Genistein, a Soybean Isoflavone, Inhibits Inward Rectifier K⁺ Channels in Rat Osteoclasts

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Abstract: Genistein, a soybean-derived isoflavone with an inhibitory effect on protein tyrosine kinases (PTKs), has been shown to suppress osteoclastic bone resorption. To clarify the mechanisms underlying this action, we investigated the effects of genistein on inward rectifier K⁺ current (I_{KIR}) in rat osteoclasts by using the whole-cell patch-clamp technique. Extracellularly applied genistein inhibited I_{KIR} in a concentration-dependent manner. Physiologically attainable concentrations of genistein inhibited I_{KIR}. IC₅₀ values obtained 5 and 10 min after the application of genistein were 54 and 27 µM, respectively. The removal of genistein partially restored the current. Daidzein, an isoflavone without PTK-inhibiting activity, also showed a weak inhibitory effect on I_{KIR}, but genistin had no effect. Other PTK inhibitors, tyrphostin A25, tyrphostin B42, and tyrphostin B46, inhibited I_{KIR}, whereas herbimycin A and lavendustin A were without effect. The inactive tyrphostin, A1, showed a similar inhibitory effect as tyrphostin A25. The tyrosine phosphatase inhibitor, orthovanadate, did not affect the inhibitory potency of genistein on I_{KIR}. The inhibitory action of genistein was unaffected by changing intracellular Ca²⁺ concentration ([Ca²⁺]) or by pretreatment of the cell with GDPβS, Rp-cAMPS, okadaic acid, or stauroporine. Therefore the inhibition of I_{KIR} by genistein does not depend on PTK inhibition, involvement of changes in [Ca²⁺], or secondary interaction with protein kinase A or protein kinase C. Genistein-induced inhibition of I_{KIR} would cause membrane depolarization, elevation of [Ca²⁺], and inhibition of osteoclastic bone resorption. [Japanese Journal of Physiology, 51, 501–509, 2001]

Key words: osteoclast, inward rectifier K⁺ current, soybean isoflavone, genistein, tyrosine kinase inhibitor.

Decreased bone mass resulting from estrogen deficiency is a serious problem in postmenopausal women. Although estrogen replacement therapy is an effective treatment for the prevention of bone loss, adverse effects on reproductive organs such as the uterus are recognized [1]. Isoflavonoids found in leguminous plants, especially soybeans, are estrogen-like substances (so called phytoestrogens), structurally and functionally similar to 17β-estradiol [2]. Kuiper et al. [3] have shown that the typical soybean isoflavone genistein binds to the intracellular estrogen receptor and suggested that genistein may produce biological effects similar to estrogen. Recently, isoflavonoids have been reported to attenuate bone loss from the lumbar spine in perimenopausal women [4]. In tissue culture, genistein has been shown to inhibit the parathyroid hormone–induced bone resorption as effectively as estrogen, and this effect was prevented by tamoxifen, an antiestrogen, supporting the notion that genistein has an estrogen-like action on bone cells [5]. Ishimi et al. [6] have demonstrated that genistein prevents bone loss caused by estrogen deficiency without exhibiting estrogenic action in the uterus of ovariectomized mice. These findings suggest that genistein might be a useful candidate for the prevention and therapy of postmenopausal osteoporosis. However, the detailed mechanisms of genistein’s effects on bone cells have not been clarified.
Several lines of evidence indicate that protein tyrosine kinases (PTKs) are important for osteoclastic bone resorption. As shown by gene knockout experiments, a disruption of PTK pp60<sup>src</sup> dramatically reduces the activity of osteoclasts [7]. Boyce <i>et al.</i> [8] noted that osteoclasts require pp60<sup>src</sup> for acid secretion which is a key process for bone resorption. In rat osteoclasts, we have shown that the inhibition of the endogenous PTK activity by genistein and herbimycin A causes an increase in intracellular Ca<sup>2+</sup> concentration that serves as an inhibitory signal for bone resorption [9].

Genistein has also been shown to modulate ion channel activity in various cells through mechanisms dependent on [10–12] or independent of [13–16] PTK. Inward rectifier K<sup>+</sup> channels expressed in osteoclasts have been suggested to play important roles in maintaining the activity of bone resorption [17]. Mammalian osteoclasts express an inward rectifier K<sup>+</sup> channel [17] with electrophysiological and pharmacological properties resembling those of the cloned IRK<sub>1</sub> [18], which is also referred to as Kir2.1 [19]. Indeed, mRNA expressing IRK1/Kir2.1 has been detected in rat osteoclasts [20]. We have reported that at physiological concentrations, estrogen acts directly on osteoclast preparations, which were obtained by the following procedure: The preparation of mixed bone cells, obtained as described above, was placed in a 35 mm tissue culture dish filled with the culture medium. The culture dish was mounted on an inverted microscope, and cells with one or two nuclei were removed mechanically from the coverslips by use of a sharp glass rod (tip diameter, 5–10 μm) mounted on a micromanipulator. The remaining cells on the coverslip were multinucleate and stained positive for TRAP. All experiments were done within 8 h after cell isolation.

**Electrophysiological measurements.** The coverslip with adherent cells was placed in a recording chamber (volume 1 ml) mounted on an inverted microscope (TE300; Nikon, Tokyo, Japan) and continuously superfused (1 ml/min) with standard extracellular solution containing (in mM) 134 NaCl, 6 KCl, 10 glucose, 0.5 MgCl<sub>2</sub>, 1.25 CaCl<sub>2</sub>, and 10 N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES), adjusted to pH 7.3 with tris-(hydroxymethyl)-aminomethane (Tris). In some experiments, BaCl<sub>2</sub> was added to the standard extracellular solution to block K<sup>+</sup> currents. The extracellular concentration of K<sup>+</sup> ([K<sup>+</sup>]o) was altered by replacing NaCl with an equimolar concentration of KCl. The osmolarity of these extracellular solutions was adjusted to the value equal to that of the patch pipette solutions with mannitol. The osmolarity of the solution was measured with a freezing-point depression osmometer (Osmometer Automatic, Knauer, Berlin, Germany).

Membrane currents were measured by using the whole-cell configuration of patch-clamp technique [23] and recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Currents were filtered at 1 kHz and digitized at a sampling frequency of 2 to 5 kHz. Membrane currents were displayed in a chart recorder and stored on a computer disk and on videotape through a pulse code modulator. Data acquisition and analysis were performed with pCLAMP 6.0 software (Axon Instruments). All
electrophysiological experiments were performed at room temperature (25 to 27°C).

Patch pipettes were prepared by using a pipette puller (P-97; Sutter Instrument, Novato, CA, USA) and heat-polished by using a microforge (MF83; Narishige Sci. Inst. Lab., Tokyo, Japan). The standard patch pipette solution contained (in mM) 140 KCl, 3 MgCl$_2$, 2 ATP (disodium salt), 0.3 EGTA, and 10 HEPES adjusted to pH 7.25 with Tris. In some experiments, 0.3 mM EGTA in the patch pipette solution was replaced with 1 mM EGTA plus 0.4 mM Ca$^{2+}$ (estimated free Ca$^{2+}$, 87 nM). To elevate [Ca$^{2+}$], 0.3 mM EGTA in the patch pipette solution was replaced with 1 mM EGTA plus 0.8 mM Ca$^{2+}$ (estimated free Ca$^{2+}$, 520 nM). Free Ca$^{2+}$ was calculated by using the computer program MAXC, which is freely available on the Web (http://www.stanford.edu/~cpatton/maxc.html). The osmolarity of these patch pipette solutions was 290 to 305 mOsm. The patch pipette resistance was 2.5 to 5 MΩ. Series resistance compensation (70 to 90%) was used to reduce the error in the potential at which the cell was clamped. The reference electrode was an Ag–AgCl wire connected to the extracellular solution through a 3 m KCl–agar salt bridge. The zero current potential before formation of the gigaseal was regarded as 0 mV.

**Chemicals.** Tyrophostin derivatives (tyrphostin A1, tyrphostin A25, tyrphostin B42, tyrphostin B46), herbimycin A, lavendustin A, and staurosporine were purchased from Calbiochem (La Jolla, CA, USA) and Wako Pure Chemical industries (Osaka, Japan). All other chemicals including genistein, daidzein, and genistin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). These drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentration in the extracellular solution just before each experiment. In some experiments, genistein, herbimycin A, and lavendustin A were added to the standard patch pipette solution. The final DMSO concentration in the solution was less than 0.1% even when a combination of more than two drugs was used. We confirmed that 0.1% DMSO applied extracellularly or intracellularly had no effect on the membrane currents. Guanosine 5′-O-(2-thiotriphosphate) trilithium salt (GDPβS) was added in the standard patch pipette solution for some experiments. Sodium orthovananate was dissolved in the standard extracellular or patch pipette solution. 4,4′-Disothiocyanostilbene-2,2′-disulfonic acid (DIDS), which was used to block Cl$^-$ currents, was dissolved in the extracellular solution.

**Statistical analysis.** Data are expressed as mean values with standard deviations (±SD) from number of cells (n). Statistical differences were analyzed by using Student’s t-test, and p values less than 0.05 were considered to be significant.

### RESULTS

**Inhibitory action of genistein on inward rectifier K$^+$ current**

Figure 1A shows the effect of genistein on whole-cell currents evoked by voltage-step pulses (from −120 to 60 mV in 30 mV increments; holding potential −50 mV). Hyperpolarizing pulses elicited inward current, whereas depolarizing pulses elicited much smaller outward current, typical for inward rectifier K$^+$ channels (I$_{Kir}$) [21]. Application of genistein (50 µM) to the bath for 5 min reduced the amplitude of inward currents evoked by hyperpolarizing pulses, and the currents were restored by the removal of genistein. The initial (at 10 ms) and steady-state (at 300 ms) amplitudes of the inward current evoked by 300 ms hyperpolarizing pulses were proportionally reduced and recovered. In 8 cells studied, the application of genistein (50 µM) for 5 min reduced the amplitude of the inward current at a membrane potential of −120 mV to 50±10% of control. Approximately 50% of osteoclasts studied (n=51) exhibited an outward rectifier Cl$^-$ current at positive membrane potentials. In these cells we made whole-cell recordings in the presence of the Cl$^-$ channel blocker DIDS (0.5 mM) to isolate I$_{Kir}$ [21]. DIDS did not change the magnitude of the inhibition by genistein (data not shown). Figure 1B shows the current/voltage (I/V) relationships obtained before (control) and after the application of genistein (50 µM), using a ramp voltage pulse (from −120 to 50 mV; holding potential −50 mV). A small outward current with its peak at membrane potentials of about −60 mV was observed in the standard extracellular solution (control). The application of genistein diminished this outward current as well as the inward current. The inhibitory effect of genistein on both the inward and outward currents occurred within 1 min after the onset of its application and were partially reversible within a few minutes of its withdrawal. The net current inhibited by genistein (Fig. 1B; inset) showed inward-going rectification with small outward current. The reversal potential of the net current inhibited by genistein was positively shifted by increasing [K$^+$]o (Fig. 1C). The mean reversal potentials were −76±5 (n=10), −24±3 (n=5), and 1±2 mV (n=4) for 6, 50, and 140 mM, respectively. A least-squares fit for the reversal potentials against [K$^+$]o on a semilogarithmic scale had a slope of 56 mV per 10-fold change in [K$^+$]o. We evaluated the inhibitory effects of genistein on the outward and
inward currents on the basis of the \( I/V \) relationships, obtained by subtracting a background current, which could be observed after a blockade of \( I_{\text{Kir}} \) by the application of \( \text{BaCl}_2 \). An application of genistein (50 \( \mu \text{M} \)) for 5 min reduced the amplitude of the outward and inward currents to 48\( \pm \)11 and 51\( \pm \)8\% of control in 5 cells examined, respectively. No significant difference was found in the inhibitory effects of genistein on the outward and inward currents. Therefore the degree of the inhibition of \( I_{\text{Kir}} \) by genistein is voltage-independent and could be evaluated from the effects on the inward current in the subsequent experiments.

The inhibitory effects of genistein on \( I_{\text{Kir}} \) were also observed by using pure osteoclast-preparations, which were made by removing unidentified cells with one or two nuclei from the coverslips (see MATERIALS AND METHODS). In experiments with these pure osteoclast preparations, the amplitude of the inward current at a membrane potential of \(-120\) mV was reduced to 51\( \pm \)11\% (\( n=4 \)) of control after the application of 50 \( \mu \text{M} \) genistein for 5 min. No significant differences were observed between the inhibitory effects of genistein obtained from the preparations of pure osteoclasts and mixed cells. This result indicates that an inhibition of \( I_{\text{Kir}} \) by genistein is not mediated through nonosteoclastic cells, suggesting a direct effect of genistein on osteoclasts.

**Effects of other isoflavones on \( I_{\text{Kir}} \)**

Figure 2 shows the relationship between the concentration of various isoflavones, including genistein and the relative amplitude of \( I_{\text{Kir}} \). In this experiment, no significant decline in the amplitude of \( I_{\text{Kir}} \) was observed within 30 min after the establishment of whole-cell configuration. Genistein inhibited \( I_{\text{Kir}} \) in a concentration-dependent manner. The concentration-response curve obtained 5 min after the application of genistein showed an IC\(_{50}\) value of 54 \( \mu \text{M} \). However, the inhibition of \( I_{\text{Kir}} \) did not reach a steady state within 5 min after the application of genistein. The IC\(_{50}\) value obtained 10 min after the application of genistein was 27 \( \mu \text{M} \). Significant inhibition of \( I_{\text{Kir}} \) was observed with genistein at concentrations higher than 1 \( \mu \text{M} \). An application of daidzein (\( \approx \)50 \( \mu \text{M} \)) for 5 min also inhibited \( I_{\text{Kir}} \), but the inhibition was less than that by genistein. No further inhibition was observed even when the application time was extended to exceed 10 min. On the other hand, genistin hardly affected \( I_{\text{Kir}} \) even during prolonged application (up to 10 min). These findings raise the possibility that genistein inhibits \( I_{\text{Kir}} \) via inhibiting PTK, since both daidzein and genistin,
unlike genistein, lack PTK inhibitory activity \[24\]. Therefore we next examined the possible involvement of PTK in the inhibitory effect of genistein on \(I_{\text{Kir}}\).

**Possible involvement of PTK in the genistein-induced inhibition of \(I_{\text{Kir}}\)**

Figure 3A shows the effects of various PTK inhibitors besides genistein on \(I_{\text{Kir}}\). Tyrphostin B46 and tyrphostin B42 had a similar inhibitory effect on \(I_{\text{Kir}}\) as genistein. A weak but significant inhibition of \(I_{\text{Kir}}\) was observed with tyrphostin A25 at concentrations higher than 50 \(\mu\)M. Tyrphostin A1, a tyrphostin analogue with no effect on PTK activity \[25\], also exhibited an inhibitory effect comparable to that of tyrphostin A25. However, both herbimycin A and lavendustin A failed to inhibit \(I_{\text{Kir}}\), even during prolonged application (up to 30 min). Moreover, no measurable effect was observed, even when a higher concentration (10 to 30 \(\mu\)M) of herbimycin A or lavendustin A was included in the patch pipette solution and applied intracellularly for 30 min (data not shown).

We next examined the effects of orthovanadate, a protein tyrosine phosphatase inhibitor \[26\], on the genistein-induced inhibition of \(I_{\text{Kir}}\). Before the application of genistein, cells were perfused either extracellularly or intracellularly for more than 10 min with orthovanadate (300 \(\mu\)M) or in the patch pipette solution (100 \(\mu\)M), respectively. Figure 3B summarizes the effects of orthovanadate on \(I_{\text{Kir}}\) inhibition by genistein (20 and 50 \(\mu\)M). The rela-
The inhibitory effects of 20 and 50 μM genistein (control) were not significantly affected by extracellular perfusion with 300 μM orthovanadate. Intracellular dialysis with orthovanadate (100 μM) for 10 min also had no effect on the inhibition of \( I_{K_{ir}} \) by 20 and 50 μM genistein. Neither was the recovery of \( I_{K_{ir}} \) from the blockade affected by extracellular or intracellular orthovanadate. Extracellularly or intracellularly applied orthovanadate itself did not significantly change the amplitude of \( I_{K_{ir}} \) (data not shown).

**Effect of intracellularly applied genistein on \( I_{K_{ir}} \)**

Externally applied genistein is likely to penetrate the cell membrane and enter the cell interior. Thus genistein contained in the patch pipette solution was allowed to diffuse directly into the cell. However, no significant change in the amplitude of \( I_{K_{ir}} \) could be detected during dialysis for 15 to 20 min with 50 μM genistein in the patch pipette solution. Since it is very likely that dialysis from the intracellular side is less efficient than direct application from the extracellular side, we dialyzed the cells with higher concentrations of genistein (100 to 200 μM). The amplitude of \( I_{K_{ir}} \), however, remained unchanged for 15 min during the dialysis. After dialysis with 100 to 200 μM genistein, extracellular application of genistein (50 μM) for 5 min still reduced the amplitude of \( I_{K_{ir}} \) (at −120 mV) to 51±7% (n=4) of control. No significant differences were observed between the inhibitory effects of genistein (50 μM) obtained from the dialyzed versus the nondialyzed cells.

**Effects of [Ca\(^{2+}\)], GDPβS, Rp-cAMPS, staurosporine, and okadaic acid on genistein-induced inhibition of \( I_{K_{ir}} \)**

To test whether the inhibitory effects of genistein on \( I_{K_{ir}} \) involve a cytosolic second-messenger pathway, the effects of [Ca\(^{2+}\)], GDPβS, Rp-cAMPS, staurosporine, and okadaic acid on the genistein-induced inhibition of \( I_{K_{ir}} \) were examined and are summarized in Fig. 4. The relative amplitude of \( I_{K_{ir}} \) was obtained 5 min after the application of genistein (50 μM). We recently reported that genistein leads to an elevation of [Ca\(^{2+}\)] in rat osteoclasts [9]. Therefore we examined the inhibitory effects of genistein with [Ca\(^{2+}\)], clamped to the resting [Ca\(^{2+}\)], levels reported in rat osteoclasts [9], using a patch pipette solution containing 1 mM EGTA plus 0.4 mM Ca\(^{2+}\) (estimated free Ca\(^{2+}\), 87 nM). Under these conditions, genistein reduced \( I_{K_{ir}} \) to 62±6% (n=6). This value was not significantly different from the one obtained when the standard patch pipette solution containing 0.3 mM EGTA was used (control; 54±8%, n=10). Moreover, the elevation of [Ca\(^{2+}\)], using patch pipette solution containing 1 mM EGTA plus 0.8 mM Ca\(^{2+}\) (estimated free Ca\(^{2+}\), 520 nM) did not affect the amplitude of \( I_{K_{ir}} \) (data not shown). To examine the involvement of GTP-binding proteins in the genistein-induced inhibition of \( I_{K_{ir}} \), GDPβS (1 mM) was added to the standard patch pipette solution. GDPβS could not prevent the inhibitory effects of genistein on \( I_{K_{ir}} \) (58±5%; n=4). Furthermore, the intracellular application of Rp-cAMPS (200 μM, 52±8%; n=4), an inhibitor of protein kinase A (PKA), and okadaic acid (1 μM, 62±8%; n=4), an inhibitor of protein phosphatase, and the extracellular application of staurosporine (10 nM, 54±7%; n=5), a protein kinase inhibitor, did not significantly change the inhibition of \( I_{K_{ir}} \) by genistein. Extracellularly applied PMA (10 nM), an activator of protein kinase C (PKC), also had no effect on the inhibition of \( I_{K_{ir}} \) by genistein (data not shown). We did not examine the effect of the PKA activator forskolin on the inhibitory action of genistein, since forskolin itself suppressed the inhibitory effect of genistein. However, forskolin itself (estimated free Ca\(^{2+}\), 87 nM) and of 1 mM GDPβS, 200 μM Rp-cAMPS, or 1 μM okadaic acid were contained in the standard patch pipette solution, then allowed to diffuse into the cell for at least 10 min before the application of genistein. Staurosporine (10 nM) was extracellularly applied for at least 10 min before and during the application of genistein. The control (open column) represents the inhibition observed with standard patch pipette solution containing 0.3 mM EGTA. Each column indicates mean±SD. The number of observations is given in parentheses. No significant difference was found in the inhibitory action of genistein on \( I_{K_{ir}} \) between the control and any other column.
has a weak inhibitory effect on $I_{\text{Kir}}$ [21]. The application of these signal modulators did not significantly change the amplitude of $I_{\text{Kir}}$.

**DISCUSSION**

In this study, we show that genistein inhibits inward rectifier K$^+$ channels in rat osteoclasts. The inhibition of $I_{\text{Kir}}$ by genistein was dependent on concentration and application time (Fig. 2). A significant inhibitory effect of genistein could be observed at concentrations above 1 $\mu$M. The plasma concentration of genistein in humans has been reported to reach approximately 2 $\mu$M by the ingestion of a commercially available soybean food containing 37 mg of genistein [16]. An important result of the present study is that physiologically relevant concentrations of genistein inhibited $I_{\text{Kir}}$ in osteoclasts. Therefore it is likely that the genistein action on $I_{\text{Kir}}$ contributes to the bone protective effect of soybean isoflavones.

It has been demonstrated that genistein, a soybean isoflavone, binds to intracellular estrogen receptors and mimics the biological responses of estrogens [3]. Oursler et al. [27] have shown that intracellular estrogen receptors exist in osteoclasts and that estrogens directly regulate osteoclast activity. In our present experiment, the inhibitory effect of genistein on $I_{\text{Kir}}$ occurred within 1 min after application and partially reversed within a few minutes of its removal (Fig. 1). To our knowledge, even the most rapid onset of gene activation by estrogen takes at least 30 min [28]. The rapidity of the genistein effect on $I_{\text{Kir}}$ appears to be inconsistent with the time course of responses mediated by intracellular estrogen receptors. A ligand-binding analysis of estrogen receptors has demonstrated that the concentration of estrogen at half-maximal specific binding is in the nanomolar range [3]. A much higher concentration (100 to 200 $\mu$M) of genistein in the patch pipette was not effective in inhibiting $I_{\text{Kir}}$. Therefore it is unlikely that intracellular estrogen receptors play a role in this inhibition.

Genistein is a broad spectrum PTK inhibitor that inhibits PTK activity by competing with ATP for its binding to PTKs [24]. Studies have reported modulating effects of tyrosine phosphorylation on several ion channels. The IC$_{50}$ values of genistein are 20 to 50 $\mu$M for the $\gamma$-aminobutyric acid type-A (GABA$_A$) receptor channel [11] and the voltage-dependent Ca$^{2+}$ channel [12]. In our present experiments, genistein at similar concentrations decreased $I_{\text{Kir}}$ (Fig. 2), and its IC$_{50}$ value (27 to 54 $\mu$M) is within the concentration range for PTK inhibition [24]. PTK inhibitors targeting the substrate binding site of PTKs (e.g., tyrphostins) have been frequently used to assess the modulating effects of tyrosine phosphorylation on ionic channels. In those studies, concentration ranges of 10 to 100 $\mu$M have been used (for tyrphostin B46 [10]; for tyrphostin B42 [12]). At similar concentrations, tyrphostins were capable of inhibiting $I_{\text{Kir}}$ (Fig. 3A). However, the soybean isoflavone daidzein and the PTK antagonist tyrphostin A1, which have no PTK inhibiting activity, also exhibited a weak inhibitory effect on $I_{\text{Kir}}$ (Figs. 2 and 3A). Furthermore, other PTK inhibitors, herbimycin A and lavendustin A, failed to inhibit $I_{\text{Kir}}$ (Fig. 3A). The lack of an inhibitory action of herbimycin A and lavendustin A may not be due to cellular impermeability of these compounds, since the intracellular application of herbimycin A (10 $\mu$M) or lavendustin A (10 $\mu$M) through the patch pipette solution had no effect on the amplitude of $I_{\text{Kir}}$. It is interesting that Wischmeyer et al. [29] have demonstrated that inward rectifier K$^+$ channels, Kir2.1, themselves are substrates of PTKs, and their activities are negatively controlled by a PTK-dependent phosphorylation. In their experiments, extracellular orthovanadate rapidly inhibited Kir2.1 currents. In our study, however, the inhibitory effects of genistein on $I_{\text{Kir}}$ were not modified by the presence of extracellular or intracellular orthovanadate (Fig. 3B). Therefore, it appears unlikely that tyrosine phosphorylation is involved in the inhibition of $I_{\text{Kir}}$ by genistein.

We recently reported that genistein increases [Ca$^{2+}$], via the activation of a Ca$^{2+}$ influx pathway in rat osteoclasts [9]. Some reports have shown that the activity of inward rectifier K$^+$ channels was reduced by elevated [Ca$^{2+}$] [30]. In our present experiments (Fig. 4), however, genistein-induced inhibition of $I_{\text{Kir}}$ was not affected by clamping the [Ca$^{2+}$], to the resting concentration level, reported in rat osteoclasts. We also observed that the amplitude of $I_{\text{Kir}}$ remained unchanged, even when [Ca$^{2+}$], was elevated to a value (520 nM) higher than that obtained by genistein [9]. These findings suggest that the inhibitory action of genistein on $I_{\text{Kir}}$ is independent of a rise of [Ca$^{2+}$]. A G protein–mediated inhibition of inward rectifier K$^+$ channels has been reported in several cells, including osteoclasts [31, 32]. The activity of inward rectifier K$^+$ channels has also been shown to be modulated by PKA and PKC [33, 34]. As shown in Fig. 4, however, Rp-cAMPS and staurosporine did not prevent the inhibitory effect of genistein on $I_{\text{Kir}}$, and okadaic acid and PMA did not change it, suggesting that the PKA and PKC pathways are not involved. Paillart et al. [14] demonstrated that genistein was able to block voltage-sensitive sodium channels through direct binding to the channel protein. This direct effect of
genistein on ionic channels has also been suggested in L-type Ca\(^{2+}\) channels [15] and delayed K\(^{+}\) channels [13]. Although the inward rectifier K\(^{+}\) channels in rat osteoclasts appear to be directly modulated by genistein, our data presented in this report cannot exclude the possibility of yet unknown intracellular signaling pathways. Therefore our evidence is insufficient to conclude with certainty that genistein acts directly on the channels. Additional experiments will be necessary to determine whether the inhibitory effect of genistein on \(I_{\text{Kir}}\) was mediated by direct action on the channel site or by modulation of some other intracellular signaling pathway.

\(I_{\text{Kir}}\) in osteoclasts has been demonstrated to set the membrane potential near \(E_K\) [17]. The \(I/V\) relationship in a standard extracellular solution shows a small outward current with its peak at around \(-60\, \text{mV}\) (Fig. 1B). This small current, which prevents further depolarization and clamps the membrane potential near \(E_K\), was reduced by genistein (Fig. 1B), resulting in depolarization of the membrane. We have recently shown that 17β-oestradiol inhibits \(I_{\text{Kir}}\) and depolarizes the membrane of rat osteoclasts [21]. Therefore genistein appears to cause membrane depolarization in a manner similar to 17β-oestradiol. Membrane depolarization because of an inhibition of inward rectifier K\(^{+}\) channels would induce [Ca\(^{2+}\)]\(_i\) elevation, since high [K\(^{+}\)]\(_o\)–induced depolarization has been shown to increase [Ca\(^{2+}\)]\(_i\), through a voltage-dependent Ca\(^{2+}\) influx pathway in chicken [35] and rat [36] osteoclasts. Although we have previously reported that in rat osteoclasts genistein activates a Ca\(^{2+}\) influx pathway by its inhibitory action on PTKs, resulting in increased [Ca\(^{2+}\)]\(_i\), that serves as an inhibitory signal for bone resorption [9], it is possible that the membrane depolarization resulting from the inhibition of \(I_{\text{Kir}}\) by genistein indirectly contributes to the genistein-induced increase in [Ca\(^{2+}\)]\(_i\). Furthermore, Sims and Dixon [17] have suggested that the inward rectifier K\(^{+}\) channels in osteoclasts may play an important role in maintaining the H\(^+\) transport to bone surface opposite ruffled border membrane, which is essential for bone mineral resorption. It thus appears that the inhibition of inward rectifier K\(^{+}\) channels by genistein impedes this important role, resulting in decreased H\(^+\) transport. Thus the inhibitory action of genistein on inward rectifier K\(^{+}\) channels in osteoclasts is an important finding, elucidating the mechanism involved in the reduction of osteoclastic bone resorption and the bone protective effect of dietary soybean isoflavones.

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