that K^+ channels dominate the membrane potential in osteoblasts as they do in most cells. Although the depolarization per se would not change $[K^+]_i$ or $[Cl^-]_i$, other channels or transport mechanisms, for example, a voltage-dependent Cl^- conductance or some other voltage-dependent transport mechanisms, need to be present and activated in parallel to do so. If a voltage-dependent Cl^- conductance or a background Cl^- conductance were present in osteoblasts, then a depolarization more positive than the reversal potential for Cl^- would allow Cl^- to enter the cell and, in combination with a K^+ conductance, could transport KCl into the cell. Our experiments with K-aspartate cannot exclude such a mechanism where Cl^- and K^+ channels work in parallel if aspartate is hardly permeable through the postulated Cl^- channel as has been shown for other Cl^- channels. Our experiments with DIOA, which could prevent the induction of the volume-sensitive Cl^- channel in isotonic high-KCl solution, however, seem to argue for the involvement of the K-Cl cotransporter that is responsible for cell swelling due to a reversed mode of action.

DIOA was thought to specifically block the K-Cl cotransporter at least compared with the Na-K-2Cl cotransporter (8). Our results shown in Fig. 4 indicating that KCl failed to induce the volume-sensitive Cl^- current in the presence of DIOA led us to speculate that DIOA blocked the K-Cl cotransporter, thereby preventing the increase in $[KCl]_i$ that would cause cell

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swelling and Cl^- current activation. To our surprise, DIOA also had a direct effect on the volume-sensitive Cl^- channels when tested with a hypotonic NaCl solution (Fig. 5). An identical concentration of DIOA that had completely prevented the induction of the volume-sensitive Cl^- conductance reduced this conductance to about two-thirds of the control conductance in the absence of DIOA. Although we assume that this partial reduction of the Cl^- conductance by DIOA is not sufficient to explain the failure to induce the activation of the Cl^- conductance by KCl demonstrated in Fig. 4, we cannot exclude the possibility that the direct effect of DIOA on Cl^- current (as presented in Fig. 5) might have at least partially affected our results. In summary, we conclude that it is very likely that DIOA prevented the induction of the Cl^- conductance shown in Fig. 4 mainly through a blockade of the K-Cl cotransporter.

Using RT-PCR, we could prove the presence of KCC1, KCC3, and KCC4 transcripts in our osteoblast cell line, verified by sequencing. The KCC1 isoform is highly homologous to KCC3, a KCC isoform that is widely but not universally distributed in tissue (19), and KCC4 shares the highest (69%) identity with KCC2. Therefore, we found KCC proteins from a separate subfamily of K-Cl cotransporters. The KCC1 isoform, belonging to a subgroup that exhibits a lower K^+ affinity compared with the KCC4 gene product, shows a higher affinity in kinetic studies (22). A very weak reaction in the KCC2 RT-PCR reaction appeared and was also proven to be KCC2 message by sequencing. This was not expected because KCC2 is the neuronal isoform (17). It is known that K-Cl cotransporters have a stoichiometry of 1:1 (15), but we cannot deduce from our data whether KCC1, KCC3, and KCC4 are present as homomeric or heteromeric dimers.

Changes in $[Cl^-]_i$. The change in $[Cl^-]_i$ as calculated in RESULTS is only a rough estimate because it takes into account neither the dilution of $[Cl^-]_i$ by water flux through the membrane nor the exchange of water and osmolytes with the pipette solution. A quantitative model describing changes in cell volume in a cell dialyzed with hyperosmotic pipette solutions (added sucrose) has been presented by Ross et al. (26). This model predicts cell volume changes as solutes enter the cell from the pipette. The situation described there is comparable to ours, although in our case we have assumed that the solutes entered the cell through the activity of a K-Cl cotransporter. Several observations from their model make this situation similar and comparable to ours. First, the sucrose diffusion out of the pipette in their experiments is such that the sucrose concentration is in equilibrium within ~100 s; second, the activation of the Cl^- conductance is only dependent on the transosmotic gradient; and third, there is only a weak correlation between the access resistance (series conductance) and the diffusion of osmolytes out of the pipette. The experiments elevating external osmolarity with sucrose also argue for an activation mechanism of the Cl^- channel by changes in cell volume rather than external ionic strength.

From the time course of the Cl^- conductance activation and assuming that all of the observed changes in $[Cl^-]_i$ are due to the K-Cl transport activity, we can estimate the transport rate to be in the order of about 10^7 cycles per second per cell. Assuming transport rates of about 10^4 per second (for review see Ref. 15), one can again estimate about 1,000 functional K-Cl cotransporters per cell. We realize that this calculation can only be a very rough estimate. In addition, it makes no predictions about the activation mechanism of the Cl^- conductance or whether the change in osmolarity has an additional effect on the K-Cl cotransporter as was described earlier (6). We conclude that human osteoblasts express functional K-Cl cotransporter in their cell membrane. These transporters seem to play an important role in the indirect activation of volume-sensitive Cl^- channels by an increase in extracellular KCl. If the extracellular KCl concentration increases, the K-Cl cotransporter shuttles KCl into the cell, thereby rising the intracellular ion concentration. This in turn is followed by water influx and cell swelling.

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