Inhibition of inwardly rectifying K^+ current by external Ca^{2+} ions in freshly isolated rabbit osteoclasts

Naohide Yamashita, Tomoe Ishii, Etsuro Ogata* and Toshio Matsumoto

*Fourth Department of Internal Medicine, University of Tokyo School of Medicine, Tokyo 112, Japan and *Japanese Foundation for Cancer Research, Cancer Institute Hospital, Tokyo 170, Japan*

1. Regulation of membrane potential by extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was examined in freshly isolated rabbit osteoclasts.
2. The resting membrane potential of osteoclasts was close to the K^+ equilibrium potential in 1 mM Ca^{2+} medium. An elevation of $[Ca^{2+}]_o$ caused membrane depolarization, accompanied by a decrease in the membrane conductance.
3. The inwardly rectifying K^+ current observed under voltage clamp was dose-dependently inhibited by an elevation of $[Ca^{2+}]_o$, which explained the membrane depolarization caused by high $[Ca^{2+}]_o$.
4. Other divalent cations also inhibited the inwardly rectifying K^+ current with the following order of potency: $Ca^{2+} < Ni^{2+} \leqslant Co^{2+} < Cd^{2+}$.
5. In the presence of intracellular GTPγS the inwardly rectifying K^+ current was irreversibly inhibited by $[Ca^{2+}]_o$, whereas the inhibition of the inwardly rectifying K^+ current was greatly attenuated by intracellular application of GDPβS.
6. Pertussis toxin (PTX) treatment did not abolish the inhibition of the inwardly rectifying K^+ current caused by $[Ca^{2+}]_o$.
7. These results suggest that inwardly rectifying K^+ channels in osteoclasts were regulated by a PTX-insensitive G-protein, which was coupled to the putative Ca^{2+} receptor or sensor on the cell membrane.

Osteoclasts resorb bone by secreting lysosomal enzymes and protons in a resorptive hemivacuole opposite their ruffled borders. The extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) in this space increases and reaches values as high as 40 mM (Silver, Murrills & Etherington, 1988). It is known that the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of osteoclasts increases and that cells contract in response to an elevation of $[Ca^{2+}]_o$. These responses may be mediated through a Ca^{2+} receptor or a sensor on the osteoclast cell membrane (Bax, Shankar, Moonga, Huang & Zaidi, 1992; Zaidi *et al.* 1993). However, little is known about how ionic channels of osteoclasts are regulated by $[Ca^{2+}]_o$. Inwardly rectifying K^+ channels are the predominant channels in osteoclasts (Ravesloot, Ypey, Vrijheid-Lammers & Nijweide, 1989; Sims, Kelly & Dixon, 1991; Kelly, Dixon & Sims, 1992). These ionic channels and other pumps may well play important roles in maintaining the homeostasis of the ionic environments in osteoclasts (Baron, Neff, Louvard & Courtoy, 1985; Anderson, Woodbury & Jess, 1986; Arisaka & Gay, 1986; Baron, Neff, Roy, Boisvert & Caplan, 1986; Tuukkanen & Väänänen, 1986; Arisaka, Yamamoto & Gay, 1988; Hunter, Schraer & Gay, 1988). Inwardly rectifying K^+ channels exist in many kinds of cells and it is known that agonists or antagonists modulate these channels through a GTP-binding protein (G-protein) (Brown & Birnbaumer, 1990). In the present study we have investigated the modulation of inwardly rectifying K^+ channels by $[Ca^{2+}]_o$ in rabbit osteoclasts and also examined the involvement of the G-protein in their regulation. A preliminary report of this work has already been published as an abstract (Yamashita, Ishii, Ogata & Matsumoto, 1992).

METHODS

Materials

Guanosine 5′-triphosphate (GTP), adenosine 5′-triphosphate (ATP), pertussis toxin (PTX), dithiothreitol (DTT) and nicotinamide–adenine dinucleotide (NAD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) were purchased from Boehringer Mannheim (Mannheim, Germany). *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes), ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and tetramethylammonium (TMA) were purchased from Wako Junyaku (Tokyo, Japan).

Cell culture

Newborn rabbits (Japanese White) were anaesthetized using ether and decapitated. Osteoclasts were isolated from long bones according to the methods reported by Tezuka *et al.* (1992). After isolation, cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and kept at 4 °C. About 30 min before recordings, the cells were seeded on glass coverslips, uncoated or coated with vitronectin, or on 35 mm plastic culture dishes. Cells were incubated at 37 °C in humidified air containing 5% CO_2 in DMEM containing 10% FCS. After incubation for 30–60 min osteoclasts attached to the bottom of the plates. Most cells were rounded in appearance although some cells appeared spread. Both forms of osteoclast were used in the present study. The cells were then washed with the standard extracellular medium (see below) and electrophysiological studies were done. All experiments were carried out within 6 h of isolation of cells.

Osteoclasts could be discriminated easily by eye when viewed by phase-contrast microscopy because of their multinucleation. In response to application of 10 mM $[Ca^{2+}]_o$ the osteoclasts contracted, and $[Ca^{2+}]_i$ increased (data not shown). To further confirm the identity of the cell under study, tartrate-resistant acid phosphatase staining was used after electrophysiological recordings in the early part of the study. All of the cells used for recording ($n = 6$) were positively stained. Thus, the morphologically identified osteoclasts satisfied all other criteria for osteoclast identification. The following experiments were done on morphologically identified cells.

Electrophysiology

The whole-cell variation of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used. The standard patch electrode solution contained (mM): 95 potassium aspartate, 47·5 KCl, 1 $MgCl_2$, 0·1 EGTA (TMA salt), 10 Hepes (TMA salt, pH 7·2), 100 μM GTP (Na salt) and 2 mM ATP (Mg salt). In the high-EGTA internal solution the EGTA concentration in the patch electrode solution was increased to 5 mM by isosmotically replacing aspartate. The standard extracellular medium was composed of (mM): 128 NaCl, 3 KCl, 1 $MgCl_2$, 1·0 $CaCl_2$, 10 Hepes (Na salt, pH 7·4). In high-Ca^{2+} media, Ca^{2+} concentration was increased by isosmotic replacement of Na^+ ions. The media containing high concentrations of divalent cations other than Ca^{2+}, such as 5 mM Co^{2+}, were prepared in a similar manner. The osteoclasts were continuously superfused during electrical recordings using a peristaltic pump. High $[Ca^{2+}]_o$ was applied by changing the perfused medium. The liquid junction potential between the standard extracellular solution and other solutions used (internal and external) was measured directly using a 3 M KCl electrode as a reference. The value of the liquid junctional potential (−8 to −2 mV) was corrected in each experiment. A List L/M EPC-7 amplifier (List Electronic, Darmstadt, Germany) was used for recording membrane current and potential. Application of voltage or current pulses, data acquisition, and analysis were done using a 486-based Gateway 2000 computer using the pCLAMP software package (Axon Instruments, CA, USA). Experiments were performed at room temperature (22–25 °C). Glass capillaries, 1·5 mm in external diameter, with a filament were used to make the electrodes. The patch electrode resistance ranged from 3 to 5 MΩ and the access resistance was 5–10 GΩ. The series resistance compensation (70~90%) was used to reduce the error in the potential at which the cell was clamped. During analysis the error was assumed to be zero.

Pertussis toxin treatment

To activate pertussis toxin (PTX), 10 μM PTX was dissolved in the patch electrode solution containing 10 mM DTT and 10 mM NAD, and was incubated at 37 °C for 20 min. It was then diluted (1:10) with the patch electrode solution, the final PTX concentration being 1 μM. Activated PTX was applied to the interior of the cell through the patch pipette. To examine the efficacy of activated PTX, we tested whether it inhibited G-protein-coupled K^+ channels in human growth hormone (GH)-producing pituitary cells (Yamashita, Shibuya & Ogata, 1988). Activated PTX inhibited the somatostatin-induced inwardly rectifying K^+ current in these cells ($n = 3$).

Values are given as means ± S.D. unless otherwise stated.

RESULTS

Membrane depolarization caused by an elevation of $[Ca^{2+}]_o$ in osteoclasts

Consistent with the reports of Kelly *et al.* (1992), we found resting membrane potentials in rabbit osteoclasts of −73·6 ± 4·5 mV ($n = 13$). Figure 1*A* shows the effect of $[Ca^{2+}]_o$ elevation on the membrane potential. The resting membrane potential in 1 mM Ca^{2+} medium was approximately −70 to −75 mV for the cell shown. A hyperpolarizing current pulse of 8 pA amplitude was applied every 10 s to monitor the membrane conductance. Application of 5 mM $[Ca^{2+}]_o$ caused the membrane potential to start switching between −75 and −20 mV (approximate values). The duration of the membrane depolarization was 1–19 s. In addition, the membrane conductance decreased from approximately 700 pS in 1 mM Ca^{2+} medium to approximately 420 pS in 5 mM Ca^{2+} medium and further decreased to approximately 100 pS during the membrane depolarization. The reduction of $[Ca^{2+}]_o$ from 5 to 1 mM restored the membrane potential to the resting level as was the case for the membrane conductance. The following application of 10 mM $[Ca^{2+}]_o$ depolarized the membrane to approximately −15 mV but in a sustained fashion, again accompanied with a decrease in the membrane conductance (approximately 88 pS). The membrane potential in 10 mM $[Ca^{2+}]_o$ was a little less negative compared with the depolarized membrane potential in 5 mM $[Ca^{2+}]_o$. The decrease in membrane conductance in 10 mM $[Ca^{2+}]_o$ was more prominent than that in 5 mM $[Ca^{2+}]_o$, indicating that the permeability to some ions decreased in high $[Ca^{2+}]_o$; this was dependent on the concentration of $[Ca^{2+}]_o$. In some cells the membrane potential remained at approximately −20 mV in 5 mM $[Ca^{2+}]_o$ (data not shown).

Inhibition of the inwardly rectifying K^+ current caused by an elevation of $[Ca^{2+}]_o$

The resting potential of rabbit osteoclasts was close to the equilibrium potential of K^+ ions. Accordingly, one might suggest that the permeability to K^+ ions decreased in

high $[Ca^{2+}]_o$. To elucidate this mechanism, voltage clamp experiments were carried out. Figure 1*B* shows a family of membrane current traces recorded in 1 mM Ca^{2+} medium under voltage clamp. The holding potential was −40 mV. When the membrane was hyperpolarized below −80 mV, the amplitude of the inward current increased. The current–voltage (*I–V*) relationship (Fig. 1*C*) showed a prominent inward-going rectification below −80 mV. This inward-going rectification is consistent with earlier reports by Kelly *et al.* (1992) and was observed in all cells examined ($n = 103$), whether rounded or spread. Between −70 and −20 mV the *I–V* relationship showed a negative slope region and the amplitude of the membrane current increased monotonically above −20 mV. In the present experiments this negative slope region was varied considerably among cells. The time-dependent inactivation of the inwardly rectifying K^+ current, which was ascribed to the inhibition of the inwardly rectifying K^+ current by extracellular Na^+ ions (Inoue & Yoshii, 1992), was observed when the membrane was hyperpolarized to values more negative than −150 mV in Na^+-containing media (data not shown).

Figure 2*A* shows the changes in membrane currents when $[Ca^{2+}]_o$ was successively increased from 1 to 5 mM and then to 10 mM. In high-Ca^{2+} medium, the amplitude of membrane currents decreased. A similar inhibition of the inwardly rectifying K^+ current was observed in all cells examined ($n = 42$). As the membrane current was attained at potential levels less negative than −150 mV in the present study, a voltage ramp pulse was used to obtain the *I–V* relationship. This voltage ramp did not interfere with the blockade of inwardly rectifying K^+ channels by extracellular Na^+ (see above). Figure 2*B* shows *I–V* relationships of the membrane current using the ramp pulse protocol. Although the negative slope region in the *I–V* relationship was not prominent in the cell shown, the inwardly rectifying K^+ current was progressively inhibited as $[Ca^{2+}]_o$ increased. After increasing $[Ca^{2+}]_o$ to 80 mM, it was decreased to 3 mM and the *I–V* relationship was restored close to the original level. The slope conductance

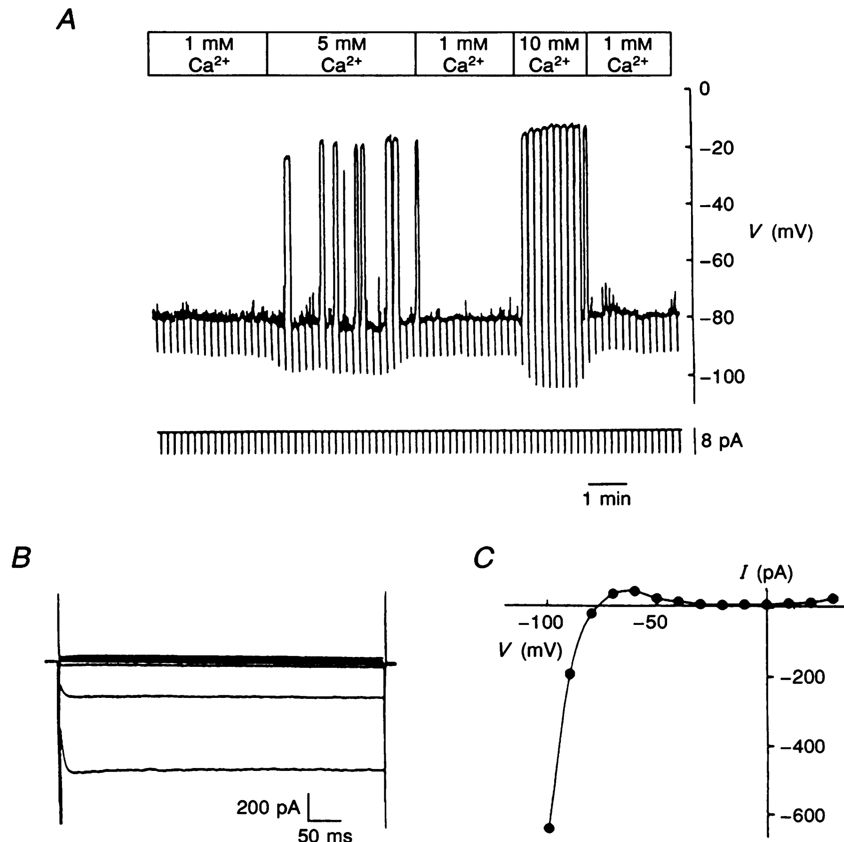

Figure 1. Membrane depolarization of osteoclasts caused by high $[Ca^{2+}]_o$
A, membrane depolarization caused by high $[Ca^{2+}]_o$ recorded under current clamp. The membrane conductance was monitored by applying a constant current pulse of 8 pA every 10 s. The patch electrode solution contained 142·5 mM K^+ and 0·1 mM EGTA. *B*, membrane currents obtained under voltage clamp. The holding potential was −40 mV and test potentials stepped from −100 to 30 mV in 10 mV increments. The extracellular medium contained 1 mM Ca^{2+} and the patch electrode solution was the same as in *A*. *C*, the corresponding current–voltage (*I–V*) relationship of the membrane currents shown in *B*.

of the $I-V$ relationship from −90 to −130 mV was measured and the inhibition of the inwardly rectifying K^+ current was plotted against $[Ca^{2+}]_o$ as shown in Fig. 2C (open circles, $n = 8$). The leakage current was subtracted and the slope conductance was normalized by taking the value measured in 1 mM $[Ca^{2+}]_o$ to be 100%. Figure 2C clearly shows that the inhibition of the inwardly rectifying K^+ current was dependent on $[Ca^{2+}]_o$.

Figure 3A shows representative changes in the negative slope region of the $I-V$ relationships. The negative slope region was prominently observed in the cell shown. In 1 mM Ca^{2+} medium the $I-V$ relationship intersects the abscissa at −78 mV, indicating the resting membrane potential of the cell shown. In 5 mM Ca^{2+} medium the magnitude of the negative slope region decreased and the $I-V$ relationship intersects the voltage axis at around −75, −40 and −20 mV in the cell shown. This phenomenon indicates that the membrane potential is unstable at approximately −40 mV, and that the membrane potential of the cell switches between −75 and −20 mV upon small depolarizing or hyperpolarizing currents from other sources. The potential levels at which the $I-V$ relationship intersected the abscissa varied even in the same cell and in some cells it only intersected the abscissa at approximately −20 mV in 5 mM Ca^{2+} medium (data not shown). In 10 and 20 mM Ca^{2+} media the $I-V$ relationship intersects the abscissa at approximately −18 or −15 mV only, which was a little depolarized compared with the most depolarized level (−20 mV) in 5 mM Ca^{2+} medium. Because the resting potential is determined by the zero-current level in the $I-V$ relationship, these changes in the $I-V$ relationships caused by high $[Ca^{2+}]_o$ are a good explanation of the membrane potential changes shown in Fig. 1A. The membrane depolarization or the inhibition of the inwardly rectifying K^+ current by $[Ca^{2+}]_o$ was consistently observed irrespective of the material on which the cells were plated (glass coverslip, uncoated or coated with vitronectin, or plastic dishes).

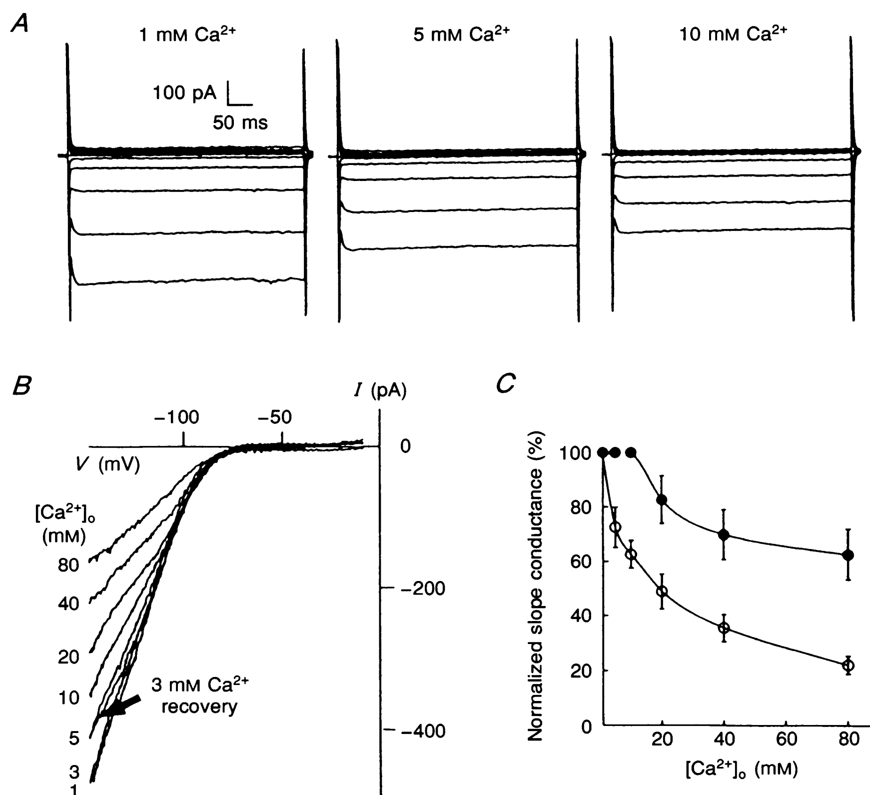

Figure 2. Inhibition of the inwardly rectifying K^+ current caused by high $[Ca^{2+}]_o$

A, inhibition of the inwardly rectifying K^+ current by high $[Ca^{2+}]_o$. The holding potential was −40 mV; the test potentials were the same as those in Fig. 1B. The patch electrode solution was the same as in Fig. 1A. *B*, $I-V$ relationships of membrane currents obtained by applying a ramp test potential from −140 to −10 mV. The holding potential was −40 mV and the duration of the ramp pulse was 400 ms. The patch electrode solution contained 145 mM K^+ and 5 mM EGTA. *C*, changes in the slope conductance of the $I-V$ relationship (from −130 to −90 mV) caused by high $[Ca^{2+}]_o$. The slope conductance was normalized by taking the value measured for 1 mM $[Ca^{2+}]_o$ to be 100%. Control values (○) were measured in standard patch electrode solution ($n = 8$) and values were also obtained with the patch electrode solution containing 200 μM GDPβS (●, $n = 4$). Vertical bars indicate ± S.D.

Effects of other divalent cations on the inwardly rectifying K^+ current

Figure 3*B* shows the effect of Co^{2+} on the *I–V* relationship of the inwardly rectifying K^+ current. As in the case of Ca^{2+}, Co^{2+} inhibited the inwardly rectifying K^+ current in a dose-dependent manner. Figure 3*C* compares the effects of Ca^{2+}, Co^{2+} and Ni^{2+} on the inwardly rectifying K^+ current. Co^{2+} and Ni^{2+} inhibited inwardly rectifying K^+ channels more potently than Ca^{2+}. Including the data from other experiments, the cations inhibited inwardly rectifying K^+ channels in the following order of potency: $Ca^{2+} < Ni^{2+} \leqslant Co^{2+} < Cd^{2+}$.

Involvement of the G-protein in the inhibition of the inwardly rectifying K^+ current

In neuronal cells and heart muscle cells, inwardly rectifying K^+ channels are regulated by various agonists and antagonists through G-proteins (Brown & Birnbaumer, 1990). In osteoclasts the increase in $[Ca^{2+}]_i$ caused by high $[Ca^{2+}]_o$ is considered to be mediated by the putative Ca^{2+} receptor or sensor (Bax *et al.* 1992; Zaidi *et al.* 1993). Because the inwardly rectifying K^+ currents in osteoclasts were inhibited by an elevation of $[Ca^{2+}]_o$, it was suggested that inwardly rectifying K^+ channels may be coupled to the Ca^{2+} receptor through the G-protein. Therefore, we examined the involvement of the G-protein in the inhibition of the inwardly rectifying K^+ current caused by high $[Ca^{2+}]_o$. Figure 4*A* shows the effect of GTPγS on inwardly rectifying K^+ channels. The upper panel depicts the data of the control experiment. The inwardly rectifying K^+ current was reversibly inhibited by high $[Ca^{2+}]_o$. When 100 μM GTPγS was intracellularly applied through the patch pipette, the inhibition of the inwardly rectifying K^+ current was not reversible (lower panel). The *I–V* relationships of the membrane current altered by $[Ca^{2+}]_o$ are shown in Fig. 4*B*. A similar irreversible inhibition of inwardly rectifying K^+ channels was observed when Co^{2+} was applied to osteoclasts with the patch pipette solution containing GTPγS (data not shown).

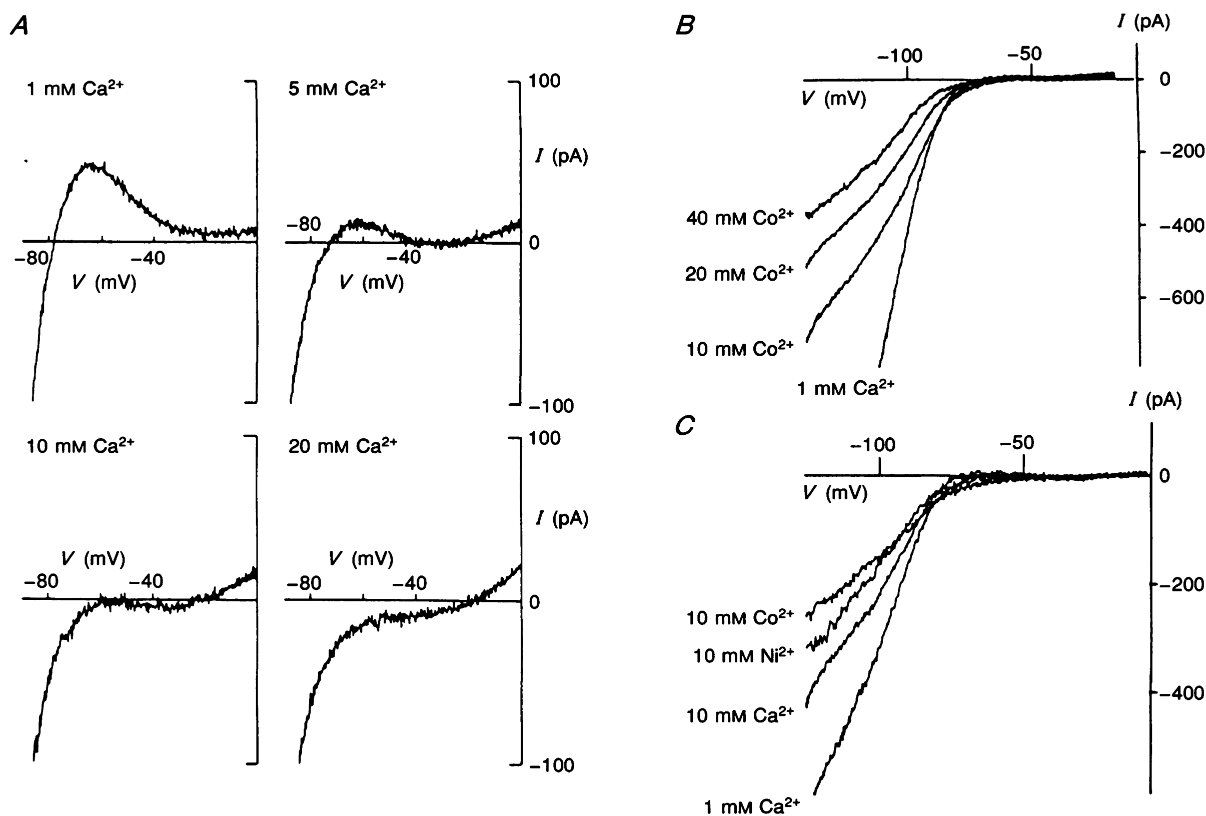

Figure 3. Changes in the *I–V* relationships caused by $[Ca^{2+}]_o$ and other divalent cations
A, changes in the negative slope region of the *I–V* relationship caused by high $[Ca^{2+}]_o$. The patch electrode solution contained 145 mM K^+ and 0·1 mM EGTA. The holding potential was −40 mV and membrane currents were obtained by applying a ramp test potential from −130 to 0 mV. The ramp duration was 400 ms. *B*, changes in the *I–V* relationship caused by external Co^{2+} ions. A ramp potential from −140 to −10 mV was applied. The holding potential was −40 mV. The patch electrode solution was the same as in *A*. *C*, comparison of effects of Ca^{2+}, Co^{2+} and Ni^{2+} on the *I–V* relationship. The holding potential, the ramp pulse protocol and the patch electrode solutions were the same as those in *A*.

Figure 4C shows the I–V relationships of the membrane current when 200 μM GDPβS was intracellularly applied through the patch pipette. The intracellular application of GDPβS greatly attenuated the inhibition of inwardly rectifying K^+ channels caused by high $[Ca^{2+}]_o$. Filled circles in Fig. 2C show the changes in the slope conductance of I–V relationships in the presence of intracellular GDPβS ($n = 4$, compare with open circles as the control). To examine whether the involved G-protein was sensitive to pertussis toxin, the preactivated PTX was applied to the cell through the patch pipette. However, this treatment did not affect the inhibition of the inwardly rectifying K^+ current by high $[Ca^{2+}]_o$ (data not shown). These results are consistent with the assumption that high $[Ca^{2+}]_o$ inhibited the inwardly rectifying K^+ current through the activation of an PTX-insensitive G-protein.

DISCUSSION

The results of the present study revealed that the membrane of osteoclasts depolarizes in response to an elevation of $[Ca^{2+}]_o$. This was ascribed to the inhibition of the inwardly rectifying K^+ current. Other divalent cations, such as Co^{2+} and Ni^{2+}, also inhibited the inwardly rectifying K^+ current. The inhibition of inwardly rectifying K^+ channels was greatly attenuated by the intracellular application of GDPβS but was not affected by PTX, suggesting that the inhibition by $[Ca^{2+}]_o$ was mediated by a PTX-insensitive G-protein. Zaidi, Datta, Patchell, Moonga & MacIntyre (1989) reported that $[Ca^{2+}]_i$ increases in response to an elevation of $[Ca^{2+}]_o$, and suggested that osteoclasts possess a Ca^{2+} receptor on the cell membrane which senses a change in $[Ca^{2+}]_o$ (Bax *et al.* 1992; Zaidi *et al.* 1993). The presence of similar $[Ca^{2+}]_o$ receptors has been suggested in parathyroid cells (Nemeth, Wallace & Scarpa, 1986), renin-secreting cells (Kurtz & Penner, 1989) and calcitonin-secreting cells (Yamashita & Hagiwara, 1990) and a parathyroid Ca^{2+} receptor has recently been cloned which appears to belong to the G-protein-coupled receptor superfamily (Brown *et al.* 1993; Rask *et al.* 1993).

It is well known that the inwardly rectifying K^+ current is inhibited by external Na^+ and Ba^{2+} (Inoue & Yoshii, 1992). However, the effect of $[Ca^{2+}]_o$ on the inwardly rectifying K^+ current has been controversial. In tunicate eggs (Ohmori, 1978) and in frog skeletal muscles (Standen & Stanfield, 1978), an elevation of $[Ca^{2+}]_o$ does not inhibit inwardly rectifying K^+ channels, whereas it inhibits inwardly rectifying K^+ channels in guinea-pig ventricular myocytes (Biermans, Vereecke & Carmeliet, 1987) and in starfish eggs (Hagiwara, Miyazaki, Moody & Patlak, 1978).

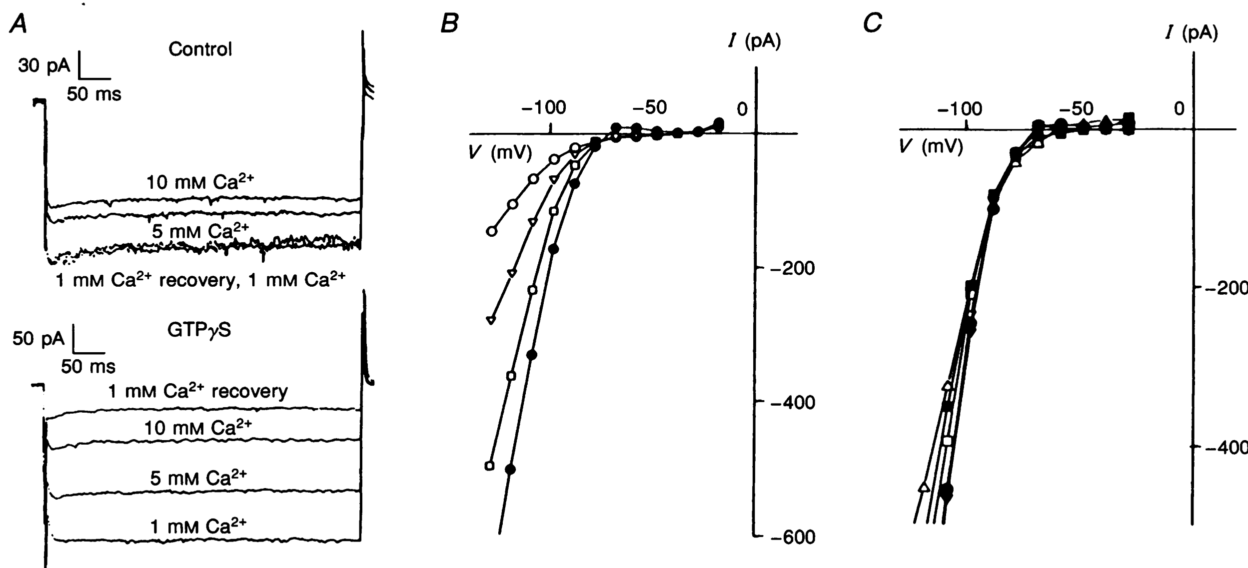

Figure 4. Involvement of the G-protein in the inhibition of the inwardly rectifying K^+ current

A, the effect of GTPγS on the inhibition of the inwardly rectifying K^+ current caused by high $[Ca^{2+}]_o$. GTPγS (100 μM) was intracellularly applied through the patch pipette. The patch pipette solution contained 145 mM K^+ and 5 mM EGTA. The upper panel shows the control records and the lower panel shows the effect of intracellular GTPγS. The holding potential was −40 mV and the test potential was −100 mV. *B*, the I–V relationship of membrane currents obtained in *A*. Symbols: ●, □ and ▽, represent 1, 5 and 10 mM Ca^{2+}, respectively; ○, 1 mM Ca^{2+} recovery. *C*, the effect of GDPβS on the inhibition of the inwardly rectifying K^+ current caused by high $[Ca^{2+}]_o$. Symbols: ●, ▽, ▼, □, ■ and △, represent 1, 5, 10, 20, 40 and 80 mM Ca^{2+}, respectively. GDPβS (200 μM) was intracellularly applied through the patch pipette. The holding potential was −40 mV. The patch pipette solution was the same as that in *A*.

It is difficult to explain these discrepancies at present. However, the involvement of a G-protein in the inhibition of inwardly rectifying K^+ channels by $[Ca^{2+}]_o$ in rabbit osteoclasts suggests that activation of the Ca^{2+} receptor or sensor was involved in the inhibition of the inwardly rectifying K^+ current. The inhibition of the inwardly rectifying K^+ current by $[Ca^{2+}]_o$ was not fully blocked by intracellular application of GDPβS. It is also possible that $[Ca^{2+}]_o$ may inhibit inwardly rectifying K^+ channels partly via a direct mechanism, such as the plugging of the channel by Ca^{2+} ions.

An elevation of $[Ca^{2+}]_o$ also causes a rise in $[Ca^{2+}]_i$ in osteoclasts, which is suggested to be associated with the contraction of osteoclasts in $[Ca^{2+}]_o$, and other divalent cations, such as Ni^{2+} and Cd^{2+}, also increase $[Ca^{2+}]_i$ (Zaidi *et al.* 1993). In the present experiment, these divalent cations inhibited the inwardly rectifying K^+ current like Ca^{2+}. It has been reported that the application of Ni^{2+} causes only a transient increase in $[Ca^{2+}]_i$, whereas the elevation of $[Ca^{2+}]_o$ causes transient and sustained increases in $[Ca^{2+}]_i$ (Zaidi *et al.* 1933). It is also suggested that sustained application of Ni^{2+} inactivates the Ca^{2+} receptor, and that membane hyperpolarization attenuates the inactivation of Ca^{2+} receptor (Zaidi *et al.* 1933). We could not find an apparent blockade of the inhibition of the inwardly rectifying K^+ current by Ni^{2+}, probably because we hyperpolarized the membrane when the $I-V$ relationship was obtained. The inhibition of the inwardly rectifying K^+ current was consistently observed when 5 mM EGTA was added to the patch electrode solution. These results suggest that the rise in $[Ca^{2+}]_i$ was not ascribed to the inhibition of the inwardly rectifying K^+ current, although there was a possibility that the inhibition of the inwardly rectifying K^+ current was caused by an increase in $[Ca^{2+}]_i$.

It has recently been reported in freshly isolated rat osteoclasts that rounded osteoclasts, which may represent a resorptive phase, exhibit an outwardly rectifying K^+ conductance with no apparent inwardly rectifying K^+ conductance, whereas spread osteoclasts, which may represent a motile phase, express an inwardly rectifying K^+ conductance with no apparent outwardly rectifying K^+ current (Arkett, Dixon & Sims, 1992). In the present experiment we used rounded rabbit osteoclasts. All of them exhibited the inwardly rectifying K^+ current with no apparent outward K^+ current. We also found the inwardly rectifying K^+ current in spreading rabbit osteoclasts. As in the present experiments rabbit osteoclasts were used within a short period (6 h) after isolation, and because recordings were done within 1 h of plating the cells, it is unlikely that the expression of ionic channels altered during incubation. Although these discrepancies between rat and rabbit osteoclasts may be ascribed to a difference in species, we do not have a good explanation at present.

Bone-free cultured osteoclasts possess voltage-gated Ca^{2+} channels on the cell membrane and $[Ca^{2+}]_i$ increases due to an application of high external $[K^+]$, which depolarizes the membrane (Miyauchi *et al.* 1990). However, freshly isolated osteoclasts or osteoclasts adherent to bone lack voltage-activated Ca^{2+} channels (Miyauchi *et al.* 1990; Kelly *et al.* 1992). In such cells high-K^+-induced membrane depolarization reduces $[Ca^{2+}]_i$ (Miyauchi *et al.* 1990), probably through the reduction of driving force for Ca^{2+} ions through voltage-independent Ca^{2+} pathways. Like Kelly *et al.* (1992) we did not find macroscopic voltage-gated Ca^{2+} channel currents in freshly isolated rabbit osteoclasts (data not shown). Therefore, it is plausible to consider that $[Ca^{2+}]_i$ in osteoclasts attached to the bone decreases when the membrane is depolarized. It is possible that the inhibition of inwardly rectifying K^+ channels, which depolarizes the membrane, damps the elevation of $[Ca^{2+}]_i$ caused by high $[Ca^{2+}]_o$ and thereby regulates $[Ca^{2+}]_i$. Sims *et al.* (1991) and Kelly *et al.* (1992) have proposed that the electrogenic H^+ pump, through which bone is resorbed, hyperpolarizes the membrane and activates inwardly rectifying K^+ channels, preventing excessive hyperpolarization by clamping the membrane at the equilibrium potential of K^+ ions. Thus, inwardly rectifying K^+ channels may play functional roles in osteoclasts by altering the membrane potential. It is therefore of interest that extracellular Ca^{2+} ions seem to regulate this important conductance.

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Acknowledgements

We thank Dr J. H. Ravesloot and Professor R. Baron for valuable comments and suggestions, and for scrutinizing our English. This work was supported by grants from the Ministry of Education of Japan, the Ministry of Health and Welfare of Japan and the Yamanouchi Pharmaceutical Company.

Received 25 November 1993; accepted 2 March 1994.