

Characterization of the Increase in $[Ca^{2+}]_i$ During Hypotonic Shock and the Involvement of Ca^{2+} -activated K^+ Channels in the Regulatory Volume Decrease in Human Osteoblast-like Cells

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Abstract. The calcium indicator fura-2 was used to study the effect of hypotonic solutions on the intracellular calcium concentration, $[Ca^{2+}]_i$, in a human osteoblast-like cell line. Decreasing the tonicity of the extracellular solution to 50% leads to an increase in $[Ca^{2+}]_i$ from ~150 nM up to 1.3 μ M. This increase in $[Ca^{2+}]_i$ was mainly due to an influx of extracellular Ca^{2+} since removing of extracellular Ca^{2+} reduced this increase to ~250 nM. After cell swelling most of the cells were able to regulate their volume to the initial level within 800 sec. The whole-cell recording mode of the patch-clamp technique was also used to study the effect of an increase in $[Ca^{2+}]_i$ on membrane currents in these cells. An increase in $[Ca^{2+}]_i$ revealed two types of Ca^{2+} -activated K^+ channels, K(Ca) channels. Current through both channel types could not be observed below voltage of +80 mV with $[Ca^{2+}]_i$ buffered to 100 nM or less. With patch-electrodes filled with solutions buffering $[Ca^{2+}]_i$ to 10 μ M both channels types could be readily observed. The activation of the first type was apparently voltage-independent since current could be observed over the entire voltage range used from -160 to +100 mV. In addition, the current was also blocked by charybdotoxin (CTX). The second type of K(Ca) channels in these cells could be activated with depolarizations more positive than -40 mV from a holding potential of -80 mV. This type was blocked by CTX and paxilline. Adding paxilline to the extracellular solution inhibited regulatory volume decrease (RVD), but could not abolish RVD. We conclude that two K(Ca) channel types exist in human osteoblasts, an intermediate conductance K(Ca) channel and a MaxiK-like K(Ca) channel. MaxiK channels might get activated either directly or by an increase in

$[Ca^{2+}]_i$ elicited through hypotonic solutions. In combination with the volume-regulated Cl^- conductance in the same cells this K^+ channel seems to play a vital role in volume regulation in human osteoblasts.

Key words: K(Ca) channels — $[Ca^{2+}]_i$ — Volume regulation

Introduction

Regulatory volume decrease (RVD) after cell swelling involves the increase in K^+ and/or Cl^- fluxes through the cell's membrane in a variety of cell types. Possible mechanisms by which the increase in cell volume is sensed and transduced involve the dilution of intracellular messengers or macromolecules or a direct or indirect mechanical activation of ion channels by changes in tension of the cell membrane or the cytoskeleton (for review *see* Lang et al., 1998). For example, lowering the osmolarity of the bath solution will cause water to enter the cell, consequently leading to cell swelling. This swelling produces membrane stretch and activates a volume-sensitive Cl^- conductance in a variety of cell types (for reviews *see* Nilius et al., 1996; Strange, Emma and Jackson, 1996) including human osteoblasts and a human osteoblast-like cell line (Steinert & Grissmer, 1997). This activation is likely to be one limb of RVD. The other limb of RVD often involves a potassium efflux. This is the case in human T lymphocytes (Cahalan & Lewis, 1988; Lee et al., 1988), where it was shown that the function of the voltage-gated potassium channel expressed in human T lymphocytes (Grissmer et al., 1990) was important in this scenario (Cahalan & Lewis, 1988; Lee et al., 1988). Therefore we investigated human osteoblast-like cells in order to find a cation ef-

flux. K^+ channels have been reported in several osteoblast-like cell lines (Ypey et al., 1992; Davidson 1993; Gofa & Davidson 1996; Yellowley et al., 1998; Kawase & Burns, 1999), including MaxiK-like K(Ca) channels (Moreau et al., 1996, 1997). The emphasis of our study was to identify cation conductances in an osteoblast-like cell line that could be responsible for the other limb of RVD complementing the volume-activated Cl^- conductance described earlier (Steinert & Grissmer, 1997). For this purpose we investigated first the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, to find out whether hypotonic extracellular solutions could indeed activate Ca^{2+} -activated conductances through a rise in $[Ca^{2+}]_i$. Simultaneously, we measured the time course of the cell volume. Having established an increase in $[Ca^{2+}]_i$ after hypotonic stress and the ability of the cells for RVD, we investigated the channel activity of the osteoblasts under conditions with high $[Ca^{2+}]_i$ (10 μM free Ca^{2+}). Under these conditions we identified two distinct types of K(Ca) channels, an IK-like and a MaxiK-like K(Ca) channel. RVD in human osteoblasts seems to depend strongly on functional MaxiK channels since blocking these channels inhibits RVD substantially.

Some of the results have been reported in preliminary communications (Weskamp & Grissmer, 1998a,b, 1999a,b).

Materials and Methods

CELLS

C1-cells (Steinert & Grissmer, 1997), immortalized human osteogenic precursor cells, were obtained from Dr. Brian A. Ashton (The Robert Jones & Agnes Hunt Orthopedic & District Hospital, Oswestry, Shropshire, UK) and cultured in Dulbecco-MEM (10% FCS) with 2 mM glutamine and maintained continuously in a humidified, 5% CO_2 incubator at 37°C.

SOLUTIONS

All experiments were done at room temperature (21–25°C). The cells under investigation were normally bathed in mammalian Na^+ solution containing (mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; adjusted to pH 7.4 with NaOH. The osmolarity was adjusted with either sucrose or mannose to 310 mOsm. Cell swelling was induced by perfusing the bath with dilutions of the Na^+ solutions to 50%, thereby reducing $[Ca^{2+}]_o$ to 1 mM. In other experiments cell swelling was induced by perfusing the bath with dilutions of the Na^+ solutions to 50% while setting $CaCl_2$ to 4 mM. The rise in $[Ca^{2+}]_i$ induced by these two different solutions, however, was not different. The resulting osmolarity for both solutions was 190 mOsm. In mammalian K^+ solutions used for electrophysiology all the NaCl was replaced by 160 mM KCl. In Ca^{2+} -free Na^+ solution no Ca^{2+} was added and the solution was buffered with 500 μM EGTA (97%, Sigma Chemie GmbH, Deisenhofen, Germany). Hypotonic Ca^{2+} -free Na^+ solution was prepared by diluting the Ca^{2+} -free Na^+ solution to 50%. The composition of the

internal pipette solution was for the “low” $[Ca^{2+}]_i$ condition as follows: (mM) 135 K^+ -aspartate, 2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2; $[Ca^{2+}]_{free} = 33$ nM, for the “moderate” $[Ca^{2+}]_i$ condition: (mM) 135 K^+ -aspartate, 2 $MgCl_2$, 7.85 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2; $[Ca^{2+}]_{free} = 500$ nM and for the “high” $[Ca^{2+}]_i$ condition: (mM) 135 K^+ -aspartate, 2 $MgCl_2$, 9.9 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2; $[Ca^{2+}]_{free} = 10$ μM . All $[Ca^{2+}]_{free}$ were calculated using Eqcal from Biosoft (Cambridge, UK). These internal solutions should optimize the electrophysiological characterization of the intermediate as well as the MaxiK channels. CTX and paxilline were purchased from Bachem (Heidelberg, Germany), dissolved in mammalian Na^+ solution (CTX) or DMSO (paxilline) as stock solutions (10 μM for CTX, 10 mM for paxilline) and applied in concentrations of 10 to 50 nM and 10 μM , respectively.

ELECTROPHYSIOLOGY

Experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981) as described earlier (Grissmer & Cahalan, 1989a,b; Grissmer, Lewis and Cahalan, 1992; Grissmer, Nguyen and Cahalan, 1993; Hanselmann & Grissmer, 1996; Rauer & Grissmer, 1996; Jäger et al., 1998). Electrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, England) in two stages and fire-polished to resistances measured in the bath of 3.5–5 M Ω . 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) and were not corrected for junction potentials. Each illustrated response was observed at least four times.

$[Ca^{2+}]_i$ -MEASUREMENTS

The $[Ca^{2+}]_i$ -measurements were similarly carried out as described previously (Lewis & Cahalan, 1989; Verheugen et al., 1997). Briefly, the cells were incubated in Dulbecco-MEM containing 4 μM fura-2 AM (Sigma Chemie GmbH, Deisenhofen, Germany) for 20 min at 37°C. Then the cells were washed three times with mammalian Na^+ solution. All video microscopic measurements of Ca^{2+} were done using a Zeiss Axiovert 100 (Zeiss, Oberkochen, Germany) with a 40 \times oil-immersion objective (Zeiss Fluor, N.A. 1.3). The excitation light was supplied by a 75 W Xenon arc lamp (Opti-Quip, New York, NY) and filtered at 350 \pm 10 nm and 380 \pm 15 nm by interference filters (Chroma Technology, Brattleboro, VT), which were changed by a filter wheel (lambda 10, Sutter Instruments, Novato, CA). Fluorescence images were collected through a 500–530 nm bandpass filter (Zeiss, Oberkochen, Germany) by an intensified CCD camera (C2400, Hamamatsu, Japan). For each wavelength, 8 video frames per time point were digitized, averaged and background-subtracted using a VideoProbe image processor (VideoProbe, ETM Systems, Irvine CA). 350/380 nm ratio images were computed pixel by pixel every three seconds and stored on a hard disk for offline analysis with VideoProbe. Then the average ratio within a square field in the cells was determined. Intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, were estimated according to the following equation $[Ca^{2+}]_i = K_D \cdot S_f \cdot (R - R_{min}) / (R_{max}) - R$ (Grynkiewicz, Poenie & Tsien, 1985), where K_D is the dissociation constant of fura-2 (assumed to be 350 nM, (Negulescu & Machen, 1990)), S_f is a device dependent scale factor and R is the ratio of the two measured fluorescence intensities 350 nm/380 nm. R_{min} and R_{max} were determined in vitro using solutions containing zero Ca^{2+} and 39.8 μM Ca^{2+} respectively (Fura-2 calcium imaging calibration kit, Molecular Probes, Eugene, OR).

VOLUME ESTIMATION

We took the area covered by the cells as a measure for the cell volume. First, the fluorescence images were converted into binary images and

then the area of the cells was determined by counting the pixels representing the cells every frame using Scion Image 1.62c, a Macintosh computer program written by Wayne Rasband, NIH. Then the cell size in isotonic solution was averaged over seven frames and set as 100% (isotonic cell volume).

Results

VOLUME REGULATION AND $[Ca^{2+}]_i$ INCREASE IN HYPOTONIC SOLUTION

The basic observation is illustrated in Fig. 1 which shows $[Ca^{2+}]_i$ of several cells in pseudocolor representation and the time course of cell volume before and during application of a hypotonic Na^+ solution. Figure 1A, *left* shows the image taken before the solution change. The cells under these conditions display a low $[Ca^{2+}]_i$ (159 ± 42 nM; $n = 7$) indicating a resting, quiescent state. Figure 1A, *middle* shows the cells 80 sec after perfusing the bath with a 50% Na^+ solution and Fig. 1A, *right* is taken after 780 sec. It is obvious that this solution change increased $[Ca^{2+}]_i$ as can be visualized through the color change in Fig. 1A, *middle* compared to Fig. 1A, *left*. Furthermore, the color change in Fig. 1A, *right* compared to Fig. 1A, *middle* shows that $[Ca^{2+}]_i$ decreased within 700 sec to the base level.

Figure 1B shows on the left the time course of $[Ca^{2+}]_i$ during hypotonic stress. Ten to 20 sec after the solution change to a hypotonic solution $[Ca^{2+}]_i$ of all cells increased and reached a maximum of $1.3 \mu M$ after ~ 100 sec. Then $[Ca^{2+}]_i$ decreased nearly to the basic level within 800 sec. The time course of the relative cell volume during hypotonic stress is visualized in Fig. 1B on the right. Upon application of hypotonic solution the cells swelled up to 150% of their initial volume. After about 100 sec RVD started and the cell volumes were regulated to their initial level within 800 sec.

Figure 1C summarizes the results shown in Fig. 1B by showing the averages of $[Ca^{2+}]_i$ and relative cell volume, respectively. Both time courses are very similar and might suggest a Ca^{2+} -dependent component of RVD. Since the rise in Ca^{2+} preceded RVD we concluded that an increase in $[Ca^{2+}]_i$ alone cannot account for RVD.

To find out whether the $[Ca^{2+}]_i$ transient was due to Ca^{2+} entering the cell from the outside or Ca^{2+} release from internal stores we preincubated the cells for 10 min in Ca^{2+} -free Na^+ solution and applied then hypotonic Ca^{2+} -free Na^+ solution. The result of this experiment is shown in Fig. 2. After changing to hypotonic Ca^{2+} -free Na^+ solution, $[Ca^{2+}]_i$ increased to about 200 nM and the volume to 150%. Then $[Ca^{2+}]_i$ decreased again within 250 sec to 300 sec to the initial level while RVD was as fast as in hypotonic Na^+ solution with 4 mM Ca^{2+} . From the comparison of the changes in volume as well as the $[Ca^{2+}]_i$ signal in the presence and absence of external

Ca^{2+} (compare Fig. 1C and Fig. 2) we conclude that most of the change in $[Ca^{2+}]_i$ induced by the hypotonic solution comes from the outside, implying a Ca^{2+} influx pathway in this osteoblast-like cell line. In addition, this comparison clearly demonstrates that RVD was hardly dependent on external and internal Ca^{2+} . The reduction of the Ca^{2+} transient was not dependent on an incubation time up to 10 min.

ACTIVATION OF K(Ca) CHANNELS

Figure 3A shows membrane currents of C1 cells bathed in Na^+ solution. The currents were elicited by voltage ramps from -120 to $+100$ mV from a holding potential of -80 mV. If the patch pipette contained solutions that buffered $[Ca^{2+}]_i$ to 33 nM, only little current could be observed at either depolarized or hyperpolarized potentials (*lower trace*). With 500 nM Ca^{2+} in the patch pipette a current could be observed which consisted of a linear portion at potentials up to ~ 30 mV and a part where the slope increased slightly at more positive potentials (*middle trace*). If the patch pipette contained solutions that buffered $[Ca^{2+}]_i$ to a much higher value, in this case to $10 \mu M$, a huge outward current was observed at potentials more positive than 0 mV (*upper trace*). In addition to this huge outward current at depolarized potentials an almost linear current component was observed between -100 and 0 mV. The current crossover obtained in Na^+ solution (containing 4.5 mM K^+) between the currents observed with the three different $[Ca^{2+}]_i$ of ~ -70 to -80 mV indicated already that the linear current component might be selective for K^+ over Na^+ . In the following experiments we wanted to characterize in more detail those channel types that caused both these current components. Since we were only interested in Ca^{2+} -activated conductances (see Fig. 1), all the following experiments were carried out with $10 \mu M$ free $[Ca^{2+}]_i$ in the pipette solution.

To confirm that the linear component of the ramp current obtained between -100 and 0 mV might be caused by current flowing through K^+ channels, we changed the external Na^+ solution to a K^+ solution. The currents obtained in these two solutions are shown in Fig. 3B. Changing the bath solution from the Na^+ solution to the K^+ solution resulted in an inward current at negative potentials. This inward current changed direction at ~ 0 mV as expected for a K^+ conductance. This change in reversal potential unequivocally indicated that the linear current component at potentials below 0 mV was due to the activation of a Ca^{2+} -activated K^+ channel. Since the current voltage relationship was almost linear there does not seem to be any voltage dependence to the activation mechanism of those channels suggesting that this current component is due to current flowing through either small or intermediate Ca^{2+} -activated K^+ channels known for

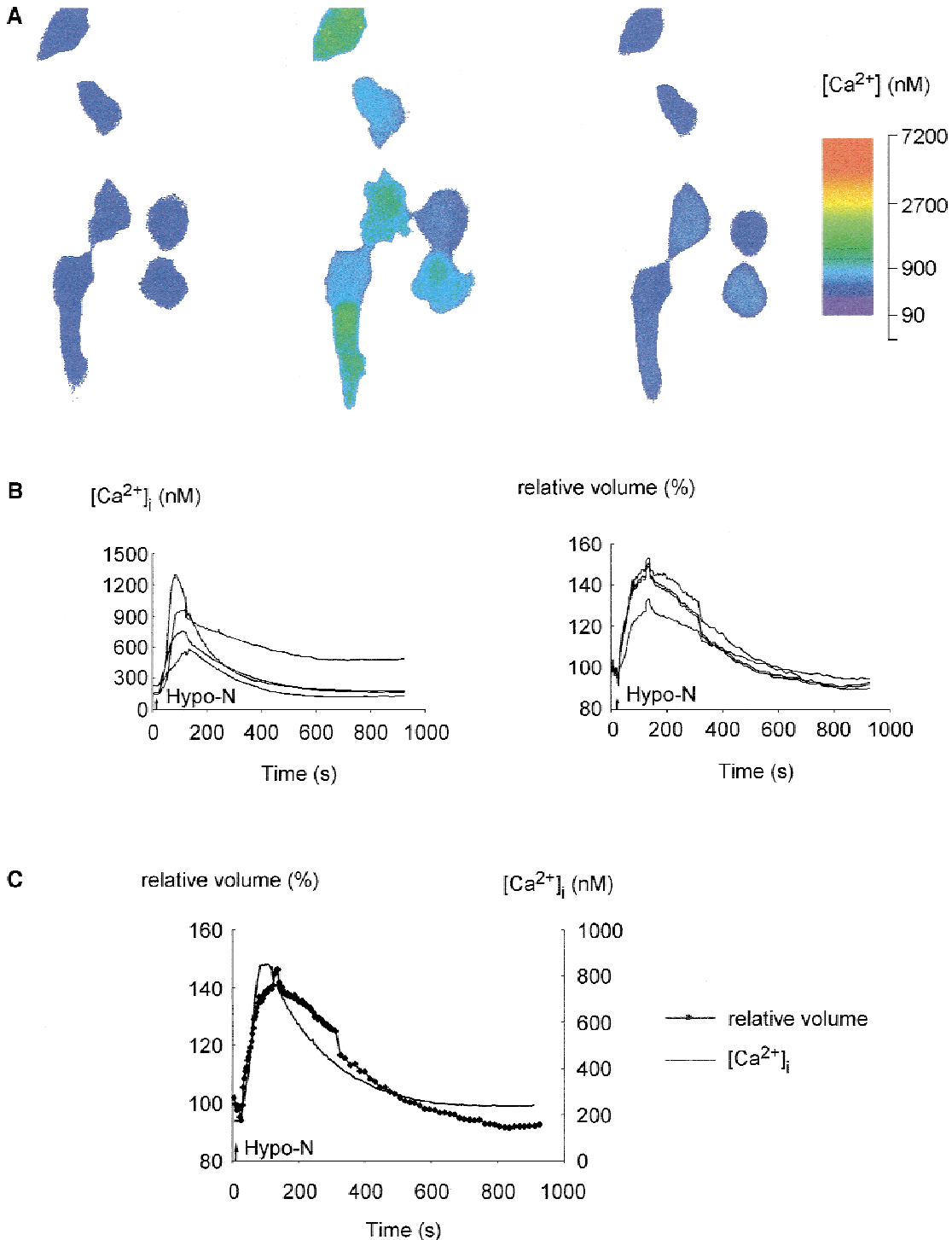


Fig. 1. Changes in $[Ca^{2+}]_i$ and cell volume in C1 cells in response to a hypotonic (50% Na^+ solution with 4 mM $CaCl_2$) external solution. (A) Shown are pseudo color representations of fura-2 ratio images. Images were obtained in normal Na^+ solution (left), 80 s (middle) and 780 s (right) after the change to the hypotonic Na^+ solution. (B) Time course of changes in $[Ca^{2+}]_i$ (left) and cell volume (right) in C1 cells shown in (A) after application of a hypotonic external solution (Hypo-N). (C) Time course of averaged changes in $[Ca^{2+}]_i$ and relative volume of the cells shown in (A). The arrows in (B) and (C), respectively, mark the beginning of the solution change.

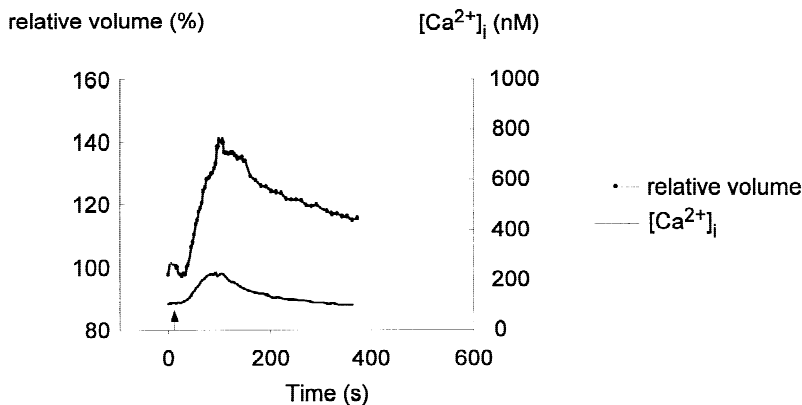


Fig. 2. Changes in $[Ca^{2+}]_i$ and relative volume in C1 cells after application of a hypotonic solution with 500 μ M EGTA and no added Ca^{2+} . Each curve represents the mean of four cells. The arrow marks the beginning of the solution change. Note the different time scale compared to Fig. 1.

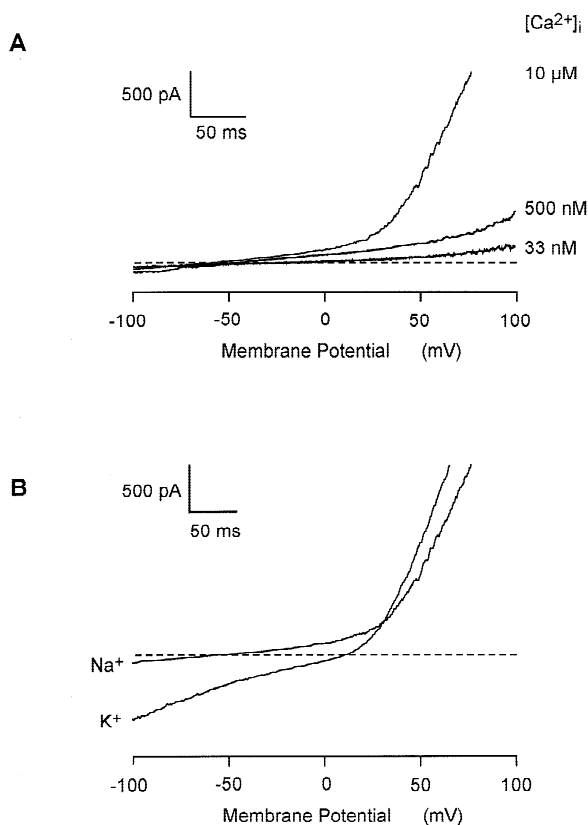


Fig. 3. Effect of $[Ca^{2+}]_i$ on membrane currents in C1 cells. (A) Membrane currents were elicited by voltage ramps from -100 to 100 mV within 400 ms. Current traces shown were measured in different cells in Na^+ solution with pipette solutions that buffer $[Ca^{2+}]_i$ to 33 nM, 500 nM or 10 μ M. (B) Membrane currents were similarly elicited as described in A (voltage ramps from -100 to 100 mV within 400 ms at 10 s intervals) in either external Na^+ or K^+ solution. The pipette solution was buffered to 10 μ M $[Ca^{2+}]_i$.

their voltage-independent activation (Grissmer et al., 1992, 1993; Hanselmann & Grissmer, 1996; Jäger & Grissmer, 1997).

We performed additional experiments to characterize in more detail the outward current at more depolar-

ized potentials obtained in Na^+ solution. To do this we activated currents through whole-cell dialysis with pipette solutions containing 10 μ M $[Ca^{2+}]_i$, changed the bath solution to the K^+ solution and applied voltage steps to different potentials from a holding potential of -80 mV. The result of such an experiment is shown in Fig. 4. Currents could be elicited at depolarizing potentials more positive than -30 mV with a maximum inward current of ~ 0.5 nA (at -10 mV) and an outward current of ~ 2 nA (at 90 mV). For a quantification of the voltage dependence of this current, the amplitude of the steady-state current, I_{ss} , was obtained from the current records shown between 10 and 190 msec after the start of the depolarizing voltage step and plotted against the applied membrane potential (Fig. 4B). The smooth curve represents a current-voltage fit as performed by the Pulsefit routine yielding a reversal potential of ~ 15 mV, a $V_{1/2}$ of ~ 13 mV and a k of 11.5 mV. The unexpected positive reversal potential could be explained only in part by the junction potential generated by the change from Na^+ solution to K^+ solution, since that was smaller than 2 mV. Alternatively, an overestimation of leak current during the leak subtraction procedure might also, at least partially, be responsible for the positive reversal potential we observed. We then calculated the conductance (g_{ss}) from the steady state currents, plotted the conductance against the applied membrane potential and fitted a Boltzmann curve of the form $g_{ss} = 1 - 1/\{1 + \exp[(V - V_{1/2})/k]\}$ to the data (Fig. 4C) using $V_{1/2}$ and k obtained through the Pulsefit routine and a g_{max} of 33 nS. Activation of the conductance started at potentials around -30 mV and reached its maximum at potentials more positive than $+30$ mV. These observations suggest that this current was mostly generated by K^+ flowing through voltage-dependent Ca^{2+} -activated K^+ channels that are most likely to be MaxiK channels.

PHARMACOLOGY

To confirm our assumption that the voltage-dependent K^+ current observed in Fig. 4 was flowing through

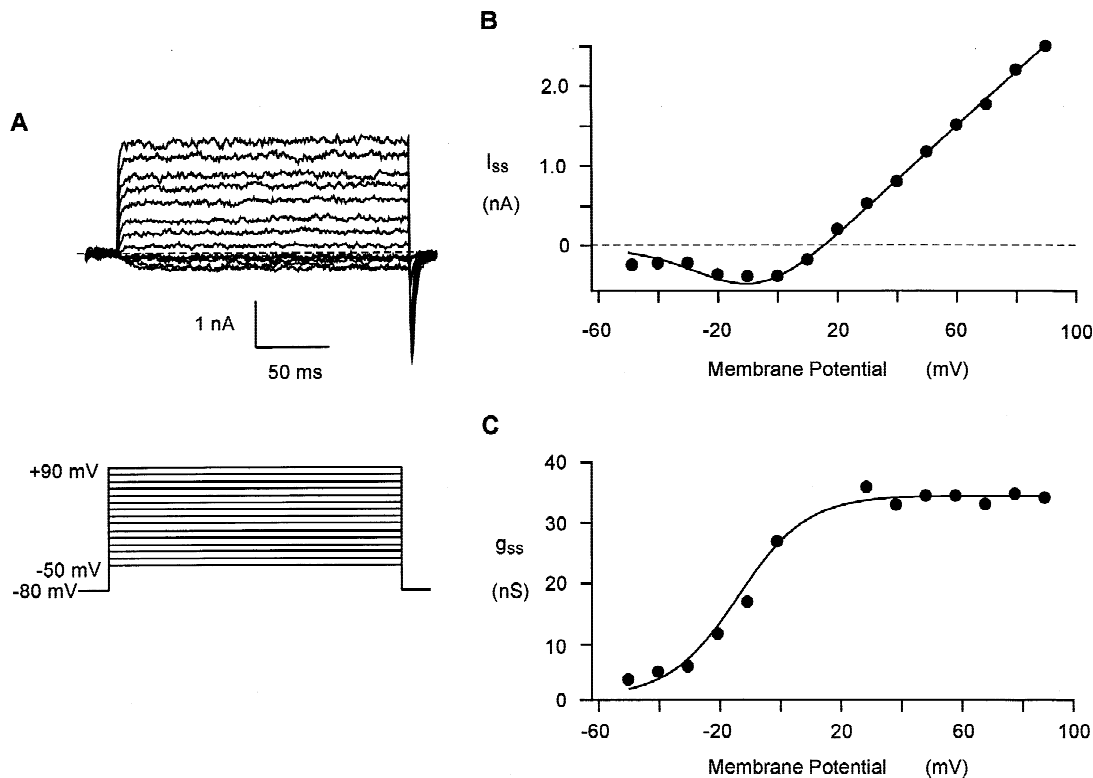


Fig. 4. Membrane currents in C1 cells with $10 \mu\text{M} [\text{Ca}^{2+}]_i$. (A) Currents were elicited by 200 ms voltage steps from -50 to $+90$ mV in 10 mV increments from a holding potential of -80 mV in K^+ solution. Time between pulses was 5 s. (B) Steady-state current-voltage relationship for the experiment shown in A. Steady-state current (I_{ss}) was determined as the plateau current measured between 10 and 190 ms after the beginning of the depolarizing voltage step. A current-voltage fit indicated a reversal potential of ~ 15 mV and a V of ~ -13 mV and $k = 11.5$ mV. (C) Steady-state conductance-voltage relationship from the experiment shown in A. The steady-state conductance (g_{ss}) was calculated from (B) and plotted against the depolarizing step potential. A Boltzmann curve was fitted to the data with $V_{1/2}$ of -13.1 mV and $k = 11.5$ mV.

MaxiK channels, we used two blockers known to block current through MaxiK channels. CTX, a 37 amino acid peptide toxin isolated from the scorpion *Leiurus quinquestriatus* was first described to be a specific blocker of MaxiK channels (Miller et al., 1985). Therefore we investigated the effect of 10 nM CTX on the whole cell current elicited by voltage steps from a holding potential of -80 to $+100$ mV. The pipette solution was buffered to $10 \mu\text{M} [\text{Ca}^{2+}]_i$ and the cells were bathed in Na^+ solution. CTX reversibly reduced the current $\sim 40\%$ indicating a current through MaxiK channels. Since CTX is also known to block current through “pure” voltage gated K^+ channels (Sands, Lewis & Cahalan, 1989; Schneider et al., 1989; Grissmer et al., 1994) we applied paxilline, a more specific blocker of MaxiK channels (Knaus et al., 1994; Sanchez & McManus, 1996). The result of this experiment is shown in Fig. 5B. The voltage was stepped from -80 to $+100$ mV in Na^+ solution and the whole cell currents were recorded. Addition of $10 \mu\text{M}$ paxilline to the bath solution completely blocked the current. This strongly indicates that this outward current was flowing through MaxiK channels.

As mentioned above the current at negative poten-

tials could be either flowing through small or intermediate Ca^{2+} -activated K^+ channels. To distinguish between these two possibilities, we used again CTX since it is also known to block the intermediate conductance Ca^{2+} -activated K^+ channel (Grissmer et al., 1993; Jäger & Grissmer, 1997) in addition to the MaxiK channels (Miller et al., 1985), but is unable to block current through the small conductance Ca^{2+} -activated K^+ channels (Grissmer et al., 1992; Grissmer & Hanselmann, 1996; Jäger & Grissmer, 1997). The result of this experiment is shown in Fig. 6. The currents were activated again through whole-cell dialysis with pipette solutions containing $10 \mu\text{M} [\text{Ca}^{2+}]_i$. The cell was bathed in K^+ solution and currents were elicited through voltage ramps before, during and after application of 10 nM CTX to the bath solution. 10 nM CTX reduced the slope of the ramp current to $\sim 50\%$ of the control value before and after the application of CTX. Due to the size and voltage dependence of the outward current more positive than 0 mV, the effect of CTX on the MaxiK channels in this type of experiment using voltage ramps seemed to be underestimated (compare Fig. 5A). The figure, however, clearly shows that the inward current at potentials more

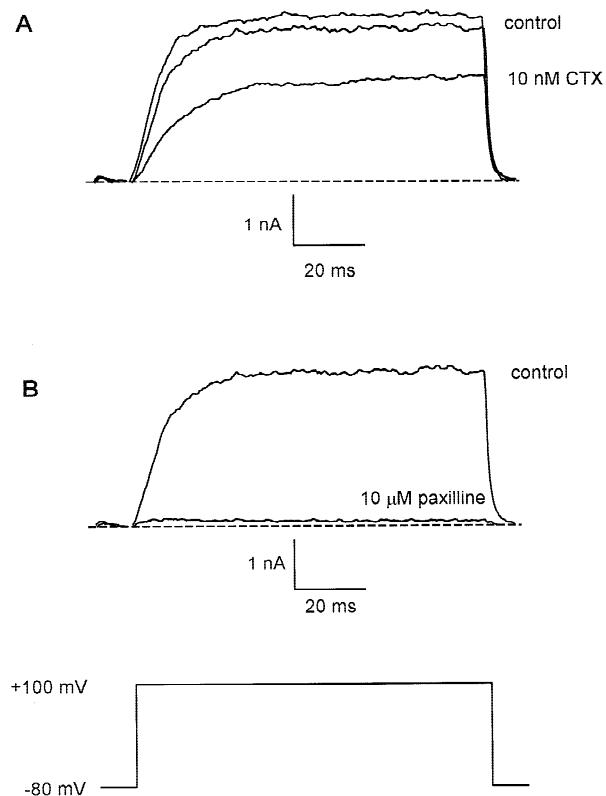


Fig. 5. Effects of CTX and paxilline on the steady state current in C1 cells with $10 \mu\text{M} [\text{Ca}^{2+}]_i$. Currents were elicited by 100 ms steps from a holding potential of -80 mV to $+100 \text{ mV}$ every 20 s in Na^+ solution. Current traces were obtained before, during, and after application of 10 nM CTX (A) to the bath solution or (B) before (control) and 80 s after application of $10 \mu\text{M}$ paxilline to the bath solution.

negative than 0 mV were carried by current flowing through intermediate conductance Ca^{2+} -activated K^+ channels. In addition, comparing Fig. 5A with this Fig. 6 points to the fact that both channels types were blocked by CTX with similar potency.

VOLUME REGULATION AND PAXILLINE

After confirming the expression of Ca^{2+} -activated K^+ channels in the human osteoblast-like cell line C1 we tested the influence of paxilline on RVD in order to clarify whether the intermediate or the big conductance Ca^{2+} -activated K^+ channel might play a role in RVD. The result of such an experiment on a single cell is visualized in Fig. 7. This figure shows the time course of the change of the relative cell volume elicited through application of a hypotonic solution containing $10 \mu\text{M}$ paxilline. After an initial increase in cell volume within the first 100 sec, RVD, as seen through the decrease in volume after the initial swelling was greatly inhibited through this solution containing $10 \mu\text{M}$ paxilline. 800

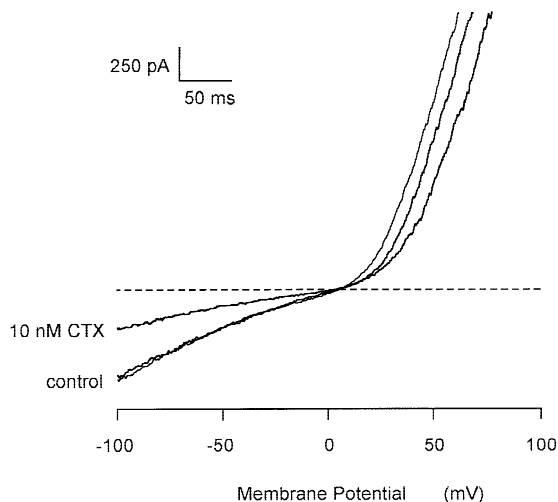


Fig. 6. Membrane currents in C1 cells with $10 \mu\text{M} [\text{Ca}^{2+}]_i$. Ramp currents were elicited by voltage ramps from -100 to $+100 \text{ mV}$ within 400 ms at 10 s intervals. Traces were obtained while bathing the cells in a K^+ solution before, during, and after application of a K^+ solution containing 10 nM charybdotoxin (CTX).

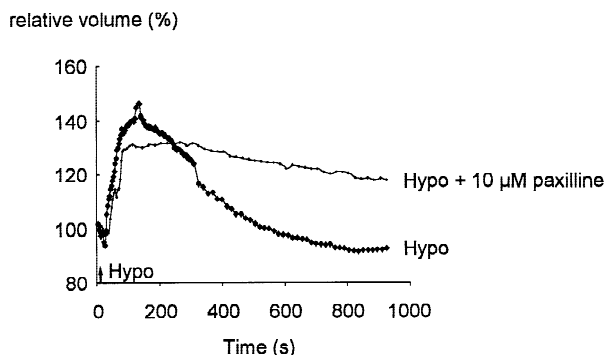


Fig. 7. Changes in relative volume in a single C1 cell after application of a hypotonic solution containing $10 \mu\text{M}$ paxilline in comparison with volume changes in hypotonic solution alone (from Fig. 1C). The arrow marks the beginning of the solution change.

sec after the solution change cell volume was decreased only by 50% of the maximal increase compared to >90% in hypotonic Na^+ solution without paxilline.

Discussion

$[\text{Ca}^{2+}]_i$ CHANGES IN RESPONSE TO HYPOTONIC STRESS AND $\text{K}(\text{Ca})$ CHANNELS

We have characterized an increase in $[\text{Ca}^{2+}]_i$ in response to cell swelling by application of hypotonic solutions. Such an increase in $[\text{Ca}^{2+}]_i$ in response to cell swelling has also been reported in various cell types including

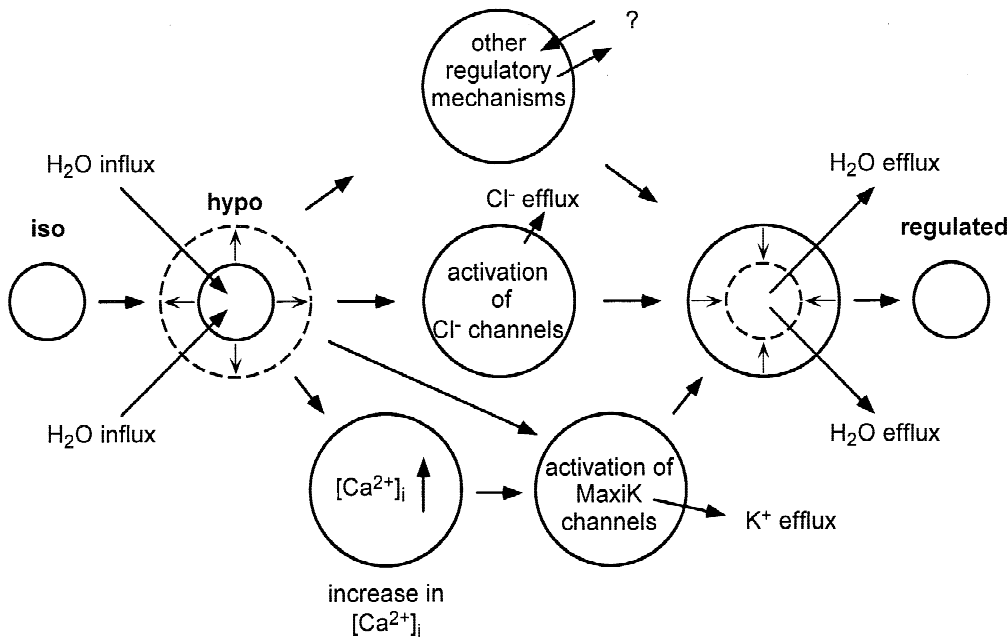


Fig. 8. Proposed model for regulatory volume decrease in osteoblasts.

isolated nerve terminals (Mongin et al., 1997), lacrimal acinar cells (Speake, Douglas and Brown, 1998), neuronal cell lines (Altamirano, Brodwick and Alvarez-Leefmans, 1998), cardiomyocytes (Taouil et al., 1998), multicellular prostate cancer spheroids (Sauer et al., 1998) and thymocytes (Ross & Cahalan, 1995). In human osteoblasts $[Ca^{2+}]_i$ reached its maximum after ~100 sec and was regulated back within 800 sec. Removal of external Ca^{2+} diminished the Ca^{2+} transient about 90% indicating that the main source of the Ca^{2+} seems to come from the extracellular solution and only a minority comes from intracellular stores, as it has been shown for GH3 cells (Chen et al., 1996).

The increase in $[Ca^{2+}]_i$ through hypotonic extracellular solutions could create conditions for the activation of two types of K(Ca) channels that we found electrophysiologically when using increased $[Ca^{2+}]_i$ in our pipette solutions during whole-cell recording. Activation of the two channel types could be observed at depolarized or hyperpolarized potentials, respectively. We clearly identified the first channel type as a MaxiK channel. Its activation was both Ca^{2+} - and voltage-dependent (see Figs. 3 and 4), it was sensitive to block by CTX (see Fig. 5A) and was completely blocked by paxilline (see Fig. 5B). The second channel type was most likely an IK channel. Its activation was Ca^{2+} dependent (see Fig. 3A), independent of voltage (see Fig. 3B) and it was blocked by CTX (see Fig. 6).

PHYSIOLOGICAL ROLE FOR CHANGES IN $[Ca^{2+}]_i$ AND ACTIVATION OF K(Ca) CHANNELS

Under $[Ca^{2+}]_o$ free conditions RVD is still present indicating that, despite the fact that 90% of the Ca^{2+} transient

is diminished during hypotonic shock, the rise in $[Ca^{2+}]_i$ might either be not important for RVD or might still be high enough to activate mechanisms involved in RVD. For example with $[Ca^{2+}]_i$ of 500 nM K(Ca) channels of the MaxiK type activate only at very depolarized potentials (> 50 mV, see Fig. 3A) while current through IK channels was already detectable at potentials < 0 mV. Previously it has been shown that 200–300 nM $[Ca^{2+}]_i$ was sufficient to activate K(Ca) channels of the IK type in T lymphocytes (Grissmer et al., 1993). Therefore we would have concluded that the loss of osmolytes under $[Ca^{2+}]_o$ free conditions would be mediated by K^+ current flowing through IK channels, however, our experiments with paxilline and RVD excluded such a mechanism.

RVD was reduced when MaxiK channels were blocked by paxilline (see Fig. 7). This indicates an important role of these channels in RVD of human osteoblasts although apparently $[Ca^{2+}]_i$ did not reach levels to activate those channels under isotonic conditions. One possibility to account for the activation of MaxiK channels in the absence of a sufficiently high enough $[Ca^{2+}]_i$ would be to assume that those channels might be either activated through stretch or that stretch influenced their sensitivity to activation by $[Ca^{2+}]_i$.

In addition, RVD is not fully abolished in the presence of paxilline. We have several explanations for this fact: (i) the block of MaxiK channels was not complete since there was a small amount of current left in the presence of 10 μ M paxilline (see Fig 5B); (ii) intermediate conductance Ca^{2+} -activated K^+ channels might also contribute to RVD, although to a much smaller extent than MaxiK channels; (iii) other transport mechanisms for osmolytes exist, like a K^+-Cl^- cotransport as shown

for many cells including red blood cells (Bize et al., 1999; Gusev, Lapin and Agualakova, 1997) and HEK-293 cells (Gillen et al., 1999). Therefore, we propose a model for volume regulation in human osteoblasts as depicted in Fig. 8.

Application of a hypotonic solution will lead to an influx of H₂O and therefore to cell swelling (*left*). This will lead to the activation of a volume-sensitive Cl⁻ conductance described earlier (Steinert & Grissmer, 1997), to an increase in [Ca²⁺]_i (this paper, *bottom*) and to the activation of other volume regulatory mechanisms. The increase in [Ca²⁺]_i or cell swelling or a combination thereof could activate the MaxiK channel. This activation will lead to an efflux of K⁺. Therefore the cell will lose these solutes and this in turn will cause H₂O efflux leading to a reduction in cell volume.

CONCLUSION

Volume-sensitive chloride 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. Since the other limb of the RVD has to involve a cation efflux, we searched for cation fluxes and found functional expression of at least two types of K(Ca) channels in the membrane of human osteoblasts. Both channel types could be activated by an increase in [Ca²⁺]_i. One channel type belonged to the classical MaxiK channel family with Ca²⁺- and voltage-dependent gating and block by CTX and paxilline, whereas the other type belonged to the IK channel family with voltage-independent gating and block by CTX. From these two different K⁺ channel types only the MaxiK channels seem to contribute significantly to the cation efflux necessary for RVD.

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References

- Altamirano, J., Brodwick, M.S., Alvarez-Leefmans, F.J. 1998. Regulatory volume decrease and intracellular Ca²⁺ in murine neuroblastoma cells studied with fluorescent probes. *J. Gen. Physiol.* **112**:145–160
- Bize, I., Guvenç, B., Robb, A., Buchbinder, G., Brugnara, C. 1999. Serine/threonine protein phosphatases and regulation of K-Cl cotransport in human erythrocytes. *Am. J. Physiol.* **277**:C926–C936
- Cahalan, M.D., Lewis, R.S. 1988. Role of potassium and chloride channels in volume regulation by T lymphocytes. In: *Cell Physiology of Blood*. R.B. Gunn and J.C. Parker, editors. pp. 281–301. Rockefeller University Press, New York
- Chen, Y., Simasko, S.M., Niggel, J., Sigurdson, W.J., Sachs, F. 1996. Ca²⁺ uptake in GH3 cells during hypotonic swelling: the sensory role of stretch-activated ion channels. *Am. J. Physiol.* **270**:C1790–C1798
- Davidson, R.M. 1993. Membrane stretch activates a high-conductance K⁺ channel in G292 osteoblastic-like cells. *J. Membrane Biol.* **131**:81–92
- Gillen, C.M., Forbush, B. 3rd. 1999. Functional interaction of the K-Cl cotransporter (KCC1) with the Na-K-Cl cotransporter in HEK-293 cells. *Am. J. Physiol.* **276**:C328–C336
- Gofa, A., Davidson, R.M. 1996. NaF potentiates a K⁺-selective ion channel in G292 osteoblastic cells. *J. Membrane Biol.* **149**:211–219
- Grissmer, S., Cahalan, M.D. 1989a. TEA prevents inactivation while blocking open K⁺ channels in human T lymphocytes. *Biophys. J.* **55**:203–206
- Grissmer, S., Cahalan, M.D. 1989b. Divalent ion trapping inside potassium channels of human T lymphocytes. *J. Gen. Physiol.* **93**:609–630
- Grissmer, S., Dethlefs, B., Wasmuth, J.J., Goldin, A.L., Gutman, G.A., Cahalan, M.D., Chandy, K.G. 1990. Expression and chromosomal localization of a lymphocyte K⁺ channel gene. *Proc. Natl. Acad. Sci. USA* **87**:9411–9416
- Grissmer, S., Lewis, R.S., Cahalan, M.D. 1992. Ca²⁺-activated K⁺ channels in a human leucemic T-cell line. *J. Gen. Physiol.* **99**:63–87
- Grissmer, S., Nguyen, A.N., Cahalan, M.D. 1993. Calcium-activated potassium channels in resting and activated human T lymphocytes. *J. Gen. Physiol.* **102**:601–630
- Grissmer, S., Nguyen, A.N., Aiyar, J., Hanson, D.C., Mather, R.J., Gutman, G.A., Kamilowicz, M.J., Auperin, D.D., Chandy, K.G. 1994. Pharmacological characterization of five cloned voltage-gated K⁺ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5, and Kv3.1, stably expressed in mammalian cell lines. *Molecular Pharmacology* **45**:1227–1234
- Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**:3440–3450
- Gusev, G.P., Lapin, A.V., Agualakova, N.I. 1997. Volume regulation in red blood cells of the frog *Rana temporaria* after osmotic shrinkage and swelling. *Membrane Cell Biology* **11**:305–317
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100
- Hanselmann, C., Grissmer, S. 1996. Characterization of apamin-sensitive Ca²⁺-activated potassium channels in human leukemic T lymphocytes. *J. Physiol.* **496**:627–637
- Jäger, H., Grissmer, S. 1997. Small Ca²⁺-activated potassium channels in human leukemic T cells and activated human peripheral blood T lymphocytes. *Cellular Physiology and Biochemistry* **7**:179–187
- Jäger, H., Rauer, H., Nguyen, A.N., Aiyar, J., Chandy, K.G., Grissmer, S. 1998. Regulation of mammalian *Shaker*-related K⁺ channels: evidence for nonconducting closed and nonconducting inactivated states. *J. Physiol.* **506**:291–301
- Kawase, T., Burns, D.M. 1999. Calcitonin gene-related peptide stimulates potassium efflux through adenosine triphosphate-sensitive potassium channels and produces membrane hyperpolarization in osteoblastic UMR106 cells. *Endocrinology* **139**:3492–3502
- Knaus, H.G., McManus, O.B., Lee, S.H., Schmalhofer, W.A., Garcia-Calvo, M., Helms, L.M., Sanchez, M., Giangiacomo, K., Reuben, J.P., Smith, A.B., Kaczorowski, G.J., Garcia, M.L. 1994. Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* **33**:5819–5828
- Lang, F., Busch, G.L., Ritter, M., Völkl, H., Waldegger, S., Gulbins, E., Häussinger, D. 1998. Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* **78**:247–306

- Lee, S.C., Price, M., Prystowsky, M.B., Deutsch, C. 1988. Volume response of quiescent and interleukin 2-stimulated T-lymphocytes to hypotonicity. *Am. J. Physiol.* **254**:C286–C296
- Lewis, R.S., Cahalan, M.D. 1989. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} currents in human leukemic T cells. *Cell Regulation* **1**:99–112
- Miller, C., Moczydlowski, E., Latorre, R., Philipps, M. 1985. Charybdotoxin, a protein inhibitor of single Ca^{2+} -activated K^+ channels from mammalian skeletal muscle. *Nature* **313**:316–318
- Mongin, A.A., Aksentsev, S.L., Orlov, S.N., Koney, S.V. 1997. Hypo-osmotic shock activates Ca^{2+} channels in isolated nerve terminals. *Neurochemistry International* **31**:835–843
- Moreau, R., Hurst, A.M., Lapointe, J.Y., Lajeunesse, D. 1996. Activation of Maxi-K channels by parathyroid hormone and prostaglandin E_2 in human osteoblast bone cells. *J. Membrane Biol.* **150**:175–184
- Moreau, R., Aubin, R., Lapointe, J.Y., Lajeunesse, D. 1997. Pharmacological and biochemical evidence for the regulation of osteocalcin secretion by potassium channels in human osteoblast-like MG-63 cells. *J. Bone Miner. Res.* **12**:1984–1992
- Negulescu, P.A., Machen, T.E. 1990. Intracellular activities and membrane transport in parietal cells measured with fluorescent dyes. *Methods in Enzymology* **192**:38–81
- Nilius, B., Eggermont, J., Voets, T., Droogmans, G. 1996. Volume-activated Cl^- channels. *Gen. Pharmacol.* **27**:1131–1140
- Rauer, H., Grissmer, S. 1996. Evidence for an internal phenylalkylamine action on the voltage-gated potassium channel $Kv1.3$. *Molecular Pharmacology* **50**:1625–1634
- Ross, P.E., Cahalan, M.D. 1995. Ca^{2+} influx pathways mediated by swelling or stores depletion in mouse thymocytes. *J. Gen. Physiol.* **106**:415–444
- Sanchez, M., McManus, O.B. 1996. Paxilline inhibition of the alpha-subunit of the high-conductance calcium-activated potassium channel. *Neuropharmacology* **35**:963–968
- Sands, S.B., Lewis, R.S., Cahalan, M.D. 1989. Charybdotoxin blocks voltage-gated K^+ channels in human and murine lymphocytes. *J. Gen. Physiol.* **93**:1061–1074
- Sauer, H., Ritgen, J., Hescheler, J., Wartenberg, M. 1998. Hypotonic Ca^{2+} signaling and volume regulation in proliferating and quiescent cells from multicellular spheroids. *Journal of Cell Physiology* **175**:129–140
- Schneider, M.J., Rogowski, R.S., Krueger, B.K., Blaustein, M.P. 1989. Charybdotoxin blocks Ca-activated K channels and Ca-independent voltage-gated K channels in rat brain synaptosomes. *FEBS Lett.* **250**:433–436
- Speake, T., Douglas, I.J., Brown, P.D. 1998. The role of calcium in the volume regulation of rat lacrimal acinar cells. *J. Membrane Biol.* **164**:283–291
- Steinert, M., Grissmer, S. 1997. Novel activation stimulus of chloride channels by potassium in human osteoblasts and human leukemic T lymphocytes. *J. Physiol.* **500**:653–660
- Strange, K., Emma, F., Jackson, P.S. 1996. Cellular and molecular physiology of volume-sensitive anion channels. *Am. J. Physiol.* **270**:C711–C730
- Taouil, K., Giancola, R., Maorel, J.E., Hannaert, P. 1998. Hypotonically induced calcium increase and regulatory volume decrease in newborn rat cardiomyocytes. *Pfluegers Arch.* **436**:656–674
- Verheugen, J.A.H., Le Deist, F., Devignot, V., Korn, H. 1997. Enhancement of calcium signaling and proliferation responses in activated human T lymphocytes. Inhibitory effects of K^+ channel block by charybdotoxin depend on the T cell activation state. *Cell Calcium* **21**:1–17
- Weskamp, M., Grissmer, S. 1998a. Calcium transients and membrane currents involved in volume regulation in human osteoblasts. *Biophys. J.* **74**:A381 (Abstr.)
- Weskamp, M., Grissmer, S. 1998b. Ca-dependent potassium currents in osteoblasts. A possible role in volume regulation. *Pfluegers Arch.* **435**:R94 (Abstr.)
- Weskamp, M., Grissmer, S. 1999a. Properties of a calcium dependent potassium current in osteoblasts. *Biophys. J.* **76**:A190 (Abstr.)
- Weskamp, M., Grissmer, S. 1999b. Calcium entry caused by hypotonic shock in human osteoblasts. *Pfluegers Arch.* **437**:R89 (Abstr.)
- Yellowley, C.E., Hancox, J.C., Skerry, T.M., Levi, A.J. 1998. Whole-cell membrane currents from human osteoblast-like cells. *Calcif. Tissue Int.* **62**:122–132
- Ypey, D.L., Weidema, A.F., Hold, K.M., Van der Laarse, A., Ravesloot, J.H., Van der Plas, A., Nijweide, P.J. 1992. Voltage, calcium, and stretch activated ionic channels and intracellular calcium in bone cells. *J. Bone Miner. Res.* **2**:377–387