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# A $Ca^{2+}$-dependent $K^+$-channel in freshly isolated and cultured chick osteoclasts

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Calcium-activated potassium channels were found in embryonic chick osteoclasts using the patch-clamp technique. The activity of the channel was increased by both membrane depolarisation and an increase in intracellular $Ca^{2+}$ concentration in the range $10^{-5}$ to $10^{-3}$ M. In the cell-attached-patch configuration the channel was only active at extreme depolarising potentials. $Ca^{2+}$ addition to the cytoplasm via ionomycin increased channel activity at the resting membrane potential of the osteoclast. The channel had a single-channel conductance of 150 pS in the inside-out patch under symmetrical $K^+$ conditions (150 mM) and was selective for potassium ions. During sustained application of increased [$Ca^{2+}$] at the cytoplasmic side of inside-out patches, channel activity sometimes decreased again after the initial increase (desensitization). The results established the properties of the single channels underlying an outward rectifying $K^+$ conductance in chick osteoclasts described previously by us.

## Introduction

Osteoclasts (OC) are large, multinuclear, bone resorbing cells which play an important role in bone remodelling and extracellular calcium homeostasis. After stimulation, OC adhere to the bone matrix and form a resorption lacuna between the bone matrix and the cell membrane. The lacuna is tightly sealed off from the extracelluar fluid by the osteoclast, causing restricted diffusion of secreted and degraded substances from the site of bone resorption. Through its ruffled border (bone-facing) membrane the OC secretes enzymes and protons into the resorption lacuna, which dissolve organic matrix components and hydroxyapatite, respectively [1,2].

Although it is well-known that the main function of the osteoclast is bone resorption, little is known about the ionic currents across the plasma membrane possibly involved in this function. Recent experiments with the whole-cell patch-clamp technique revealed the existence of three $K^+$ conductances in chick OC [3], one of which was also found in rat OC [4]. Furthermore, the $K^+$ gradient across the membrane was found to be important for several osteoclast functions, such as bone resorption [5], enzyme production and acidification [7]. These studies suggest that $K^+$ ions may play a key role in various functions of the osteoclast. As potassium channels play an important role in secretory cells as well [8], especially $Ca^{2+}$-dependent maxi $K^+$ channels, we presume that these channels may also be functional in secretory functions of osteoclasts.

We, therefore, applied the patch-clamp technique to characterize the ionic channels, underlying the outward rectifying $K^+$ conductance ($G_{K_o}$) described before by Ravesloot et al. [3] for chick osteoclasts. Here we report that these channels are $Ca^{2+}$-dependent maxi $K^+$ channels. They were identified in membrane patches of intact osteoclasts and in excised membrane patches. It is the dominant channel found in cell-attached membrane patches of chick osteoclasts. The activity of the channel is regulated by intracellular $Ca^{2+}$ and by the membrane potential. This implies that this channel is a candidate to be involved in the activation of those cell functions, which are accompanied by intracellular [$Ca^{2+}$] elevations and membrane depolarisation.

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## Material and Methods

*Cell isolation and culture*

Osteoclasts (OC) were isolated from the leg long bones of 18-day-old chick embryos. The femora and tibiae were stripped of their periosteum and other tissues, and placed in Hanks' balanced salt solution (Hanks) on ice. The bones were cut into pieces of approx. 1 mm and firmly rinsed in a large siliconized pipette. The acquired cell suspension was filtered through a sieve of 20 $\mu$m pore size. The cells remaining on the sieve were resuspended in Hanks, seeded on glass coverslips, and allowed to adhere at 37°C. After 60 min the coverslips were thoroughly washed to remove all non adhering cells from the coverslip, which was then placed in culture medium consisting of 10% fetal calf serum (Gibco), 85 $\mu$g/ml gentamicin and 584 $\mu$g/ml glutamine in $\alpha$-MEM (Difco) (pH 7.2). Cells were cultured for a maximum of three days.

6 h after isolation the cells were flattened enough to be identified as osteoclasts. Only large, flat cells with granular cytoplasm, containing 3 or more nuclei were used in the experiments. The osteoclastic nature of these multinuclear cells was firmly established by their tartrate-resistant acid phosphatase activity and their reaction with the osteoclast specific antibody OC6.9 [9]. Osteoclasts could also be found in 1- and 2-day-old cultures, though in smaller numbers. After three days most of the OC had disappeared from the culture. No differences in electro-physiologic properties were found between freshly isolated and cultured (1–2 days) osteoclasts.

*Electrophysiology*

Prior to the experiment the cells were washed in Hanks and then placed in a standard extracellular fluid-like solution (S-ECS, Table I). The glass coverslip was mounted in a teflon culture dish [10] allowing high-resolution phase-contrast microscopy on an inverted microscope. All experiments were performed at room temperature (21–24°C).

Channel currents were measured in the cell-attached-patch (CAP) and inside-out-patch (IOP) configuration of the patch-clamp technique [11] with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Patch pipettes were pulled from borosilicate glass (GC150TF-15, Clark, Reading, UK) and had resistances in the range of 2–6 M$\Omega$ after fire polishing. The software package 'pClamp' (version 5.1, Axon Instruments, Foster City, USA) run on an AT-IBM computer equipped with a Labmaster analog-digital converter, was used to simultaneously apply voltage protocols and measure channel currents. The currents were filtered with a low pass RC-filter (24 dB/octave) at a cut-off frequency of half the sampling frequency before A–D conversion. Both pClamp and Asystant (Asyst Software, Rochester, NY, USA) were used to analyze the data after recording on hard disk. For fast analysis of the channel currents, voltage ramps (25–100 mV/s) were applied instead of the usual steps. Controls showed no significant differences in single-channel properties measured upon steps and ramps.

TABLE I

*Concentrations (in mM) of the solutions used in the experiments*

The numbers in front of the ICS indicate the negative logarithm of the calculated free [$Ca^{2+}$] according to Fabiato and Fabiato [12].

| | NaCl | KCl | $MgCl_2$ | $CaCl_2$ | EGTA | Hepes | K-gluconate |
|---|---|---|---|---|---|---|---|
| 8-ICS | 8.0 | 143.0 | 1 | 0.60 | 10.00 | 10 | – |
| 7-ICS | 5.5 | 140.5 | 1 | 4.00 | 10.00 | 10 | – |
| 5-ICS | 10.5 | 145.5 | 1 | 0.02 | 0.01 | 10 | – |
| 4-ICS | 10.8 | 145.8 | 1 | 0.20 | 0.10 | 10 | – |
| 3.2-ICS | 10.0 | 145.0 | 1 | 0.75 | 0.10 | 10 | – |
| S-ECS | 150.0 | 5.0 | 1 | 1.00 | – | 10 | – |
| S-ECS.2 | 126.0 | 29.0 | 1 | 1.00 | – | 10 | – |
| K-Glu | 0.0 | 30.0 | 1 | 1.00 | – | 10 | 120 |
| Na-Glu | 30.0 | 0.0 | 1 | 1.00 | – | 10 | 120 |
| K-ECS | 0.0 | 155.0 | 1 | 1.00 | – | 10 | – |

The compositions of the intracellular fluid-like pipette solutions are given in Table I. Corresponding to their pCa, the solutions are designated 8-, 7-, 5-, 4- and 3.2-ICS. The free $Ca^{2+}$ concentrations were calculated according to Fabiato and Fabiato [12]. A pneumatic drug ejection system (PDES-03T, NPI, FRG) was used for local drug application. Pressure pulses were applied to patch pipettes (2–6 M$\Omega$), filled with a specific solution, which were placed near (50 $\mu$m) the cell.

Outward current is defined as the flow of positive ions from the inside to the outside of the membrane patch and is indicated by upward deflection in every record. Results are given as means $\pm$ standard deviations for given numbers ($n$) of cells.

## Results

*Cell-attached-patch (CAP) measurements*

In order to identify the channels underlying the whole-cell conductances of the embryonic chick osteoclast [3], the presence and properties of ionic channels in cell-attached-patches (CAP) of intact cells were examined. Pipettes were filled with 7-ICS or S-ECS while the cells were bathed in S-ECS. Tight seals with seal resistances ($R_s$) > 5 G$\Omega$, were obtained on 166 out of 235 cells of various days (0–2) of culture. The mean tight seal resistance was $14.8 \pm 8$ G$\Omega$ ($n = 36$). The seal resistance increased during the first minutes after formation of the seal. After this initial increase the seal was quite stable for up to 40 min. Of the 60 cell-at-

TABLE II

*Appearance of ion channels in embryonic chick osteoclasts in different configurations of the patch-clamp technique*

Total number of patches was 60. All CAPs preceding the 60 IOPs were considered. Note that different types of channels may occur in the same patch.

| | CAP number | IOP number |
|---|---|---|
| 150-pS Channel | 26 (43%) | 46 (77%) |
| Very high conductance channel (370 pS) | 1 (2%) | 37 (62%) |
| Other channels (30–60 pS) | 7 (12%) | 12 (20%) |

tached patches (CAP) analyzed, the majority (53) contained voltage-dependent single-channel currents. In the remaining seven patches, no channel activity was observed.

*A voltage activated CAP-channel*

The most frequent channel found in CAP (43%, see Table II) had a unit conductance of 150 ± 17 pS ($n = 7$) under (presumed) symmetrical [$K^+$] conditions. In 14% of the CAPs other channels were active (Table II), mostly at hyperpolarising potentials ($V_{pip} > 40$ mV) and with a smaller conductance (30–60 pS). A very high conductance channel (370 pS), similar to the anionic channel found in chick osteoblast [28], was only observed once. These channels are not analyzed here.

This 150-pS channel (Fig. 1A,C) was activated upon depolarisation of the patch membrane, i.e., by changing the applied pipette voltage ($V_{pip} \leqq 0$ mV).

We concluded from the rectangular shape of the single channel currents that a change in the pipette potential ($V_{pip}$) had no effect on the resting membrane potential ($V_r$) of the cell [11]. The open probability ($P_o$) of the channel was determined as the time the channel was open divided by the total recording time. If necessary, $P_o$ was corrected for the number of channels present in the patch assuming a Poisson distribution. The open probability of the channel increased when the membrane was depolarized (Fig. 1C). On average ($n = 4$) $P_o$ increased $e$-fold per −18 mV change in $V_{pip}$

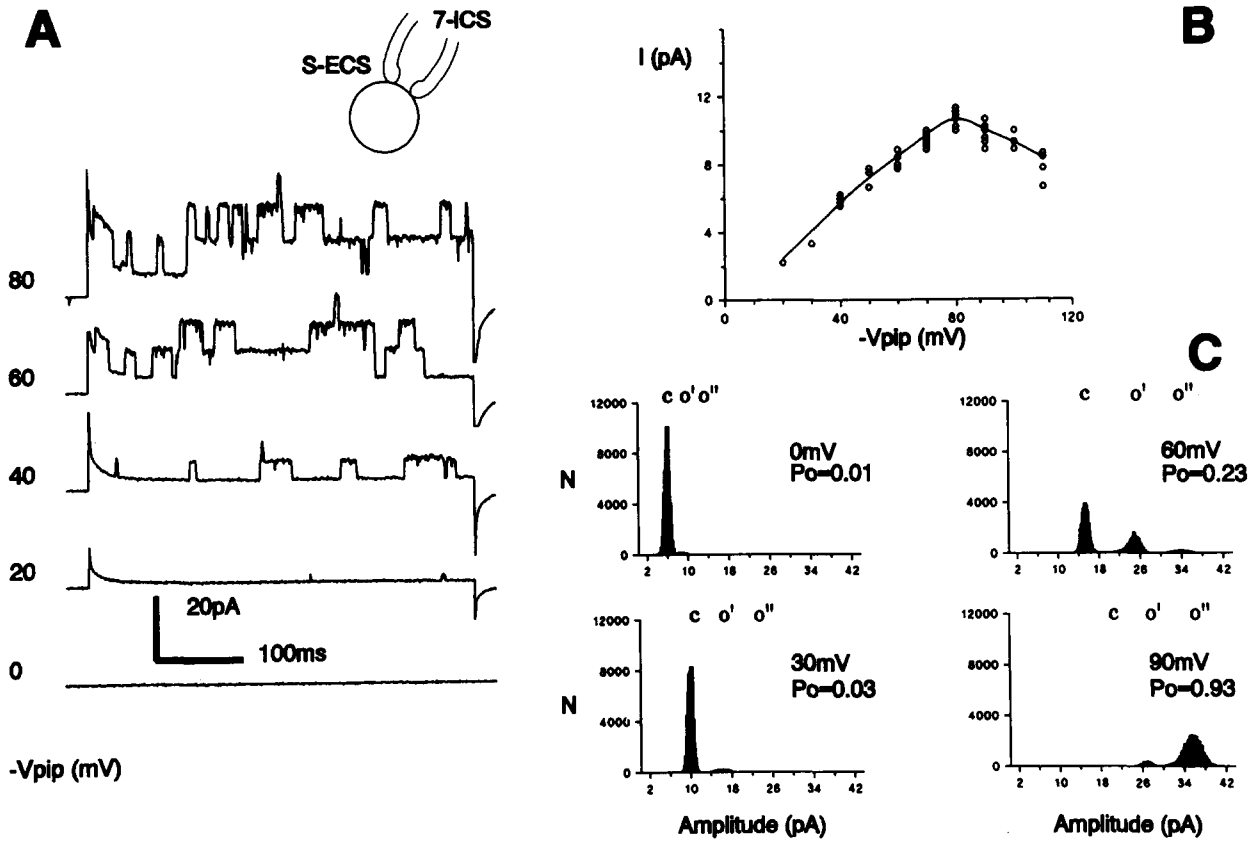


Fig. 1. Properties of the 150-pS channel in the cell-attached patch configuration of embryonic chick osteoclasts after 1 day of culture. (A) Single-channel current recordings, with at least 3 channels, during voltage steps from 0 mV pipette potential ($V_{pip}$) to various potentials (indicated at each trace). No channel currents were observed at positive $V_{pip}$ (data not shown). The pipette contained 7-ICS en the bath S-ECS. (B) Single-channel $I-V$ relationship of the same channel as in A. The amplitude of the outward channel-current decreases if the pipette potential $\leqq -80$ mV. The solid line is fitted through the data points by eye. (C) Current amplitude histograms of a different cell showing the voltage-dependence of the probability ($P_o$) that a channel is open. A 25-s trace was sampled with 2 kHz after a change in the pipette potential, indicated at the right. Not more than three distinct current amplitude levels (c, closed; o', one open and o", two open channels) were observed, meaning that there were two channels present in the patch membrane.

Directly after formation of the seal, 150-pS channel activity was sometimes observed at 0 mV pipette-potential, but this activity usually decreased progressively after several seconds. The activation potential ($V_{act}$) of the 150-pS channel was estimated as the potential at which channel activity (with an open time $\geqq 20$ ms) appeared (or disappeared) when changing the membrane potential in the depolarising (or hyperpolarising) direction. Several minutes after obtaining a giga-seal all $V_{act}$ values were more positive than $V_r$, while 57% of the $V_{act}$ values in the CAP were 70 mV more positive than the unknown $V_r$. Under whole-cell current-clamp conditions, Ravesloot et al. [3] found $V_r$ values around 0 mV for most of the osteoclasts. Channel activity did not or not completely inactivate upon depolarisation during periods of 450 ms (Fig. 1A) or longer (data not shown).

The reversal potential ($V_{rev}$) of the $I$–$V$ curve could usually not be determined in the CAP configuration, due to the limited voltage range in which the channel was active (Fig. 1B).

In 25 out of 26 measurements, the $I$–$V$ relationship of the channel in the CAP became saturated at high depolarisation of the patch membrane (60 mV more positive than $V_r$). The slope of the $I$–$V$ relationship even became negative at higher potentials (see Fig. 1B). The channel current was almost reduced to 40% of the maximal channel current at extreme depolarisation ($V_{pip} = -100$ to $-120$ mV). It was not always possible to measure the single-channel conductance accurately because of the nonlinearity and the limited voltage range of channel activation. An estimation could sometimes be made by fitting the most linear part of the $I$–$V$ relation using the least-squares method or by eye.

The voltage-dependent behaviour of the 150-pS channel in the CAP is consistent with that of the outward rectifying $K^+$ conductance ($G_{ko}$) found in OC whole-cells, but inconsistent with two other OC whole-cell $K^+$ conductances, a transient outward rectifier ($G_{Kto}$) and an inward rectifier ($G_{K_i}$) activating at hyperpolarising membrane potentials [3].

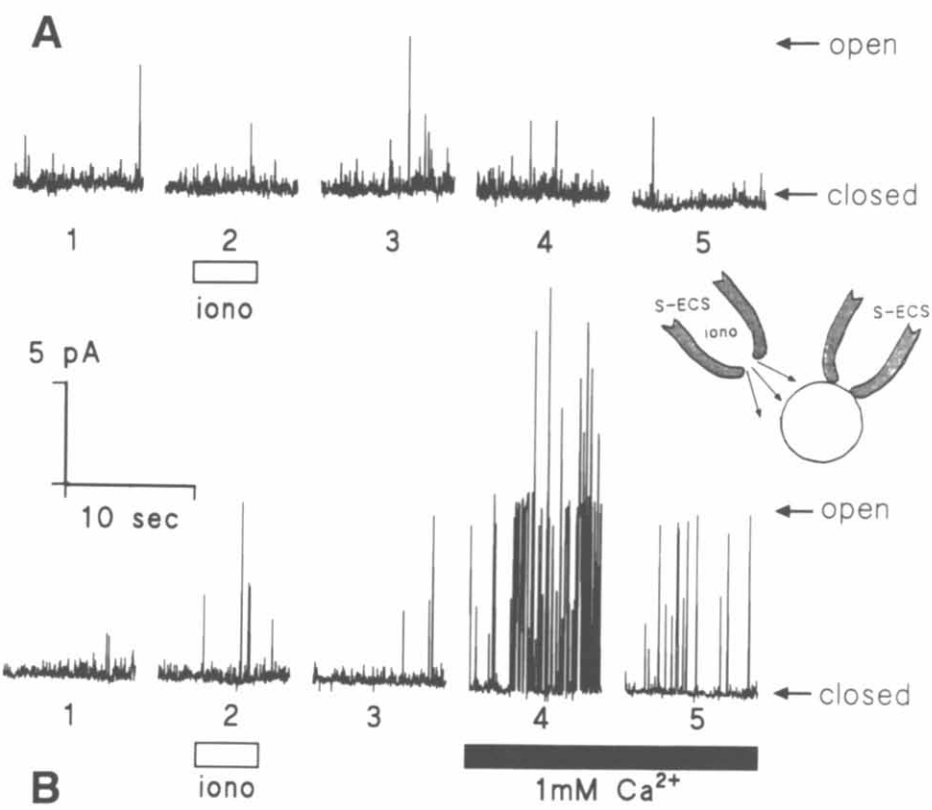


Fig. 2. Calcium-induced activation of the 150-pS channel in a cell-attached-patch. Bath and pipette contained a low [$Ca^{2+}$] (S-ECS + 10 mM EGTA, pCa approx. 7.5). The channel currents were filtered at 50 Hz before storage to a computer (sample frequency was 100 Hz). Channel activity, at a pipette potential of 0 mV, was measured continuously during 300 s. Traces of 10 seconds (labelled 1–5) are shown separated by 40, 40, 40 and 80-s intervals. In both experiments (A and B) ionomycine ($5 \cdot 10^{-6}$ M) was added locally to the same cell by pressure ejection from a nearby pipette (5 s, indicated by open bar) 50 s after the beginning of the experiment. (A) Control experiment showing the effect of an ionomycin pulse on channel activity in the absence of external $Ca^{2+}$. No increase of activity was observed in a period of 250 s after the pulse. (B) Same patch membrane as in Fig. 2A. A repeated pulse of ionomycin has no effect on channel activity in the absence of external $Ca^{2+}$. After 150 s $Ca^{2+}$ was added to the bath giving a final $[Ca^{2+}]_{out}$ of 1 mM (solid bar). Channel activity increased dramatically after 20 s (trace 4) followed by a decrease after 80 s (trace 5).

*$Ca^{2+}$-activation of the voltage-activated CAP channel*

To investigate whether the 150-pS channel in the CAP was a $Ca^{2+}$-activated channel we tested the calcium-dependence of this channel in the CAP, thus for the intact cell. In order to increase the intracellular [$Ca^{2+}$] we applied ionomycin, a calcium ionophore, to the cell during CAP measurements.

Ionomycin application was done by 5-s pressure pulses to an ejection pipette, filled with $5 \cdot 10^{-6}$ M ionomycin in S-ECS and placed close (50–80 $\mu$m) to the cell. With an extracelluar [$Ca^{2+}$] of 1 mM, 3 out of 8 CAPs showed increased and prolonged channel activity and the activation potential was shifted from −75 mV $V_{pip}$ to +10 mV $V_{pip}$ of the CAP. Two patches showed transient effects of ionomycin on channel activity and three showed no effect. The effect was due to external calcium entering the cell caused by the addition of ionomycin, since control experiments ($n = 3$) with no external $Ca^{2+}$ added showed no effect of pulsed ionomycin application (Fig. 2A). Increasing the external calcium concentration to 1 mM raised the channel activity considerably (Fig. 2B). During the experiments the channel current amplitude did not change, indicating that $V_r$ did not change. High channel activity in $Ca^{2+}$-permeabilized cells could be decreased by perfusing the bath with low-[$Ca^{2+}$] solutions. Calcium addition (1 mM) in the absence of preceding ionomycin pulses gave no significant increase in channel activity ($n = 3$, data not shown).

*Evidence for intracellular sodium block*

From the literature [18,19] it is known that intracellular $Na^+$-ions cause saturation and negative $I$–$V$ behaviour of $Ca^{2+}$-dependent $K^+$ channels, as in Fig. 1B and Fig. 3A, by blocking the channels at extreme depolarisation. We established this property of our channels by testing if the washout of internal $Na^+$-ions would give a more linear $I$–$V$ curve. Nystatin, a pore forming protein, was used to make the cell membrane selectively permeable for $K^+$ and $Na^+$ [30]. Because of a small $Cl^-$ permeability of Nystatin ($P_K/P_{Cl} = 9$), 120 mM NaCl was replaced by 120 mM potassium gluconate (Na-Glu, Table I). When, subsequently, the remaining 30 mM external sodium was replaced by potassium (K-Glu), no effect was observed on the $I$–$V$ curve of the channel with respect to the saturation and negative $I$–$V$ behaviour (data not shown) compared to the control curve (S-ECS, Fig. 1B, 3A). When Nystatin

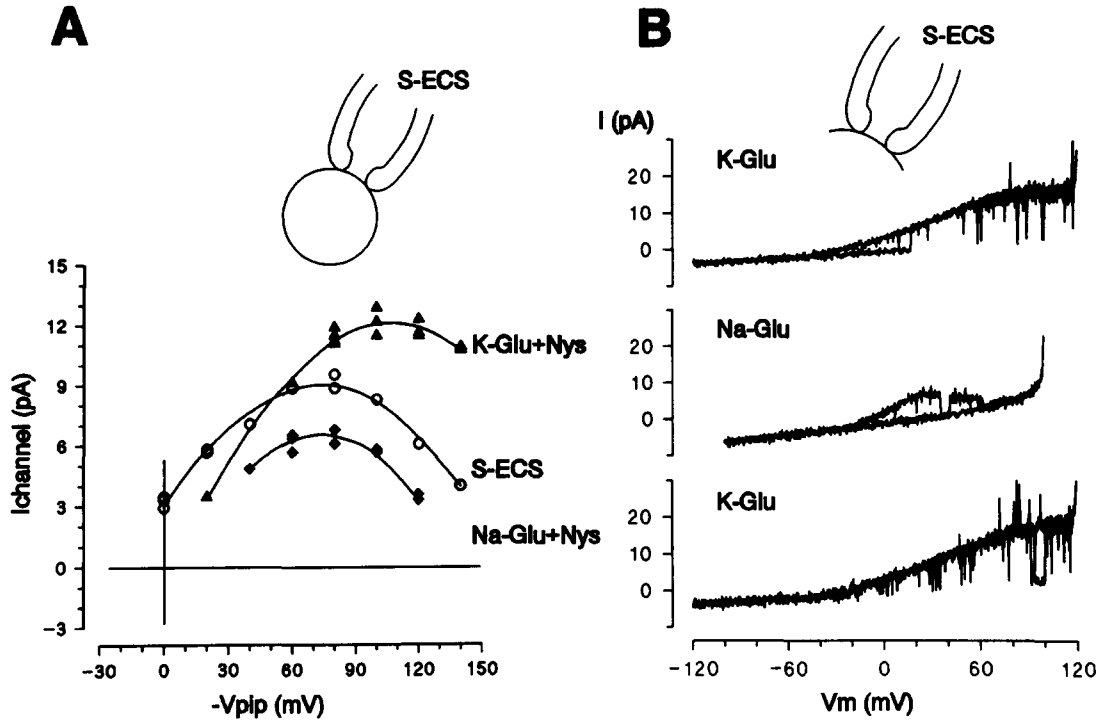


Fig. 3. The effect of internal sodium ions on the single-channel current of the 150 pS channel at depolarizing membrane potentials. (A) shows the nonlinearity in the $I$–$V$ curve of the channel in the cell-attached patch configuration at $-V_{pip} > 80$ mV under normal bath conditions (○, S-ECS). When internal sodium was washed out by replacing the bath medium by high $K^+$ and Nystatin (▲, K-Glu + Nys (125 $\mu$g/ml)), the decline in current is almost absent. When sodium (◆, Na-Glu + Nys) is added to the bath, the decline is even stronger than under control conditions (S-ECS). The lines were fitted by eye. (B) Effect of internal $Na^+$-ions on single channel current in an IOP. Top trace, two superimposed current records in response to a voltage ramp (80 mV/s). The pipette contained S-ECS and the bath K-Glu. Middle trace, the effect of replacing 30 mM $K^+$ by 30 mM $Na^+$ (Na-Glu) in the bath, on the single-channel current. At $V_m = 60$ mV the channel current is decreased to 40% of the control value. Bottom trace, replacing 30 mM $Na^+$ by 30 mM $K^+$ (K-Glu) increases the current again to the original amplitude. Currents are not leak corrected, no inward channel currents are observed at negative membrane potentials.

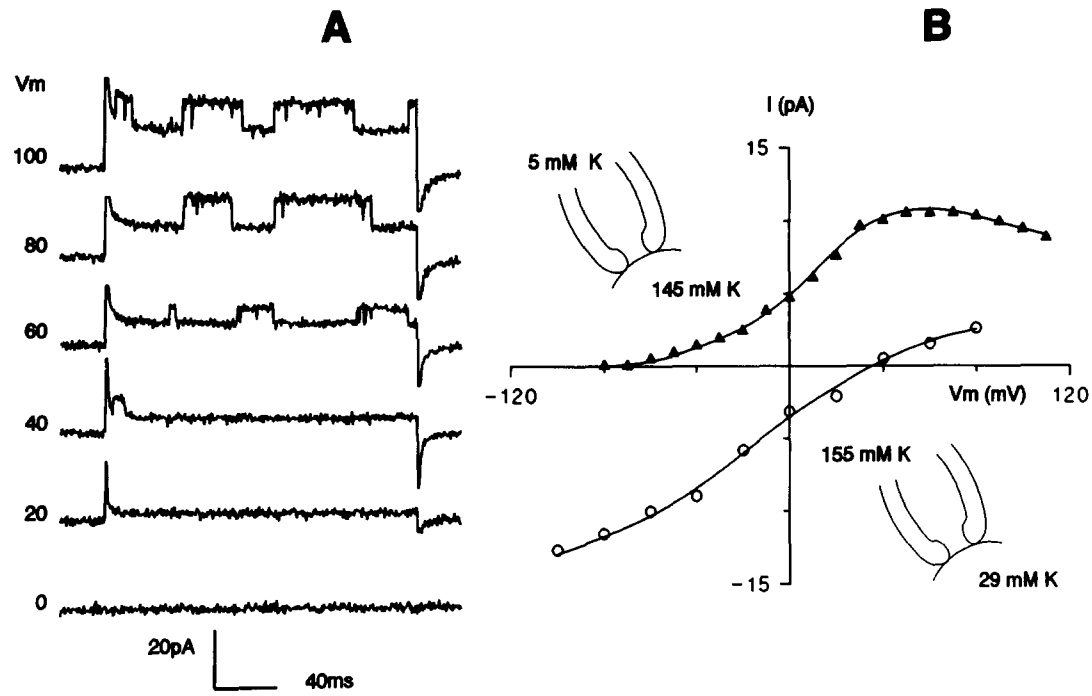


Fig. 4. Voltage-activation and selectivity properties of the 150-pS channel found in an inside-out patch from a chick osteoclast. (A) Effect of changes in membrane potential ($V_m$) on single channel currents. $V_m$ (in mV) is indicated at the left of each trace, channel openings are upward deflections. The pipette and bath were filled with 7-ICS. (B) Single channel $I-V$ plots with different [$K^+$] gradients across the patch membrane, as indicated in the insets. With S-ECS (5 mM $K^+$) in the pipette and 3.2-ICS (145 mM $K^+$) in the bath (▲) the reversal potential was around −70 mV. Saturation and a negative $I-V$ slope can be seen at membrane potentials higher than +50 mV. With K-ECS (155 mM $K^+$) in the pipette and S-ECS.2 (29 mM $K^+$) in the bath (○) the reversal potential was close to +40 mV, indicating that $K^+$-ions are the main permeant ion through the channel (calculated Nernst $E_K$ = +40 mV,). The curves through the data points are fitted by eye.

(125 μg/ml) was added to the bath (K-Glu + Nys in Fig. 3A), the decline in channel current with depolarisation was almost absent or shifted to the right. The increase in channel current amplitude was complete, 1–2 min after the application of Nystatin. Nystatin had no effect on the leak resistance (patch and seal resistance in parallel). When 30 mM of $K^+$ was replaced by 30 mM $Na^+$ the channel current amplitude decreased again to values below the control amplitudes (Na-Glu + Nys in Fig. 3A). The $Na^+$-effect was reversible within several minutes after washout of external sodium. This $Na^+$-effect was found in 4 out of 8 cells. In the other four cells it was not possible to measure single channel currents after $Na^+$ application to the bath. However, in these four cells the channel current initially decreased, until the single channels could not be discriminated anymore from the background noise.

Taken the results so far together, the CAP measurements suggest that the 150-pS CAP channel is the intracellular $Ca^{2+}$-dependent maxi channel and underlies the $K^+$-conductance activated at depolarising membrane potentials.

*Inside-out-patch (IOP) measurements*

The properties of the 150-pS channel in CAP of OC were also studied under well-controlled conditions in inside-out patches (IOP). Pulling the pipette up was often enough to remove the patch from the cell membrane. The conditions at the intracellular side of the membrane were then controlled by perfusion of the bath solution.

After excision of the IOP in S-ECS with 1 mM $Ca^{2+}$, channel activity was high, but decreased within several seconds. Usually the bathing solution was changed to a low-[$Ca^{2+}$] ICS (e.g., 7-ICS) within 1 min after excision and the activity of 150-pS CAP-channels could be followed in the IOP. Channel activation was voltage-dependent (Fig. 4A) and the single-channel conductance was almost the same as in the CAP (165

TABLE III

*Single channel (slope) conductances and reversal potentials ($V_{rev}$) of the 150 pS channel in I–O patches of chick osteoclasts with different [$K^+$] gradients across the patch membrane*

Slope conductances were determined in the linear range of the $I-V$ curve (usually around $V_m$ = 0 mV).

| Conditions Pipette/Bath | Single-channel conductance | measured $V_{rev}$ | $K^+$ Nernst potential |
|---|---|---|---|
| K-ECS/S-ECS | 90 ± 5 pS ($n$ = 4) | +65 ± 8 mV | +87 mV |
| S-ECS/3.2-ICS | 113 ± 11 pS ($n$ = 4) | −64 ± 9 mV | −85 mV |
| S-ECS/K-Glu | 141 ± 7 pS ($n$ = 4) | −64 ± 7 mV | −87 mV |
| K-ECS/3.2-ICS | 165 ± 22 pS ($n$ = 4) | −3 ± 4 mV | +1 mV |

$\pm$ 22 pS, $n = 4$) under symmetrical $K^+$ conditions (155 mM, Table III). Inactivation of the channel was not observed upon depolarisation up to 60 s (data not shown). The *I–V* relationship of the 150-pS channel was also nonlinear (Fig. 4B).

In the IOP configuration more channels were active than in the CAP, as can be seen in Table II. One explanation could be the increased $[Ca^{2+}]$ at the intracellular side of the membrane during IOP experiments. The 150-pS channels were active in 77% of the IOPs. In 20% of the IOPs, small conductance (30–60 pS), hyperpolarisation-activated channels were found, which were not analyzed. A very high conductance channel (VHCC, single-channel conductance of 370 pS) was found in many IOPs (62%). This type of channel was not active in the cell-attached mode (same patch) and resembles the anion channel described by Ravesloot et al. [28] for osteoblasts.

From the observations we conclude that the channels studied in the IOP are most likely to be the same as the 150-pS channels found in the CAP. This implies that the 150-pS CAP channel, normally present in intact cells can be studied under the more defined conditions of the excised inside-out patch.

*The 150-pS IOP channel is a $K^+$-channel*

To determine the selectivity properties of the 150-pS IOP channel, single-channel *I–V* curves were determined for different $[K^+]$ ratios across the channel.

Under asymmetrical $K^+$ conditions, with S-ECS in the pipette and 3.2-ICS in the bath, the channel *I–V* relationship was not linear (Fig. 4B). No inward current was observed at negative $V_m$ and the maximum current through the channel at positive $V_m$ (50 mV) was 10 pA, but the channel current decreased again at more positive membrane potentials. The nonlinear *I–V* relationship makes it difficult to determine the characteristic conductance of the channel. The most linear middle part of the *I–V* relationship could, however, be used to provide an estimation of the single-channel conductance. Single-channel conductances and reversal potentials with different $[K^+]$ gradients over the IOP membrane are given in Table III.

The reversal potential of the channel current under asymmetrical $[K^+]$ (S-ECS in the pipette and ICS in the bath) was $-64 \pm 9$ mV ($n = 4$), indicating that $K^+$ is indeed the main permeant cation. The Nernst potential for $K^+$ was $-85$ mV under these conditions. The Nernst potentials for $Cl^-$ and $Na^+$ under these conditions were approx. 0 mV and approx. +80 mV, respectively. Fig. 4B shows the effect of reversing the $K^+$-gradient across the patch membrane. By changing $[K^+]_i$ and $[K^+]_o$ and $[Na^+]_i$ and $[Na^+]_o$ we found that the channel is more permeable for $K^+$ ions than for $Na^+$

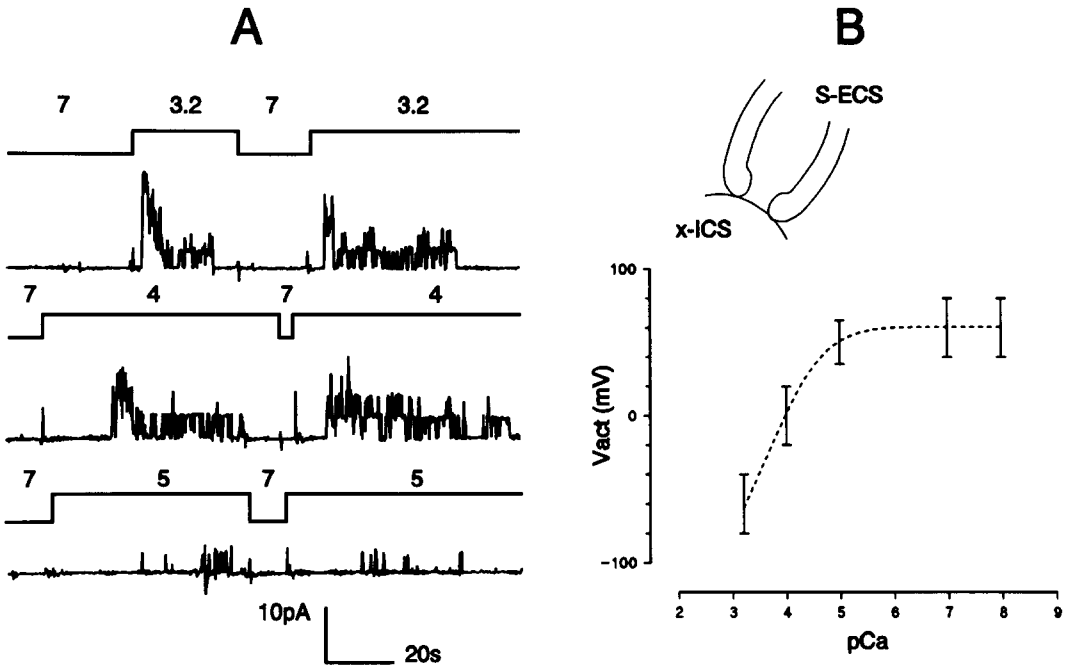


Fig. 5. Effect of different 'intracellular' $[Ca^{2+}]$ on 150-pS channel activity in an inside-out-patch (IOP). The pipette was filled with S-ECS and the intracellular side of the IOP exposed to the bath was perfused with an intracellular-like solution (×, ICS) with different $[Ca^{2+}]$. The numbers above the line indicate the pCa values of the different ICS (see Table I). (A) Transient activation by $Ca^{2+}$ of 150-pS channels (maximal six simultaneous openings can be seen) in an IOP after changing the $[Ca^{2+}]$ in the bath from $10^{-7}$ M to $10^{-3.2}$ M. The channels could only be reactivated again by first lowering the bath $[Ca^{2+}]$ to $10^{-7}$ M. (B) Effect of $[Ca^{2+}]_i$ on the activation potential ($V_{act}$) of the channel. Bars represent $\pm$S.E. for $n = 4$.

ions. The permeability ratio $P_{Na}/P_K$ was in the range of 0.12–0.04 (assuming that no other cations or anion are involved). Therefore, we call this channel a $K^+$-channel.

*$Ca^{2+}$-activation*

The calcium-dependence of the 150-pS $K^+$-channel was studied in IOP by perfusion of the bath with solutions containing different $[Ca^{2+}]$. Raising the $[Ca^{2+}]$ at the intracellular side of the IOP membrane resulted in a shift of the activation potential ($V_{act}$) to more negative patch potentials. This could only be measured in a few IOP ($n = 4$, see Fig. 5B) due to interference with other channel currents.

Very high conductance (VHCC) channels (370 pS) [28] appeared in 62% of the IOP patches within minutes (1–10 min) after excision of the patch, which made it impossible to study the $Ca^{2+}$-dependence of the 150-pS channel in great detail. However, differences in reversal potential of the channels ($V_{rev}$ (VHCC) approx. 0 mV, $V_{rev}$ (150-pS channel) approx. −64 mV) made it possible to record the 150-pS channel currents at a membrane potential of 0 mV, where the VHCC channel currents were near zero. In this way we could study the 150-pS channel only when the activation potential was more negative than 0 mV.

The 150-pS channel in the IOP could be activated by increasing the $[Ca^{2+}]$ at the cytosolic side of the membrane. In 7 out of 12 IOPs the activation of these channels by increasing the $[Ca^{2+}]_i$ had a transient character, four showed a sustained activation, while in one cell the activation was sometimes transient and sometimes sustained. Reactivation of the 150-pS channel by $Ca^{2+}$-ions was only possible by exposing the cytosolic side of the membrane first to low $[Ca^{2+}]$ (Fig. 5A).

Because of the burst kinetics and the transient character of the channel activity it was not possible the determine a stationary $P_o$ of the channel. The changes in $[Ca^{2+}]$ at the intracellular side of the membrane did not affect the single-channel conductance (Fig. 5A), since the channel amplitude stayed the same.

*Sodium block*

We also tested if internal $Na^+$-ions are responsible for the nonlinearity in the $I$–$V$ curve of the 150 pS IOP channel. In 5 out of 6 IOP experiments the nonlinearity disappeared when all $Na^+$-ions were replaced by $K^+$-ions (K-Glu in Fig. 3B, top trace). Substitution of 30 mM $K^+$ by 30 mM $Na^+$ reduced the channel-current amplitude to almost zero at depolarizing membrane potentials (Na-Glu in Fig. 3B, middle trace). In all five experiments the effect was reversible when $Na^+$ was washed out (K-Glu in Fig. 3B, bottom trace). This property has been described before for maxi, calcium-dependent $K^+$ channels [18,19].

## Discussion

The potassium channel found in embryonic chick osteoclasts shares many properties with other calcium-activated potassium channels that have been found in various tissues (see Ref. 13 for a review). These properties are: (1) large single-channel conductance (150 pS under symmetrical $K^+$ conditions (155 mM)); (2) activation by intracellular $Ca^{2+}$; (3) high selectivity for potassium ions; (4) activation by depolarising membrane potentials and (5) block by internal sodium ions at high positive potentials.

Our results indicate that the calcium-activated potassium channels found in embryonic chick osteoclasts belong to the maxi-type $K^+$ channels [13–15,29]. The channel could be found in cell-attached patches (CAP), as well as in cell-free inside-out patches (IOP). In the CAP configuration the channel was not active at resting membrane potentials, indicating that either the membrane potential was negative and/or the internal $[Ca^{2+}]$ was low.

Earlier experiments [7,16] have shown that the osteoclast is a polarised cell, even if cultured on glass. We were unable to test if there were any channels present in the membrane adhering to the glass.

The characteristics of the 150-pS channel found in CAP did not differ much of those in IOP. This made it possible to study the channel features under more controlled conditions in an IOP [17]. Comparison of the CAP-channel and the IOP-channel $I$–$V$ relations provides evidence that the internal $[K^+]$ of the intact cell is quite high (approx. 150 mM). The single-channel conductance of the CAP-channel measured with an ICS-filled pipette is equal to the IOP-channel conductance measured with ICS in the pipette and in the bath. Furthermore, the reversal potential in both cases is close to 0 mV, consistent with a resting potential of osteoclasts close to 0 mV [3].

The channel presented here is the most likely candidate for the channel underlying the outward rectifying $K^+$ conductance ($G_{k_o}$) found in whole-cell experiments [3]. Several observations indicate so. Like the whole-cell conductance, the channels activate at depolarising membrane potentials and are $K^+$-selective. The channel does not inactivate upon depolarisation like the transient outward conductance ($G_{kto}$) [3].

The decrease of outward channel current at extreme depolarising potentials was due to sodium ions, blocking the channel from the inside. This flickering block is too fast to be seen with our time resolution and will result in a decrease of the mean current through the channel. The block by sodium ions is voltage-dependent and is characteristic for this type of channel [18,19].

Calcium was shown to activate the channel from the cytosolic side of the membrane in CAP as well as in

IOP. Compared to maxi $K^+$ channels found in other cells [13,15], relatively high $Ca^{2+}$ concentrations ($> 10^{-5}$ M) were needed to shift the activation potential of the channel in IOP. However it is known that the $Ca^+$-sensitivity of maxi $K^+$ channels can vary over a wide range [13–15] and that calcium-dependent calcium release from internal stores may locally amplify cytoplasmic $Ca^{2+}$ elevations.

An interesting point is the apparent transient activation by calcium. This desensitisation was not due to concentration differences during the change of solutions. A similar $K^+$-channel found in embryonic chick osteogenic cells [20] showed no inactivation upon $Ca^{2+}$ increase, using the same setup. Calcium is known to block $Ca^{2+}$-activated potassium channels at high concentrations ($> 10^{-4}$ M) but this type of block [21] does not last more then a few seconds. The 150-pS channel described here is not active over much longer periods 10–60 s (data not shown).

The transient activation of the channel by a change in $[Ca^{2+}]_{in}$ may explain previous observations that the activation potential, measured in the whole cell configuration (WC), of the outward rectifying $K^+$-conductance ($G_{K_o}$) was similar when measured with an pipette $[Ca^{2+}]$ of $10^{-7.8}$ M or $10^{-5.1}$ M [3]. After making WC it took more than 2 min to measure the activation potential of the $G_{K_o}$.

It remains a question of interest how the maxi $K^+$-channel can be functional in chick osteoclasts. Earlier experiments [3] have shown that chick osteoclasts may have a resting membrane potential of around 0 mV. Assuming an intracellular $[Ca^{2+}]$ of $10^{-7}$ M, the channels would only be activated at extreme depolarisation of the membrane ($\geqq +60$ mV). However, when the OC is actively resorbing, the increase in $Ca^{2+}$ concentration in the resorption lacuna may cause an increase in the intracellular $Ca^{2+}$ concentration [5,6,22], thereby activating the calcium-dependent $K^+$-channel. This, however, seems inconsistent with the observations of Miyauchi et al. [5] that $[Ca^{2+}]_{in}$ is not sustained increased in bone adherent chick OC, which is suprising given the maintained response of $[Ca^{2+}]_{in}$ to a step increase of $[Ca^{2+}]_{out}$ in bone adherent OC [5]. Other intracellular messengers, like cAMP, are known to control the maxi $K^+$-channel activity [23]. Phosphorylation of the channel, by cAMP-dependent protein kinases, increased the $Ca^{2+}$-sensitivity in other cells (cf., Ref. 15). Furthermore, other intracellular components are able to shift the $Ca^{2+}$-sensitivity to a more physiological range [15].

The role of maxi $K^+$-channels in non-excitable animal cells is not clear yet. There is evidence that these channels play a role in the secretion mechanism of exocrine and endocrine glands [8]. Others report that Ca-dependent $K^+$-channels are involved in absorption and secretion in renal tissue [15].

We speculate that activation of the maxi $K^+$-channels caused by an increased intracellular $Ca^{2+}$-concentration during the process of bone resorption, may inactivate resorption activity.

Activation of the maxi $K^+$-channel will cause a membrane potential change towards $E_K$ which in turn may interfere with the extrusion of protons via the action of an electrogenic $H^+$-pump [24–27]. In this way the $Ca^{2+}$-dependent $K^+$-channel may supply a feedback system for OC resorption activity.

However, a better understanding of the role of the calcium-dependent $K^+$-channel in osteoclast functions requires more knowledge about the distribution of the various types of ion channels and transporters over the basal membrane and the apical secretory, bone-facing, membrane of the osteoclast.

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## References

1 Nijweide, P.J., Burger, E.H. and Feyen, J.H.M. (1986) Phys. Rev. 66, 855–886.
2 Vaes, G. (1988) Clin. Orthop. Relat. Res. 231, 239–271.
3 Ravesloot, J.H., Ypey, D.L., Vrijheid-Lammers, T. and Nijweide, P.J. (1989) Proc. Natl. Acad. Sci. USA 86, 6821–6825.
4 Sims, S.M. and Dixon, S.J. (1989) Am. J. Physiol. 256, C1277–C1282.
5 Miyauchi, A., et al. (1990) J. Cell Biol. 111, 2543–2552.
6 Datta, H.K., MacIntyre, I. and Zaidi, M. (1989) Biosci. Rep. 9, 747–751.
7 Anderson, R.E., Woodbury, D.M. and Jee, W.S. (1986) Calcif. Tissue 39, 252–258.
8 Petersen, O.H. and Maruyana, Y. (1984) Nature 307, 693–696.
9 Nijweide, P.J., Vrijheid-Lammers, T., Mulder, R.J.P. and Blok, J. (1985) Histochemistry 83, 315–324.
10 Ince, C., Dissel, J.T. and Diesselhof, M.M.C. (1985) Pflüg. Arch. 403, 240–244.
11 Hamill, O.P., Marty, A., Neher, E. Sakmann, B. and Sigworth, F.J. (1981) Pflüg. Arch. 391, 85–100.
12 Fabiato, A. and Fabiato, F. (1979) J. Physiol. 75, 317–330.
13 Latorre, R., Oberhauser, A., Labarca, P. and Alvarez, O. (1989) Annu. Rev. Physiol. 51, 385–399.
14 Latorre, R. and Miller, C. (1983) J. Membr. Biol. 71, 11–30.
15 Marty, A. (1989) Trends Neurosci. 12, 420–424.
16 Silver, A., Murrills, R.J. and Etherington, D.J. (1988) Exp. Cell Res. 175, 266–276.
17 Palotta, B.S., Hepler, J.R, Oglesby, S.A. and Harden, T.K. (1987) J. Gen. Physiol. 89, 985–997.
18 Kawahara, K., Hunter, M. and Giebisch, G. (1990) Pflüg. Arch. 416, 422–427.
19 Marty, A. (1983) Pflüg. Arch. 396, 179–181.

20 Ravesloot, J.H., Van Houten, R.J., Ypey, D.L. and Nijweide, P.J. (1991) J. Bone Miner. Res. 5, 1201–1210.
21 Vergara, C. and Latorre, R. (1983) J. Gen. Physiol. 82, 543–568.
22 Zaida, M., Datta, H.K., Pattchell, A., Moonga, B. and MacIntyre, I. (1989) Biochem. Biophys. Res. Commun. 163, 1461–1465.
23 Gray, M.A., Greenwell, J.R., Garton, A.J. and Argent, B.E. (1990) J. Membr. Biol. 115, 203–215.
24 Blair, H.C., Teitelbaum, S.L., Ghiselle, R. and Gluck, S. (1989) Science 245, 855–857.
25 Bekker, P.J. and Gay, C.A. (1990) J. Bone Miner. Res. 5, 569–579.
26 Sundquist, K. Lakkakorpi, P., Wallmark, B. and Vaananen, K. (1990) Biochem. Biophys. Res. Commun. 168, 309–313.
27 Vaananen, H.K., Karhukorpi, E.K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J. and Lakkakorpi, P. (1990) Cell. Biol. 111, 1305–1311.
28 Ravesloot, J.H., Van Houten, R.J., Ypey, D.L., Nijweide, P.J. (1991) J. Bone Miner. Res. 6, 355–363.
29 Marty, A. (1981) Nature 291, 497–500.
30 Horn, R. (1991) Biophys. J. 60, 329–333.