Original Paper

Evidence for Mitoxantrone-induced Block of Inwardly Rectifying K⁺ Channels Expressed in the Osteoclast Precursor RAW 264.7 Cells Differentiated with Lipopolysaccharide

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Background/Aims: Mitoxanthrone (MX) is an anthracenedione antineoplastic agent. Whether this drug and other related compounds have any effects on ion currents in osteoclasts remains largely unclear. **Methods:** In this study, the effects of MX and other related compounds on inwardly rectifying K⁺ current ($I_{K(IR)}$) were investigated in RAW 264.7 osteoclast precursor cells treated with lipopolysaccharide. **Results:** The $I_{K(IR)}$ in these cells are blocked by BaCl₂ (1 mM). MX (1-100 μM) decreased the amplitude of $I_{K(IR)}$ in a concentration-dependent manner with an IC₅₀ value of 6.4 μM. MX also slowed the time course of $I_{K(IR)}$ inactivation elicited by large hyperpolarization. Doxorubicin (10 μM), 17β-estradiol (10 μM) and tertiapin (1 μM) decreased the $I_{K(IR)}$ amplitude in these cells. In bafilomycin A₁-treated cells, MX-mediated block of $I_{K(IR)}$ still existed. In cell-attached configuration, when the electrode was filled with MX (10 μM), the activity of inwardly rectifying K⁺ (Kir) channels was decreased with no change in single-channel conductance. MX-mediated reduction of channel activity is accompanied by a shortening of mean open time. Under current-clamp conditions, addition of MX resulted in membrane depolarization. Therefore, MX can interact with the Kir channels to decrease the $I_{K(IR)}$ amplitude and to depolarize the membrane in these cells. **Conclusion:** The block by this drug of Kir2.1 channels appears to be one of the important mechanisms underlying its actions on the resorptive activity of osteoclasts, if similar results occur *in vivo*. Targeting at Kir channels may be clinically useful as an adjunctive regimen to anti-cancer drugs (e.g., MX or doxorubicin) in influencing the resorptive activity of osteoclasts.

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Introduction

The Kir2.1 channel, a member of the inwardly rectifying K⁺ channel (Kir) family, is recognized to show strong inward rectification and to function as a stabilizer of the resting potential in many types of cells including neurons, heart cells, smooth muscle cells and osteoclasts [1-3]. Mutations in the *KCNJ2* gene (chromosome 17q23), which encodes the α -subunit of Kir2.1 channel, have been demonstrated to cause developmental defects in bony structures which is one of clinical manifestations in Andersen syndrome [2]. Notably, the knockdown of Kir channels was recently demonstrated to suppress tumorigenesis [4]. It is thus tempting to speculate that the activity of Kir channels contributes to the functional activities in different types of cells *in vivo*.

Mitoxantrone (MX), a synthetic anthracenedione, is an established, antineoplastic agent. It can intercalate into DNA, thereby inhibiting the topoisomerase II enzyme and preventing the ligation of DNA strands and delaying cell-cycle progression. Its therapeutic efficacy has been reported in numerous malignancies, including advanced prostate or breast cancers with osseous metastasis [5-7]. Despite its ability to disrupt DNA synthesis and repair, this drug was demonstrated to exert some effects on ion currents and membrane potential in heart cells [8]. It thus prompts us to propose that this line of drugs has any effects on membrane-mediated ionic conductance in other types of cells including neoplastic cells [9]. However, little information with regard to the effects of MX and other structurally related compounds on ion currents in osteoblasts or osteoclasts is available.

RAW 264.7 cells, an Abelson leukemia virus-transformed cell line, are known to possess the characteristics of monocytes [10, 11]. Specially, because RAW 264.7 cells act as osteoclast progenitors and can differentiate into osteoclasts in response to either lipopolysaccharide (LPS) or receptor activator of nuclear factor κ B ligand [10, 12-15], they have attracted growing interest as a suitable model for studies of functional activities in osteoclasts. Several reports have described the electrical properties of these cells [3, 11, 16-20]. To our knowledge, how MX and other structurally related compounds interact with ion currents in these cells is still not fully understood.

Therefore, in this study, we attempted to characterize electric and pharmacological properties of ion currents, to examine the effects of MX on ion currents in LPS-treated RAW 264.7 cells and to address the issue of how this drug has any effects on ion currents, particularly on inwardly rectifying K⁺ current ($I_{K(IR)}$), in these cells. Our results revealed that MX can interact directly with Kir channels to reduce the amplitude of $I_{K(IR)}$ via a manner not limited to the inhibition of topoisomerase II activity.

Materials and Methods

Drugs and solutions

Mitoxantrone (MX; 1,4-Dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione dihydrochloride; C₂₂H₂₈N₄O₆·2HCl; novantrone®) was obtained from Enzo Life Sci. (Plymouth Meeting, PA). Bafilomycin A₁, doxorubicin (adriamycin®), lipopolysaccharide (LPS), spermine, tertiapin and tetraethylammonium chloride were obtained from Sigma-Aldrich Chemical (St. Louis, MO), and nonactin was from Tocris Cookson (Bristol, UK). Charybdotoxin and apamin were purchased from Alomone (Jerusalem, Israel). Tissue culture media, fetal bovine serum, L-glutamine, penicillin-streptomycin, fungizone, and trypsin were obtained from Invitrogen (Carlsbad, CA). All other chemicals, including BaCl₂ and CsCl, were commercially available and of reagent grade. Reagent water used in this study was deionized using a Milli-Q water purification system (Millipore, Bedford, MA).

The composition of the bath solution (i.e., normal Tyrode's solution) was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5 and Hepes-NaOH buffer 5.5 (pH 7.4). To record membrane potential or K⁺ current, a patch pipette was filled with the solution (in mM): K-aspartate 130, KCl 20, KH₂PO₄ 1, MgCl₂ 1, EGTA 0.1, Na₂ATP 3, Na₂GTP 0.1 and Hepes-KOH buffer 5 (pH 7.2). To preclude the possible contamination of Cl⁻ currents, Cl⁻ ions inside the pipette solution was replaced with aspartate. In some

experiments, EGTA (0.1 mM) in the pipette solution was replaced with 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA; 10 mM) to chelate intracellular Ca^{2+} . To measure voltage-gated Ca^{2+} currents, K^+ ions inside the pipette solution were replaced with equimolar Cs^+ ions, and the pH was adjusted to 7.2 with CsOH.

Cell preparations

RAW 264.7 cells were originally obtained from the American Type Culture Collection (TIB-71; Manassas, VA). Cells were routinely grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin. They were maintained in a 95% air and 5% CO_2 humidified atmosphere at 37 °C [10-13, 21]. The clonal strain MG-63 cell line, an osteoblast-like cell line, was also obtained from American Type Culture Collection (CRL-14271). MG-63 cells were maintained in minimum essential medium Eagle supplemented with 2 mM L-glutamine and Earle's balanced salt solution containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, and 10% fetal bovine serum [22]. Cell viability was evaluated using a WST-1 assay and an ELISA reader (Dynatech, Chantilly, VA).

To observe cell growth, a Nikon Eclipse Ti-E inverted microscope (Li Trading, Taipei, Taiwan) equipped with a 5-megapixel cooled digital camera was used. The digital camera was connected to a personal computer controlled by NIS-Elements BR3.0 software (Nikon, Kanagawa, Japan). To induce differentiation, RAW 264.7 cells were treated with lipopolysaccharide (0.5 $\mu\text{g}/\text{ml}$) for 24 hours [10-12]. In a separate series of experiments, RAW 264.7 cells after treatment with LPS for 1 day were further incubated with bafilomycin A_1 (200 nM) at 37 °C for 5 hours.

Electrophysiological measurements

Shortly before the experiments, cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of an inverted fluorescent microscope (CKX-41; Olympus, Tokyo, Japan). Cells were bathed at room temperature (20-25 °C) in normal Tyrode's solution which contained 1.8 mM CaCl_2 . The patch electrodes were made of Kimax-51 glass capillaries (Kimble; Vineland, NJ) using a PP-830 electrode puller (Narishige, Tokyo, Japan) or a P-97 Flaming/Brown micropipette puller (Sutter, Novato, CA), and their tips were then fire-polished with an MF-83 microforge (Narishige). The pipettes used had a resistance of 3-5 M Ω when filled with different intracellular solutions described above. Patch-clamp recordings were made in standard patch-clamp technique using an RK-400 (Bio-Logic, Claix, France) or an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) [11, 22]. The offset potential between the pipette and bath solution was compensated with amplifier after the pipette entered the bath but immediately before a seal was made.

Data recordings

The signals consisting of voltage and current tracings were stored online in a TravelMate-6253 laptop computer (Acer, Taipei, Taiwan) at 10 kHz through Digidata-1322A interface (Molecular Devices). The latter device was equipped with an Adaptec SlimSCSI card (Milpitas, CA) and then controlled by pCLAMP 9.2 (Molecular Devices). Current signals were low-pass filtered at 1 or 3 kHz. The pCLAMP-generated profiles for either square or ramp pulses were used to evaluate the current-voltage (*I-V*) relationships for ion currents (e.g., $I_{\text{K(IR)}}$). For further analyses, the data were exported to Origin 8.0 (OriginLab, Northampton, MA) or a spreadsheet in Microsoft Excel (Redmond, WA). The inactivation time constants of $I_{\text{K(IR)}}$ in response to step hyperpolarizations with a duration of 1 sec were measured by fitting an one-exponential function.

Data analyses

To calculate the concentration-dependent inhibition of MX on the amplitude of $I_{\text{K(IR)}}$, cells were bathed in normal Tyrode's solution and the ramp pulses from -120 to +40 mV with a duration of 1 sec at a rate of 0.05 Hz were applied. The $I_{\text{K(IR)}}$ amplitude at the level of -100 mV during cell exposure to different concentrations (1-100 μM) of MX was compared with the control value. The concentration-dependent relation of MX on the inhibition of $I_{\text{K(IR)}}$ was fitted using a modified form of the Hill equation. That is, where [MX] represents the MX concentration; IC_{50} and n_{H} are the concentration required for a 50% inhibition and Hill coefficient, respectively; *A* and *a* are the maximal fraction of total current which is MX sensitive and insensitive, respectively.

$$\frac{I_{MX}}{I_{control}} = A \times \left(\frac{[MX]^{n_H}}{IC_{50}^{n_H} + [MX]^{n_H}} \right) + a,$$

Single-channel currents recorded from RAW 264.7 or MG-63 cells were analyzed using pClamp 9.2 (Molecular Devices). Single-channel amplitudes were commonly determined by fitting Gaussian distributions to the amplitude histograms of the closed and the open state. Channel activity was defined as $N \cdot P_o$, a product of channel number (N) and open probability (P_o). Single-channel conductance of Kir channels was calculated by a linear regression using mean values of current amplitudes measured at different potentials. Open lifetime distributions in the absence and presence of MX were fitted with logarithmically scaled bin width. Fits reported here were commonly made with nonlinear, least-squares, curve-fitting algorithm.

The data of macroscopic or single-channel currents are presented as means $\pm$ standard error of the mean (SEM) with sample sizes (n) indicating the number of cells examined. The paired or unpaired Student's t test and one-way analyses of variance with a least-significance difference method for multiple-group comparisons were used for the statistical evaluation of difference among means. Statistical analyses were generally performed using either SPSS 16.2 (SPSS Inc., Chicago, IL) or R programming language [23]. Differences were considered statistically significant when $P < 0.05$ or $P < 0.01$.

Results

Electrical properties of inwardly rectifying K^+ current ($I_{K(IR)}$) in LPS-treated RAW 264.7 cells

In the initial set of experiments, a whole-cell configuration was used to characterize the electrical properties of macroscopic $I_{K(IR)}$ in these cells. Cells were bathed in normal Tyrode's solution which contained 1.8 mM $CaCl_2$, 5.4 mM KCl and 0.53 mM $MgCl_2$, and the recording pipette was filled with a K^+ -containing pipette solution, as described in Materials and Methods. When the cell was held at -50 mV, the ramp pulses from -120 to +40 mV with a duration of 1 sec were then delivered. As shown in Fig. 1A, the I - V relationship for these currents was found to exhibit a strong inward rectification and identified as an $I_{K(IR)}$. When cells were exposed to normal Tyrode's solution, the rectification started at around -62 mV and became progressively increased at more negative potentials. It is noted that as the extracellular K^+ was elevated, the amplitude of $I_{K(IR)}$ was drastically increased. For example, when the concentration of extracellular K^+ was raised from 5.4 to 10 mM, current amplitude measured at the level of -80 mV significantly increased from 31 ± 5 to 148 ± 11 pA ($n = 9$). The slope of the linear fit of current amplitude to voltages between -80 and -60 mV was elevated to 6.1 ± 1.9 nS from a control value of 1.9 ± 0.5 nS ($n = 9$). Furthermore, the reversal potential at the level of zero current shifted positively following the K^+ equilibrium potential (E_K) as extracellular K^+ was increased (Fig. 1). As shown in Fig. 1B, the line of best fit through the mean values of reversal potentials revealed a slope of 52.7 mV per tenfold increase in extracellular K^+ . Consistent with these observations, application of nonactin (10 μ M), a K^+ ionophore, increased $I_{K(IR)}$ amplitude in these cells (data not shown). The results thus indicate that in these cells, these currents with strong inward rectification in response to the ramp pulses are selective for K^+ ions and that they can shift its reversal potential with extracellular K^+ in a manner similar to those previously described for the Kir channels [24-26].

We next examined the effect of $BaCl_2$ on $I_{K(IR)}$ in LPS-treated RAW 264.7 cells. As shown in Fig. 2, when the cell was depolarized from -50 to a series of voltage steps ranging from -130 to +90 mV in 10-mV increments, a family of ion currents with a strongly inwardly-rectifying property was elicited [3, 20]. Notably, within 1 min of exposing the cells to 1 mM $BaCl_2$, current amplitudes were greatly reduced particularly at the voltages ranging between -130 and -70 mV. This inhibitory effect was readily reversed after removal of $BaCl_2$. Figure 2B illustrates the averaged I - V relationship for $I_{K(IR)}$ obtained in the control and following the exposure to $BaCl_2$ (1 mM). The results clearly showed that the $I_{K(IR)}$ recorded from these cells is sensitive to block by $BaCl_2$.

Fig. 1. Effect of different concentrations of extracellular K⁺ on $I_{K(IR)}$ recorded from LPS-treated RAW 264.7 cells. In these experiments, cells were bathed in normal Tyrode's solution and the recording pipette was filled with a K⁺-containing solution. The ramp pulses from -120 to +40 mV with a duration of 1 sec were applied to the cell. (A) Original current traces obtained when cells were exposed to different concentrations of extracellular K⁺: a: 5.4 mM extracellular K⁺; b: 10 mM extracellular K⁺; and c: 20 mM extracellular K⁺. In (B), the reversal potentials measured as zero-current membrane potentials were plotted as a function of different extracellular K⁺. Note that abscissa is on logarithmic scale. The blue line indicates the best-fit curve with a slope of 52.7 mV for 10-fold change in extracellular K⁺. Each point indicates the mean $\pm$ SEM (n = 5–8).

Fig. 2. Effect of BaCl₂ on $I_{K(IR)}$ in LPS-treated RAW 264.7 cells. In these experiments, each cell examined was held at -50 mV and the different potentials ranging from -130 to +90 mV in 10-mV increments were applied. (A) Superimposed current traces obtained in the absence (left panel) and presence (right panel) of 1 mM BaCl₂. (B) Averaged I - V relationships of $I_{K(IR)}$ obtained with or without application of BaCl₂ (mean $\pm$ SEM; n = 7–10 for each point). Current amplitude was measured at the end of the voltage pulses (-130 mV). $\square$: control; $\circ$: in the presence of 1 mM BaCl₂.

Effect of MX on $I_{K(IR)}$ in LPS-treated RAW 264.7 cells

In the next series of experiments, we investigated whether MX has any effects on macroscopic $I_{K(IR)}$ in these cells. As shown in Fig. 3A, when the cell was held at -50 mV and ramp pulses from -120 to +40 mV were applied, the I - V relationship for these currents displayed a strong inward rectification. When cells were exposed to MX, current amplitude at hyperpolarized potentials was progressively diminished. For example, when the ramp

Fig. 3. Concentration-response relationship for MX-induced inhibition of $I_{K(IR)}$ in LPS-treated RAW 264.7 cells. In these experiments, each cell was held at -50 mV and the ramp pulses from -120 to +40 mV with a duration of 1 sec were applied to the cell. The $I_{K(IR)}$ amplitude at the level of -100 mV during cell exposure to different concentrations of MX was measured. (A) Original current traces showing the amplitude of I_{MEP} in the absence and presence of MX. Current trace labeled a is control, and those labeled b, c and d were obtained in the presence of 3, 10 and 30 μ M MX respectively. (B) Concentration-response curve for MX-induced inhibition of $I_{K(IR)}$ (mean $\pm$ SEM; $n=8-14$ for each point) MX at various concentrations (1 μ M-100 μ M) was added to the bath. The red smooth lines represent the best fit to the modified Hill equation as described under Materials and Methods. The IC_{50} value, maximally inhibited percentage of $I_{K(IR)}$, and Hill coefficient for MX-induced inhibition of $I_{K(IR)}$ were 6.4 μ M, 95% ($A=0.95$), and 1.3, respectively.

pulses from -120 to +40 mV with a duration of 1 sec were applied, MX (10 μ M) significantly decreased current amplitude at the level of -100 mV from 78 ± 7 to 41 ± 4 pA ($n=8$). At the same voltage profile, the slope conductance at the voltage ranging from 100 to -80 mV was reduced to 1.2 ± 0.4 nS from a control value of 2.6 ± 0.8 nS ($n=8$), when cells were exposed to MX. This inhibitory effect was readily reversed when MX was washed out. The relationship between the MX concentration and the relative amplitude of $I_{K(IR)}$ was then constructed and plotted (Fig. 3B). The half-maximal concentration required for its inhibition of $I_{K(IR)}$ was calculated to be 6.4 ± 0.5 μ M ($n=13$). Therefore, results from these observations reflect that, in LPS-treated RAW 264.7 cells, MX has a depressant action on the amplitude of $I_{K(IR)}$.

MX-induced slowing in the time course of $I_{K(IR)}$ inactivation

Hyperpolarizing voltage pulses were noted to induce a time-dependent inactivation of K^+ inward currents. These inward currents decayed at potential above -100 mV in an exponential fashion and became faster with greater hyperpolarizations (Fig. 2). Therefore, we further evaluated whether MX can produce any effects on the time course of $I_{K(IR)}$ inactivation in LPS-treated RAW 264.7 cells. As shown in Fig. 4, raising MX concentration produced a significant reduction in the rate of $I_{K(IR)}$ inactivation in response to large hyperpolarization. For example, when the cell was hyperpolarized from -50 to -130 mV with a duration of 1 sec, the time constant of $I_{K(IR)}$ inactivation during cell exposure to 10 μ M MX was significantly increased to 253 ± 12 msec ($n=7$) from a control value of 208 ± 8 msec ($n=10$). Therefore, the results allowed us to indicate that in addition to the decreased current amplitude, the presence of MX slowed the time course of $I_{K(IR)}$ inactivation elicited by large hyperpolarization.

Comparison between the effects of increased extracellular calcium, 17 β -estradiol, tetraethylammonium chloride, doxorubicin, and tertiapin

Effects of other relevant compounds on the amplitude of $I_{K(IR)}$ in RAW 264.7 cells were examined and compared. As shown in Fig. 5, when extracellular Ca^{2+} was raised to 3.6 mM or cell exposure to 10 μ M 17 β -estradiol, the $I_{K(IR)}$ amplitude was diminished by approximately

Fig. 4. Effect of MX on the time course of $I_{K(IR)}$ inactivation in LPS-treated RAW 264.7 cells. In these experiments, the cell was hyperpolarized from -50 to -130 mV with a duration of 1 sec. (A) a: control; b: 10 mM MX. Blue smooth curves in a and b show the single-exponential fits of $I_{K(IR)}$ trajectories with inactivation time constants of 208 and 253 msec, respectively. (B) Summary of the data showing effect of MX (3 and 10 μ M) on inactivation time constant of $I_{K(IR)}$ (mean $\pm$ SEM; n=7 for each bar). *Significantly different from control ($P < 0.05$).

35%. Similarly, either tetraethylammonium chloride (10 mM) or doxorubicin (10 μ M) was capable of producing a reduction of these currents. Similar to MX in the chemical structure, doxorubicin is another anthracycline compound and recognized as a DNA topoisomerase II poison. Moreover, tertiapin (1 μ M) was noted to have an inhibitory effect on $I_{K(IR)}$ in LPS-treated RAW 264.7 cells. Tertiapin, a bee venom peptide, has been described as an inhibitor of acetylcholine-activated K^+ current and inwardly rectifying K^+ current in heart cells [27]. However, neither charybdotoxin (200 nM) nor apamin (200 nM) produced any effects on $I_{K(IR)}$ in these cells (data not shown).

Effect of MX on $I_{K(IR)}$ in LPS-treated RAW 264.7 cells which were further preincubated with bafilomycin A_1

It has been shown that changes in endosomal pH can alter MX-mediated cytotoxicity [28]. Proton currents which are subject to inhibition by bafilomycin A_1 , were reported in differentiated RAW 264.7 cells [19]. To this aim, the effect of MX on $I_{K(IR)}$ was further evaluated in cells treated with bafilomycin A_1 for 5 hours. However, unexpectedly, in these RAW 264.7 cells preincubated with bafilomycin A_1 for 5 hours, the inhibitory effect of MX on the I - V relationship of $I_{K(IR)}$ was little altered (Fig. 6). For example, in cells pretreated with bafilomycin (200 nM), MX (10 μ M) significantly increased the $I_{K(IR)}$ amplitude at the level of -100 mV from 119 ± 15 to 45 ± 7 pA (n = 8). There was no significant difference in the inhibitory effect of MX on $I_{K(IR)}$ between control cells and cells treated with bafilomycin A_1 . Therefore, the results led us to suggest that the ability of MX to inhibit $I_{K(IR)}$ observed in LPS-differentiated RAW 264.7 cells is not necessarily associated with a mechanism linked to changes in proton currents.

Electric properties of MX-induced inhibition of inwardly rectifying K^+ (K_{ir}) channels recorded from LPS-treated RAW 264.7 cells

The MX-induced inhibition of $I_{K(IR)}$ described above could be due to either the decreased open probability, a reduction in the number of functional active channels, or changes in the channel gating. The effects of this compound on the activity of K_{ir} channels were further investigated in these cells. In these experiments, RAW 264.7 cells were bathed in normal

Fig. 5. Inhibitory effects of increased extracellular Ca^{2+} , β -estradiol (10 μM), doxorubicin (10 μM), tetraethylammonium chloride (10 mM) and tertiapin (1 μM) on the amplitude of $I_{\text{K(IR)}}$ in LPS-treated RAW 264.7 cells (mean $\pm$ SEM; $n=6-10$ for each bar). In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl_2 . The hyperpolarizing pulse from -50 to -120 mV with a duration of 300 msec was applied to each cell examined. Current amplitude measured at the end of voltage pulses in the control was considered to be 1.0 and the relative amplitude of $I_{\text{K(IR)}}$ after application of each agent was compared and plotted. Extracellular Ca^{2+} : increasing extracellular Ca^{2+} to 3.6 mM; β -estradiol: 10 μM 17 β -estradiol; TEA: 10 mM tetraethylammonium chloride; DOXO: 10 μM doxorubicin; and tertiapin: 1 μM tertiapin. *Significantly different from control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

Fig. 6. Effect of MX on the averaged I - V relations of $I_{\text{K(IR)}}$ in RAW 264.7 cells treated with bafilomycin A_1 . LPS-treated RAW 264.7 cells were further preincubated with bafilomycin A_1 (200 nM) for 5 hours. In each cell examined, $I_{\text{K(IR)}}$ was elicited from -50 mV to different potentials ranging from -120 to 0 mV with 10-mV increments. Each point represents the mean $\pm$ SEM ($n=5-8$). $\square$: control; $\circ$: in the presence of MX (10 μM). Notably, the presence of MX remains effective at suppressing $I_{\text{K(IR)}}$ in these cells at hyperpolarized potentials.

Tyrode's solution, and single-channel recordings were performed. The recording pipette was filled with a K^+ -containing solution in the absence or presence of 10 μM MX. In cell-attached patches from the recording pipette filled without MX, the activity of Kir channels was clearly observed. It was noted that when the pipette was filled with 10 μM MX, there was a drastic decrease in Kir-channel activity at the same level (Fig. 7). A representative example of Kir-channel activity in the absence and presence of MX is illustrated in Fig. 7A. The probability of channel openings measured in the absence of MX (0.127 ± 0.011 , $n = 8$) was significantly greater than that during exposure to MX (0.015 ± 0.006 , $n = 7$). However, single-channel amplitude at the level of -50 mV between the absence and presence of MX did not differ significantly (2.55 ± 0.09 pA [in the absence of MX], $n = 8$ versus 2.52 ± 0.09 pA [in the presence of MX], $n = 7$) (Fig. 7B). Figure 7C illustrates an ohmic I - V relationship of Kir channels between the absence and presence of MX in LPS-treated RAW 264.7 cells. The values of single-channel conductance for these channels between the absence and presence of 10 μM MX are virtually indistinguishable (25.5 ± 1.1 pS [in the absence of MX], $n=7$ versus 20.3 ± 1.0 pS [in the presence of MX], $n=7$). The results indicate that this compound decreases the activity of Kir channels in LPS-treated RAW 264.7 cells; however, no modification in single-channel conductance was observed.

Another important finding is that, in the inside-out configuration, when MX (10 μM) was applied to the intracellular surface of the detach patch, the activity of Kir channels remained

Fig. 7. Inhibitory effect of MX on the activity of Kir channels in LPS-treated RAW 264.7 cells. The experiments were conducted in cells bathed in normal Tyrode's solution containing 1.8 mM CaCl_2 . Cell-attached configuration was made and the recording pipette was filled with or without MX (10 μM). The attached cell was held at -50 mV relative to the bath. (A) Original current traces obtained in the absence (left panel) and presence (right panel) of 10 μM MX. Channel opening gives an upward deflection in current. (B) Amplitude histograms measured in the absence (left panel) and presence (right panel) of 10 μM MX. Notably, the amplitude of single-channel current did not differ between the absence and presence of MX. (C) Averaged I - V relationships of single Kir-channel currents obtained in the control ($\square$) and after application of 10 μM MX ($\blacksquare$). Single-channel amplitude was plotted as a function of potential (i.e., $\Delta\text{voltage}$). The voltage is given as the difference between the resting potential. The value of K^+ equilibrium potential (E_{K}) was shifted in the depolarizing direction due to a high K^+ in the pipette (145 mM). Since the patch potential is the sum of the resting potential plus the pipette potential, these currents reverse at approximately +69 mV. Each point represents the mean $\pm$ SEM ($n=9-13$). Notably, no significant modification in single-channel conductance was demonstrated during exposure to MX.

unaltered, although spermine (50 nM) effectively suppressed channel activity (data not shown). Therefore, it is possible that the α -subunit of the Kir2.1 channel, which is encoded by the *KCNJ2* gene, is functionally expressed in RAW 264.7 cells [29]. MX induces block of Kir channels possibly by acting at a site that is accessible from the extracellular leaflet of the channel.

Effect of MX on kinetic behavior of Kir channels in LPS-treated RAW 264.7 cells

The MX effect on the kinetic behavior of Kir channels was examined and analyzed during recordings from patches showing only single-channel openings. As shown in Fig. 8, in a cell-

Fig. 8. Mean open time of Kir channels in the absence and presence of MX recorded from LPS-treated RAW 264.7 cells. Open-time histograms obtained in the absence (upper panel) and presence (lower panel) of 10 μ M MX are illustrated. The abscissa and ordinate indicate the logarithm of open time (msec) and the square root of the number of events ($\sqrt{n}$), respectively. The smooth line (blue) shown in each lifetime distribution was well fitted by two-exponential function, and the dashed line is placed at the value of the time constant in the open state. Notably, as the cell was exposed to MX, there was a decrease in the slow component of mean open times.

attached patch of control cell (i.e., in the absence of MX), open-time histogram of Kir channels at the level of -50 mV can be well fitted by a two-exponential curve. The time constants for the fast and slow components of open-time histogram were 4.6 ± 0.9 and 32 ± 5 msec ($n = 8$), respectively. MX (10 μ M) significantly decreased the slow time constant of the open state to 12 ± 2 msec ($n = 8$). Conversely, no significant difference in the fast time constant between the absence and presence of MT was demonstrated (4.6 ± 0.9 msec [in the absence of MX] versus 4.4 ± 0.8 msec [in the presence of MX], $n = 8$). Moreover, we did not find the difference in kinetic behavior of Kir channels in RAW 264.7 cells treated with and without LPS. Therefore, the inhibitory effect of MX on the activity of Kir channels can be due to a shortening in the slow component of the open times, despite no notable change in single-channel amplitude. The binding of this compound to the membrane at the extracellular surface is capable of allowing the Kir channel to spend less time in the open state.

Effect of MX on membrane potential of LPS-treated RAW 264.7 cells

The final series of experiments were designed to test the effect of MX on changes in membrane potential in RAW 264.7 cells. Current-clamp condition was made in these experiments and cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl_2 . The typical effects of MX on membrane potential in these cells are illustrated in Fig. 9. The frequency histograms of membrane potentials derived from RAW 264.7 cells were noted to display two distinct groups (Fig. 9B) [18]. In control cells, the histogram of membrane potential comprised two broad distributions; one falls within a distribution ranging between -56 and -53 mV with a mean value of -54.4 ± 1.1 mV ($n = 8$), and the other is between -54 and -50 mV with a mean value of -52.3 ± 1.3 mV ($n = 8$). Notably, during cell exposure to 10 μ M MX, the resting potential was shifted and became less negative, along with changes in the frequency histogram of membrane potential. One is a distribution between -51 and -49 mV with a mean value of -49.5 ± 1.0 mV ($n = 7$), while the other falls within a range between -50 and -46 mV with a mean value of -48.3 ± 0.9 mV ($n = 7$). A further application of nonactin (10 μ M) reversed MX-induced membrane depolarization. The results indicate that application of MX can depolarize resting potential as well as alter the frequency histogram of membrane

Fig. 9. Effect of MX on the resting potential in LPS-treated RAW 264.7 cells. In these experiments, cells were bathed in normal Tyrode's solution, the patch pipette was filled with K⁺-containing solution and changes in the resting potential were measured under a current-clamp mode. (A) Original potential trace obtained in the absence (upper panel) and presence (lower panel) of 10 μM MX. (B) Frequency histograms obtained in the control (upper panel) and during exposure to 10 μM MX (lower panel). The blue and red smooth lines shown in (B) indicate the fitted Gaussian function. Notably, during cell exposure to MX, the resting potential becomes less negative.

potential recorded from LPS-treated RAW 264.7 cells. As the $I_{K(IR)}$ is involved in maintaining the resting potential in many cell types like osteoclasts [2, 16, 24, 25], MX-induced changes in membrane potential can be in great part explained by its inhibition of Kir channels.

Discussion

The major findings of this study are as follows. First, MX, an anthracenedione anti-neoplastic agent, was effective at decreasing the amplitude of $I_{K(IR)}$ in LPS-treated RAW 264.7 cells. Second, the presence of MX decreased the rate and extent of $I_{K(IR)}$ inactivation. Third, the $I_{K(IR)}$ of RAW 264.7 cells is blocked not only by MX, but also by elevated extracellular Ca²⁺, 17β-estradiol, or doxorubicin. Fourth, MX-induced inhibition of Kir channels was due to the shortening of mean open time, but not to changes in single-channel conductance. Fifth, application of MX caused membrane depolarization in these cells. Therefore, it is anticipated from these data that the inhibitory effect of MX on Kir channels is an important mechanism through which MX and its structurally related compounds interfere with the resorptive activity of osteoclasts, if similar findings occur *in vivo*.

The IC₅₀ value required for MX-mediated inhibition of $I_{K(IR)}$ is about 6.4 μM. The effect of MX shown here is expected to occur at a concentration achievable in humans because of a higher tissue affinity [30]. When mesoporous silica nanoparticles or the polyethylene glycol-modified liposomes which are loaded with MX or doxorubicin are delivered to the patients [31-33], a significant fraction of liposomes can be accumulated within the osseous tissues and their concentrations around the microenvironment of osteoclasts even become higher.

Therefore, there would be a link between the effect of MX on osteoclasts and its inhibitory action on Kir-channel activity.

MX-mediated reduction of Kir-channel activity may arise from the different types of kinetic behavior. A lack of its effect on single-channel conductance indicates that the site of action within the central pore cavity is unlikely. However, this drug reduces channel activity most likely by gaining access to its binding on channel gating, because in its presence, the mean open time of the channel was significantly shortened. Our results can thus point to this drug as a Kir channel blocker characterized by a greater affinity for the channel in the open state. Moreover, in our study, application of MX into the intracellular face of the channel was not found to have any effects on the activity of Kir channels. Therefore, these findings make it unlikely that the inhibitory effects of MX on $I_{K(IR)}$ in RAW 264.7 cells result from a direct action on PIP2-channel interaction as described in heart cells [34]. On the other hand, because Kir2.1 channels can be functionally coupled to G protein(s) [35], whether MX and other structurally related compounds interact directly with G protein(s) to alter Kir-channel activity is necessarily explored.

Increasing extracellular Ca^{2+} was found to decrease the amplitude of $I_{K(IR)}$ in RAW 264.7 cells. One would expect that the inhibition by extracellular Ca^{2+} of this current could be associated with the level of intracellular Ca^{2+} . However, when the recording pipette was filled with a high concentration (10 mM) of BAPTA, the $I_{K(IR)}$ amplitude measured under whole-cell mode was also suppressed by elevation of extracellular Ca^{2+} . Extracellular Ca^{2+} -mediated inhibition of $I_{K(IR)}$ is thus unrelated to changes in the level of intracellular Ca^{2+} and can be due to the ability of extracellular Ca^{2+} to reduce the affinity of K^+ ions to the binding to a site within the Kir channel. The results could notably have an important impact, because components of the plasma membrane in osteoclasts *in vivo* are and dynamically exposed to a microenvironment enriched in high concentrations of Ca^{2+} ions [15, 36].

Unlike MG-63 osteoblast-like cells [22], RAW 264.7 cells with or without treatment of LPS were not found to display voltage-gated Ca^{2+} currents. The Kir channel activity was not functionally expressed in MG-63 osteoblast-like cells; however, we did find the presence of Kir channels in BV-2 microglial cells (data not shown). Therefore, in LPS-treated RAW 264.7 cells, MX-mediated inhibition of $I_{K(IR)}$ can result in membrane depolarization, which decreases in the inwardly directed driving force for Ca^{2+} and subsequently diminishes the amplitude of Ca^{2+} -activated K^+ currents [17]. It is anticipated that such positive feedback of membrane depolarization on Kir channels in LPS-treated RAW 264.7 cells is unique and provides an ideal tool for fine tuning of Kir-channel activity. It also needs to be noted that increased K^+ efflux through Ca^{2+} -activated K^+ channels in osteoblasts embedded in the osseous lacunae [22] can be another intriguing mechanism of increased $I_{K(IR)}$ amplitude in osteoclasts under the extracellular milieu, as increased extracellular K^+ enhanced the $I_{K(IR)}$ amplitude [25, 26].

Earlier work has demonstrated that the activity of Kir channels in osteoclasts play a role in maintenance of the H^+ transport to bone surface opposite to ruffled border membrane, which is essential for bone mineral resorption [1]. A previous report revealed the presence of plasmalemmal V-ATPase in RAW 264.7 cells [19]. Moreover, elevating endosomal pH was found to decrease sequestration in endosomes of anticancer drugs such as doxorubicin and MX, thereby increasing cytotoxicity of these drugs [28]. However, in our study, the $I_{K(IR)}$ present in RAW 264.7 cells was not sensitive to block by bafilomycin A_1 , although MX or doxorubicin had inhibitory effect on it. Bafilomycin A_1 is recognized as a blocker of proton currents [19]. On the other hand, in bafilomycin A_1 -preincubated RAW 264.7 cells, MX-induced inhibition of $I_{K(IR)}$ remained unaltered. Therefore, it is clear from our experiments that the magnitude of proton currents does not contribute to MX-mediated block of $I_{K(IR)}$ observed in LPS-treated RAW 264.7 cells.

In our study, the open-time histogram of Kir channels needs to be fitted by a two-exponential function. The result is somewhat different from that reported recently [37], although the single channel conductance of Kir channel presented herein is similar (about 20 pS). The reason is currently unknown; however, it could be due to the different

concentrations of internal spermine, Mg^{2+} , or other second messengers inside RAW 264.7 cells [34]. Nevertheless, there tends to be at least two states in the Kir channel observed in RAW 264.7 cells. Addition of MX can shorten the open time of Kir channels as well as slow the rate of $I_{K(IR)}$ inactivation. Despite the detailed mechanism of the inhibitory actions, MX or doxorubicin at clinically relevant concentrations can effectively suppress the activity of Kir channels in the osteoclast precursor RAW 264.7 cells differentiated with LPS.

Estrogen replacement therapy is recognized as an effective treatment for the prevention of decreased bone mass in postmenopausal women. In our study, 17β -estradiol was found to inhibit the amplitude of $I_{K(IR)}$ within a few minutes in LPS-treated RAW 264.7 cells. Given that the inhibitory effect is rapid in onset and tends to be inconsistent with the binding to cytosolic receptors, such an action could be direct and mediated through a non-genomic mechanism [16, 38, 39]. To what extent the block by 17β -estradiol of Kir channels contributes to the inhibition of bone resorption or remodeling remains to be further defined.

Conclusions

MX is a blocker of $I_{K(IR)}$ measured from in RAW 264.7 cells differentiated with LPS. MX is able to interact with Kir channels to decrease the amplitude of $I_{K(IR)}$, thereby producing membrane depolarization in these cells. The Kir channel would be expected to be a potential target for MX or other structurally related compounds (e.g., doxorubicin) to influence the resorptive activity of osteoclasts, if similar findings presented here are obtained *in vivo*. The potential for block of Kir channels enriched in osteoclasts may have important clinical implications in treating various disorders where mineralized tissues are disrupted.

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