Original Paper

The Inhibition of Inwardly Rectifying K⁺ Channels by Memantine in Macrophages and Microglial Cells

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Key Words

Memantine • Inwardly rectifying potassium channel • Channel kinetics • Membrane potential • Macrophage • Microglial cell

Abstract

Background/Aims: Memantine (MEM) can block *N*-methyl-D-aspartate receptors non-competitively and is recognized to exert anti-inflammatory action. Whether MEM and other related compounds produce any effects on K⁺ currents in macrophages and in microglial cells is largely unknown. In this study, we investigated the effects of MEM and other related compounds on inwardly rectifying K⁺ current ($I_{K(IR)}$) in RAW 264.7 macrophages and in BV2 microglial cells. **Methods:** Patch-clamp recordings under whole-cell, cell-attached or inside-out configuration were performed in standard patch-clamp technique. MEM suppressed the $I_{K(IR)}$ amplitude in a concentration-dependent manner with an IC₅₀ value of 12 μM. **Results:** This agent significantly slowed the inactivation time rate of $I_{K(IR)}$ evoked with membrane hyperpolarization. In cells dialyzed spermine (10 μM), MEM-mediated inhibition of $I_{K(IR)}$ no longer existed. MEM-suppressed activity is associated with a decrease in the slow component of mean open time and an increase in mean closed time, despite no detectable change in single-channel conductance of inwardly rectifying K⁺ (Kir) channels. Under current-clamp conditions, the addition of MEM resulted in membrane depolarization of RAW 264.7 cells. Similarly, in BV2 microglial cells, addition of MEM suppressed $I_{K(IR)}$ as well as depolarized the membrane. However, neither C6 astrocytic cells nor Jurkat T-lymphocytes were noted to display $I_{K(IR)}$. **Conclusion:** The block by MEM of Kir2.1 channels is thus one of the important mechanisms underlying its actions on the functional activities of either macrophages or microglial cells, if similar findings occur *in vivo*.

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Introduction

The strong inwardly rectifying K^+ (K_{IR}) channels belong to a family of K^+ channels that have only two membrane-spanning domains and are responsible for stabilization of the resting membrane potential (V_{rest}) near to the K^+ equilibrium potential (E_K). Inward currents through K_{IR} channels can be measured at potentials negative to E_K , while at potentials positive to E_K , outward currents are almost blocked [1-3]. Block of K_{IR} channels depolarizes the cell and decreases the driving force for inwardly transported Ca^{2+} in non-excitable cells like macrophages or microglial cells, for example, via store-operated channels, Ca^{2+} -release-activated channels or other Ca^{2+} -permeable nonselective channels [4-6]. It is also known that block by intracellular polyamines such as spermine and spermidine is functionally important [3].

Memantine (MEM) is a derivative of amantadine and has been used to treat a variety of neurological or psychiatric disorders associated with excitotoxic cell death, including Parkinson's disease and vascular dementia [7-9]. The therapeutic effect of this compound is due primarily to its ability to bind to *N*-methyl-D-aspartate (NMDA) receptor-operated cation channels [10, 11]. Despite the fact that NMDA receptors constitute the main target of MEM, recent studies have shown that it could inhibit ATP-sensitive K^+ (K_{ATP}) channels in substantia nigra neurons [12] and suppress electroporation-induced inward currents [13]. MEM has been reported to exert anti-inflammatory actions [7, 8, 14, 15]; however, such mechanisms remain unclear. Additionally, little information is available concerning the mechanisms involved in effect of MEM or structurally similar compounds on K_{IR} channels, while amantadine was reported to block viral K^+ channels [16].

Microglial cells constitute the main leukocyte-dependent source of reactive oxygen species in the brain tissue, the spinal cord and the retina. They are highly reactive, mobile and multifunctional immune cells and recognized to play an essential role in many pathological conditions including multiple sclerosis, viral infections like AIDS, and in lethal or sublethal injuries of neurons where the blood-brain barrier is left intact [17-19]. An earlier study has also reported the presence of NMDA receptors in RAW 264.7 macrophages [20]. On the other hand, the activity of K_{IR} channels has been previously described in macrophages and microglial cells [5, 21-23]. These K_{IR} channels in microglial cells can play a role in the maintenance of extracellular K^+ by absorbing excess K^+ ions released from excited neurons.

Therefore, we hypothesized that MEM can exert anti-inflammatory action by altering the activity of K_{IR} channels in macrophages or microglial cells. In this study, unlike astrocytes or T lymphocytes, BV2 microglial cells were clearly found to exhibit the activity of K_{IR} channels as described recently in RAW 264.7 macrophages [23]. Addition of MEM was found to inhibit the amplitude of inwardly rectifying K^+ current ($I_{K(IR)}$) though the K_{IR} channels in RAW 264.7 macrophages and BV2 microglial cells. It is thus anticipated that block of macrophagic or microglial K_{IR} channels by MEM contributes to the therapeutic actions of this compound.

Materials and Methods

Drugs and solutions

Amantadine, memantine (MEM, 1-amino-3,5-dimethyl-adamantane), diazoxide, dibutyryl cyclic AMP, spermine, tetraethylammonium chloride, and theophylline were obtained from Sigma-Aldrich (St. Louis, MO). Iberiotoxin and apamin were purchased from Alomone (Jerusalem, Israel). Tissue culture media, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, fungizone and trypsin were obtained from Invitrogen (Carlsbad, CA). All other chemicals, including $BaCl_2$, CsCl and CsOH, were commercially available and of reagent grade. Reagent water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA).

The normal Tyrode's solution was composed of the following (in mM): NaCl, 136.5, KCl, 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and Hepes-NaOH buffer 5.5 (pH 7.4). To record membrane potential or K^+ currents, a patch pipette was filled with the solution (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, EGTA

0.1, Na_2ATP 3, Na_2GTP 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, the pipette was filled with a solution containing spermine (10 μM). To ensure the existence of 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 . To measure single-channel currents of Kir channels, the pipette was filled with the following solution (in mM) KCl 145, MgCl_2 2 and Hepes-KOH buffer (pH 7.2).

Cell preparations

RAW 264.7 murine macrophage cells were obtained from the American Type Culture Collection (TIB-71; Manassas, VA). The clonal strain BV2 cell line was kindly provided from Professor Dr. Jau-Shyong Hong (National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC) [19]. Both of cells were maintained in phenol red-free Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin [6, 19, 24].

The C6 rat astrocytes were originally obtained from the American Type Culture Collection (CCL107) and grown to confluence in HAM's F10 media containing 15% horse serum, 2.5% FBS and 1% glutamine. For differentiation, C6 cells were serum-starved with a medium containing 1 mM dibutyryl cyclic AMP and 0.25 mM theophylline. The Jurkat T cell line, a human T cell lymphoblast-like cell line (clone E6-1), was obtained from the Bioresource Collection and Research Center (BCRC-60255; Hsinchu, Taiwan). They were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin [25]. All cells reported here were maintained in a 95% air and 5% CO_2 humidified atmosphere at 37 °C.

Assay of cell proliferation

The cells (2×10^4 per ml) were cultured at 37 °C in a 96-well microplate and treated with different concentrations of MEM. After exposure to MEM for 24, 48 and 72 hours, respectively, 20 μl of methyl tetrazolium (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solutions (5 mg/ml) was added to each well and cells were incubated for another 4 hours. The formazan crystals were dissolved in 150 μl dimethylsulfoxide (DMSO) and the absorbance of samples was measured at 490 nm by a microplate reader (Model 3550, Bio-Rad Laboratories, CA) [26].

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To detect the expression of Kir2.1 (KCNJ2) mRNA in RAW 264.7 and BV2 cells, a semi-quantitative RT-PCR assay was performed. Total RNA samples were extracted from these cells with TRIzol reagent (Invitrogen) and reverse-transcribed into complementary DNA using Superscript II reverse-transcriptase (Invitrogen). The sequences of oligonucleotide primers used for Kir2.1 (NM_008425.4) were 5'-CTCTCTGGCTGTCTTTGG-3' and 5'-GAAGACAGCAATTGGGCACT-3'. Amplification of Kir2.1 was made using PCR SuperMix from Invitrogen under the following conditions: 35 cycles composed of 30 sec denaturation at 95 °C, 30 sec primer annealing at 62 °C, 1 min extension at 72 °C, and followed by 72 °C for the final extension for 2 min. PCR products were analyzed on 1.5% (v/v) agarose gel containing ethidium bromide and then visualized under ultraviolet light. Optical densities of DNA bands were scanned and quantified by AlphaImager 2200 (ProteinSimple; Santa Clara, CA).

Electrophysiological measurements

Shortly before the experiments, the cells examined were dissociated and a small aliquot of cell suspension was placed in a recording chamber mounted on the stage of an inverted 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 (Narishige, Tokyo, Japan) or a P-97 Flaming/Brown electrode puller (Sutter; Novato, CA), and their tips were fire-polished with an MF-83 microforge (Narishige). Their resistances commonly ranged between 3 and 5 M Ω when the pipettes were filled with different internal solutions described above. Patch-clamp recordings under whole-cell, cell-attached or inside-out configuration were performed in standard patch-clamp technique using either an RK-400 (Bio-Logic, Claix, France) or an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) [23, 25]. The offset potential between the pipette and bath solution was generally 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 controlled by pCLAMP 9.2 software (Molecular Devices). Current signals were low-pass filtered at 1 or 3 kHz. The pCLAMP-generated profiles with long-lasting rectangular or ramp pulses were utilized to evaluate the current-voltage (I - V) relationships for ion currents (e.g., $I_{K(IR)}$). For further analyses, the digital data were exported to Origin 8.0 (OriginLab, Northampton, MA) or Excel 2007 (Microsoft, Redmond, WA). The inactivation time courses of $I_{K(IR)}$ in response to large hyperpolarizations with or without addition of MEM were adequately measured by fitting an one-exponential function [23].

Data analyses

To calculate the concentration-dependent inhibition of MEM on the amplitude of $I_{K(IR)}$, cells were bathed in normal Tyrode's solution and the ramp pulses from -120 to +40 mV with of 1 sec at a rate of 0.05 Hz were applied. The $I_{K(IR)}$ amplitude measured at the level of -100 mV during cell exposure to different concentrations (1-100 μ M) of MEM was compared with the control value. To ensure accurate fitting, the concentration-dependent relation of MEM on the inhibition of $I_{K(IR)}$ was fit using a modified form of sigmoidal Hill equation:

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

where [MEM] is the MEM concentration; IC_{50} and n_H are the concentration required for a 50% inhibition and Hill coefficient, respectively; A and a indicate the maximal fraction of total current which is MEM sensitive and insensitive, respectively.

Single-channel amplitudes of Kir channels were determined by fitting Gaussian distributions to the amplitude histograms of the closed and the open state. The channel open probability in each patch 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 and closed lifetime distributions with or without addition of MEM were fitted with logarithmically scaled bin width [27]. Fits reported here were commonly made with nonlinear, least-squares, curve fitting algorithms [28].

The data of macroscopic or single-channel currents are presented as the means $\pm$ standard error of the mean (SEM) with sample sizes (n) indicating the number of cells examined. Statistical evaluation of difference among means was conducted using paired or unpaired Student's t test, followed by one-way analyses of variance (ANOVA) with a least-significance difference method for multiple-group comparisons. If the assumption of normality underlying ANOVA was violated, nonparametric Kruskal-Wallis test was used. Statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC). Differences were considered statistically significant when $P < 0.05$ as indicated in the figure legend.

Results

Effect of MEM on the inwardly rectifying K^+ current ($I_{K(IR)}$) in RAW 264.7 murine macrophages

In the initial set of electrophysiological experiments, whole-cell recordings were conducted to evaluate the possible effects of MEM on ion currents in RAW 264.7 cells. To record K^+ currents, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the recording pipette was filled with K^+ -containing solution described in Materials and Methods. As the whole cell mode was established, the cell examined was held at -50 mV and the upsloping ramp pulses from -120 to +40 mV with a duration of 1 sec were then applied. Under this voltage profile, a unique population of K^+ currents was evoked and the trajectory of these currents was noted to exhibit a strong inward rectification. The rectification started at around -60 mV and was progressively increased at hyperpolarized potentials. These K^+ currents found in RAW 264.7 cells, which were subject to block by $BaCl_2$ and mitoxantrone

Fig. 1. Concentration-response relationship for MEM-induced inhibition of $I_{K(IR)}$ in RAW 264.7 murine macrophage-like cells. In this set of experiments, a holding potential was set at -50 mV and the long-lasting ramp pulses from -120 to +20 mV with a duration of 1 sec were applied to the cell examined. (A) Original current traces showing the amplitude of $I_{K(IR)}$ with or without addition of MEM: a: control; b: 3 μM MEM; c: 10 μM MEM. (B) Concentration-response curve for MEM-induced inhibition of $I_{K(IR)}$ (mean±SEM; n=7-13 for each point). The $I_{K(IR)}$ amplitude at the level of -100 mV obtained in the absence and presence of different concentration of MEM was measured during these experiments. The red smooth line represents the best fit of data to the sigmoidal Hill equation as described under Materials and Methods. The values for IC_{50} , maximally inhibited percentage of $I_{K(IR)}$, and Hill coefficient in these cells were 12 μM, 80% ($A=0.8$), and 1.2, respectively.

Fig. 2. Effect of MEM on the time course of $I_{K(IR)}$ inactivation in RAW 264-7 cells. In these experiments, each cell was hyperpolarized from -50 to -130 mV with a duration of 1 sec. (A) Current trajectories obtained in the absence and presence of MEM: a: control; b: 10 μM MEM; c: 30 μM MEM. Smooth curves overlaid and labeled a, b and c depict the single-exponential fits of $I_{K(IR)}$ trajectories in which the inactivation time constants with 85, 192 and 307 msec were respectively obtained. The inset in (A) indicates the voltage profile used. Note that the number of datum points indicated in open circles was reduced for the sake of clarity. (B) Summary of the data showing effect of MEM on inactivation time constant of $I_{K(IR)}$ (mean±SEM; n=8 for each bar). *Significantly different from control ($P<0.05$).

but not by iberiotoxin or apamin, have been referred to as $I_{K(IR)}$ [21, 23]. Of interest, as these cells were exposed to MEM, the amplitude of $I_{K(IR)}$ became progressively reduced (Fig. 1). For example, when the ramp pulse was applied, MEM (10 μM) significantly decreased $I_{K(IR)}$ amplitude at the level of -120 mV by $35\pm2\%$ from 124 ± 15 to 83 ± 11 pA (n=9). Accordingly, whole-current conductance of $I_{K(IR)}$ measured between -100 and -80 mV was significantly reduced from 2.58 ± 0.12 to 2.11 ± 0.08 nS (n=9), as cells were exposed to MEM. After washout of

Fig. 3. Effect of MEM and $BaCl_2$ in the presence of intracellular perfusion with spermine in RAW 264.7 cells. In these experiments, cells were bathed in normal Tyrode's solution and the recording pipettes were filled with a solution containing spermine (10 μ M). (A) Original current traces obtained in the absence (a) and presence (b) of MEM (10 μ M). In these experiments, the ramp pulse was applied from -180 to +40 mV with a duration of 1 sec. Records shown in (A) were obtained in the experiments where the cell was dialyzed with spermine (10 μ M). Note that these two current traces in response to ramp pulse are superimposable. (B) Summary of the data showing the effect of MEM (10 μ M) and $BaCl_2$ (1 mM) on $I_{K(IR)}$ amplitude in cells loaded with spermine (mean $\pm$ SEM; $n=6-7$ for each bar). Current amplitudes were measured at the level of -120 mV. Notably, as intracellular perfusion with spermine was made, addition of MEM had minimal effects on $I_{K(IR)}$ amplitude, although subsequent application of $BaCl_2$ (1 mM) effectively suppressed current amplitude. *Significantly different from control ($P<0.05$).

drug, $I_{K(IR)}$ amplitude was partially returned to the control. In addition, amantadine (10 μ M), a compound structurally similar to MEM, could significantly suppress the amplitude of $I_{K(IR)}$ in RAW 264.7 cells (data not shown).

The relationship between the MEM concentration and the relative amplitude of $I_{K(IR)}$ was constructed and is illustrated in Fig. 1B. In these experiments, the ramp pulse from -120 to +40 mV was applied to the cell. The $I_{K(IR)}$ amplitudes measured at the level of -100 mV were then compared with those obtained in the presence of different concentration (1-100 μ M) of MEM. Notably, addition of MEM was effective at suppressing the amplitude of $I_{K(IR)}$ with a dose-response pattern. The half-maximal concentration required for the inhibitory effect of MEM on $I_{K(IR)}$ was calculated to be 12 μ M, and at a concentration of 100 μ M, it suppressed almost all the $I_{K(IR)}$ amplitude. Results from these observations reflect that this compound has a suppressive effect on $I_{K(IR)}$ inherent in RAW 264.7 murine macrophage.

MEM-induced slowing of the time course of $I_{K(IR)}$ inactivation in RAW 264.7 cells

These inward K^+ currents elicited by long-lasting hyperpolarizations tend to exhibit a time-dependent decline particularly at the voltages below -100 mV, and the decay became faster with greater hyperpolarizations [23]. Therefore, we next evaluated whether MEM can alter the time course of $I_{K(IR)}$ inactivation in response to large rectangular hyperpolarizations. As shown in Fig. 2, raising MEM concentration produced a progressive reduction in the rate of $I_{K(IR)}$ inactivation when the cell was hyperpolarized from -50 to -130 mV with a duration of 1 sec. For example, addition of MEM (10 μ M) significantly increased the time constant of $I_{K(IR)}$ inactivation from 86 ± 15 to 192 ± 23 msec ($n=8$). These data allowed us to indicate that in addition to the decreased current amplitude, addition of MEM is capable of slowing the time course of $I_{K(IR)}$ inactivation elicited by large hyperpolarization.

Lack of effect of MEM on $I_{K(IR)}$ in cells loaded with spermine

Spermine, a polyamine compound, was known to modify the kinetics of $I_{K(IR)}$ in RAW 264.7 cells [23, 29]. Another series of experiments was conducted in cells that were loaded with spermine (10 μ M) by including the compound in the pipette solution. As shown in Fig. 3, addition of MEM was found to have little effects on $I_{K(IR)}$ amplitudes evoked with long-lasting ramp pulses in cells dialyzed with spermine. However, a subsequent application of

Fig. 4. Inhibitory effect of MEM on the activity of Kir channels recorded from RAW 264.7 cells. The cell-attached recordings were conducted in cells bathed in normal Tyrode's solution containing 1.8 mM CaCl_2 . (A) Original single-channel currents obtained in the absence (upper panel) and presence (lower panel) of 10 μM MEM. The attached cell was held at -70 mV relative to the bath. Channel opening shown in (A) gives a downward deflection in current. (B) Amplitude histograms measured in the absence (upper panel) and presence (lower panel) of 10 μM MEM. Note that the amplitude of single-channel current did not differ between the absence and presence of MEM. (C) Averaged I - V relationships of single Kir-channel currents obtained in the control (■) and during the exposure to 10 μM MEM (□). Each point represents the mean $\pm$ SEM ($n=8-10$). Notably, as straight line is overlaid, the linear I - V relationship of Kir channels with or without addition of MEM is almost superimposed.

BaCl_2 (1 mM) suppressed the amplitude of $I_{K(IR)}$ effectively. The results suggest that the effect caused by spemidine and MEM tends to be not additive and that the two agents may exert their major effects on the same component.

Electric properties of MEM-inhibited Kir channels recorded from RAW 264.7 cells

The MEM-induced inhibition of $I_{K(IR)}$ described above could be associated with either the decreased open probability, a decrease in the number of functional active channels, or changes in the channel gating. For these reasons, the effects of this compound on the activity of Kir channels inherent in these cells were further investigated. In these experiments, RAW 264.7 cells were bathed in normal Tyrode's solution, and single-channel recordings with cell-attached configuration were thereafter performed. As shown in Fig. 4, when the cell was held at -70 mV relative to the bath, addition of MEM (10 μM) caused a drastic reduction of Kir-channel activity (Fig. 4A) despite no discernible change in single-channel amplitude (Fig. 4B). For example, a MEM concentration of 10 μM significantly decreased the probability of channel openings at -70 mV relative to the bath from 0.762 ± 0.024 to 0.217 ± 0.012 ($n=12$).

We further constructed the plot of single-channel amplitude as a function of holding potential. Figure 4C illustrates an ohmic I - V relationships of Kir channels in the control and during cell exposure to MEM. When data were pooled across the experiments, a fit of the data using a linear I - V relationship yielded the single-channel conductance and reversal potential of 29.6 ± 2.1 pS and 72.1 ± 1.4 mV ($n=14$). The values did not differ significantly from those obtained during exposure to 10 μM MEM (29.2 ± 1.8 pS and 71.9 ± 1.3 mV, $n=12$). Similarly, in inside-out patch configuration, when MEM (30 μM) was applied to the internal surface of

Fig. 5. MEM-induced changes in mean open and closed time of Kir channels recorded from RAW 264.7 cells. Open- and closed-time histograms obtained in the absence (left panel) and presence (right panel) of 10 μ M MEM are illustrated in (A) and (B), respectively. Notably, the abscissa and ordinate shown in this figure indicate the logarithm of open or closed time (msec) and the square root of the number of observations, respectively. The smooth lines overlaid in each lifetime distribution were fitted by one- or two-exponential function, whereas the dashed vertical lines indicate the value of the time constant in the open (A) or closed (B) state. As the cell was exposed to MEM, there was a notable decrease in the slow component of mean open time accompanied by a lengthening in mean closed time.

the detached patch, the activity of Kir channels was also decreased. However, in cell-attached patches from a recording pipette filled with MEM (10 μ M), the activity of Kir channels still existed.

Effects of MEM on kinetic behavior of Kir channels measured from RAW 264.7 cells

In order further to evaluate the kinetic properties of Kir channels modified by cell exposure to MEM, we further analyzed the mean lifetime distributions of open and closed time for these channels from patches showing the presence of only single-channel openings. As shown in Fig. 5, in control cells (i.e., in the absence of MEM), the distribution of open-time durations of Kir channels measured at -70 mV relative to the bath was composed of two components and well fitted by a two-exponential curve with a mean open time of 6.4 ± 0.8 and 58 ± 3 msec ($n=7$). The closed-time histogram in controls was fitted by a single-exponential curve with a mean closed time of 16.2 ± 1.4 msec ($n=7$). Additionally, it was noted that during exposure to MEM (10 μ M), the mean duration in the slow component

Fig. 6. Effect of MEM on the V_{rest} in RAW 264.7 cells. In these experiments, cells were bathed in normal Tyrode's solution which contained 1.8 mM $CaCl_2$ and changes in V_{rest} were measured under current-clamp configuration. (A) Original potential traces obtained in the absence (left panel) and presence (right panel) of 10 μ M MEM. (B) Frequency histograms of membrane potentials obtained in the absence (left panel) and presence (right panel) of MEM. The smooth line shown in each panel indicate the fitted Gaussian function.

of open time distribution was shortened to 24 ± 3 msec ($n=7$), while the fast component of mean open time remain unaltered in the presence of this agent (6.2 ± 0.9 msec, $n=7$). The addition of MEM (10 μ M) also significantly prolonged mean closed time to 36.2 ± 2.7 msec ($n=7$). These data suggest that despite the inability to modify single-channel conductance, the inhibition by this compound of Kir channels in RAW 264.7 cells arises largely from the decrease of channel open probability caused by the decrease in the slow component of mean open time and the lengthening of mean closed time. It thus seems likely that the binding of this compound to the membrane existing in these cells renders the Kir channel to spend more time in the closed state.

Effect of MEM on membrane potential of RAW 264.7 cells

Next, we explored the effect of MEM 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 effect of MEM on membrane potential in these cells is illustrated in Fig. 6. The frequency histogram of membrane potentials derived from RAW 264.7 cells was found to display two distinct groups. In control cells, the histogram of membrane potential comprised two broad distributions. That is, one falls within a distribution ranging between -55 and -51 mV with a mean value of -53.3 ± 0.9 mV ($n=8$), and the other is between -51 and -49 mV with a mean value of -49.6 ± 0.8 mV ($n=8$). However, addition of 10 μ M MEM caused an upward shift in V_{rest} and cells became depolarized, along with significant changes in the frequency histogram of membrane potential. One is a distribution between -52 and -49 mV with a mean value of -50.5 ± 1.1 mV ($n=8$), while the

Fig. 7. Effect of MEM on $I_{K(IR)}$ and V_{rest} in BV2 microglial cells. In each cell examined, $I_{K(IR)}$ was elicited from a holding potential of -50 mV and various potentials ranging from -140 to +40 mV with 20-mV increments were applied. (A) Original current traces obtained in the control (upper panel) and during exposure to 10 μ M MEM (lower panel). The uppermost part in (A) indicates the voltage protocol used. (B) Averaged I - V relations of $I_{K(IR)}$ in BV2 microglial cells (mean $\pm$ SEM; n=5-10 for each point). ■: control; □: in the presence of 10 μ M MEM. Note that addition of MEM is effective at suppressing $I_{K(IR)}$ at hyperpolarized potentials. (C) Summary of the data showing effect of MEM (10 and 30 μ M) on V_{rest} in BV2 microglial cells (mean $\pm$ SEM; n=6-8 for each bar). Membrane potential was measured under current-clamp configuration. *Significantly different from control ($P<0.05$).

other falls within a range between -49 and -45 mV with a mean value of -47.5 ± 1.1 mV (n=8). Taken together, the results indicate that application of MEM can significantly depolarize the cell as well as alter the frequency histogram of membrane potentials measured from RAW 264.7 cells. MEM-induced changes in the recorded membrane potential are almost certainly explained by the suppression of Kir-channel activity in these cells, because the K^+ conductance in V_{rest} is largely determined by Kir-channel activity. However, in cells dialyzed with spermine, cell exposure to MEM had little effects on membrane potential of these cells.

Effects of MEM on $I_{K(IR)}$ and membrane potential in BV2 microglial cells

In another set of experiments, we evaluated whether MEM has any effect on $I_{K(IR)}$ (i.e., Kir2.1-encoded current) in different types of immune cells (e.g., BV2 cells). In these experiments, BV2 cells Jurkat T-lymphocytes were bathed in normal Tyrode's solution and the recording pipette was filled with K^+ -containing solution. The $I_{K(IR)}$ was evoked as the cell examined was held at -50 mV and various potentials ranging from -140 to +40 mV with 20-mV increments were applied [5, 22]. As illustrated in Fig. 7, the data showed that MEM at a concentration of 10 μ M was able to suppress the amplitude of $I_{K(IR)}$ significantly in these cells. Neither iberiotoxin (200 nM) nor apamin (200 nM) caused any effects on $I_{K(IR)}$ in BV2 cells. Thus, it is unlikely that the observed decrease in K^+ currents by MEM is due primarily to its inhibition of K^+ currents that are sensitive to block by iberiotoxin or apamin. Moreover, similar to findings seen in RAW 264.7 cells, MEM was effective at slowing the inactivation rate of $I_{K(IR)}$ elicited by large hyperpolarizations. Fig. 7B illustrates the averaged I - V relationships of $I_{K(IR)}$ obtained with or without addition of MEM in BV2 cells. The whole-cell conductance

of $I_{K(IR)}$ ranging between -120 and -80 mV was clearly reduced to 3.18 ± 0.09 nS ($n=8$) from a control value of 6.22 ± 0.11 nS ($n=9$). However, we were unable to detect the presence of $I_{K(IR)}$ in Jurkat T-lymphocytes or C6 astrocytic cells, despite they displayed voltage-gated K^+ currents [25].

The effect of MEM on membrane potential in BV2 microglial cells was further investigated. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the recording pipette was filled with K^+ -containing solution. This type of macrophages had a V_{rest} of -62 ± 5 mV ($n=19$) under current-clamp recordings. As shown in Fig. 7C, addition of MEM (10 and 30 μM) caused membrane depolarization significantly. However, MEM did not have any effect on the membrane potential of C6 astrocytic cells.

Discussion

The main findings of this study are as follows. First, MEM was effective at decreasing the amplitude of $I_{K(IR)}$ in both RAW 264.7 macrophages and BV2 microglial cells. Second, the presence of MEM reduced the rate and extent of $I_{K(IR)}$ inactivation. Third, Intracellular inclusion of spermine blocked the inhibitory effects of MEM on $I_{K(IR)}$. Fourth, MEM-induced inhibition of Kir channels was due primarily to the decrease of single-channel conductance. Fifth, application of MEM caused membrane depolarization in both RAW 264.7 and BV2 cells, but not in astrocytic C6 cells. Therefore, it is anticipated that the suppressing action of MEM on Kir channels is an important mechanism through which MEM or other structurally similar compounds can interfere with the functional activities of macrophages or microglial cells, if similar findings occur *in vivo*.

The $I_{K(IR)}$ of RAW 264.7 and BV2 cells can be observed following the hyperpolarizing pulses to -90 mV and were increased in amplitude with more negative membrane potentials. The amplitude of $I_{K(IR)}$ became inactivated as a result of prolonged hyperpolarization. This major ion conductance present in these macrophages was identified as a classical $I_{K(IR)}$ with strong inward rectification, the kinetics of which are similar to Kir2.1-encoded current [1, 3]. It is possible that the Kir channels in RAW 264.7 or BV2 cells comprise various homo- and heteromultimers of Kir2.x subunits. Addition of MEM reported here was also noted to decrease the amplitude of $I_{K(IR)}$ and to slow current inactivation through Kir channels which are constitutively active in macrophages and microglial cells.

A previous report has shown the ability of MEM to suppress the activity of K_{ATP} channels in substantia nigra neurons [12]. Similar to $I_{K(IR)}$, the macroscopic K^+ currents which K_{ATP} channels constitute are noted to be inwardly rectifying [27]. However, diazoxide, an opener of K_{ATP} channels, was not found to reverse MEM-inhibited amplitude of $I_{K(IR)}$ in RAW 264.7 cells. Moreover, the pipette solution used for whole-cell recordings contained 3 mM ATP which adequately suppresses K_{ATP} -channel activity [27]. Therefore, it seems unlikely that MEM-mediated inhibition of $I_{K(IR)}$ described herein arises from the suppression of K_{ATP} channels, while this type of K^+ channels also display an inwardly rectifying property.

Unlike RAW 264.7 or BV2 cells, differentiated astrocyte C6 cells did not display $I_{K(IR)}$, while we did find the presence of large-conductance Ca^{2+} -activated K (BK_{Ca}) channels in C6 astrocytic cells (data not shown). MEM was not found to exert any direct effects on the activity of BK_{Ca} channels in C6 astrocytic cells. However, the increased K^+ efflux through BK_{Ca} channels of astrocytes inherently existing in brain tissues can be another intriguing mechanism through which the $I_{K(IR)}$ amplitude in microglial cells would be increased. The main reason is that the increase of extracellular K^+ due to elevated K^+ efflux occurring under the narrowed extracellular milieu is able to enhance $I_{K(IR)}$ amplitude in neighboring microglial cells [2, 3, 30, 31].

MEM in a low dose (0.02-2.0 mg/kg) was reported to fully suppress cocaine-induced CPP behavior in rats via its interleukin-6-modulating effect in the medial prefrontal cortex [32]. MEM at a low dose can also exert anti-inflammatory and neuroprotective actions which are beneficial for chronic opioid-induced addiction [15]. In this study, the IC_{50} value required

for MEM-induced inhibition of $I_{K(IR)}$ in RAW 264.7 cells was measured to be about 12 μM . This value tends to be higher than the human plasma concentration of MEM, which ranges from 0.05 to 0.2 μM (or from 10 to 50 ng/ml). However, it needs to be noted that MEM distributes preferentially to the brain, with brain-to-plasma ratios ranging from around 3 to >20 over time for all dose levels and routes [33]. Following 7 days of infusion of MEM, the whole-brain concentrations were found to be 44-times higher than free serum concentrations [34]. Therefore, the effect of MEM on Kir channels in microglial cells is mostly likely to occur at a concentration achievable in humans.

Earlier work has demonstrated the existence of NMDA receptors in RAW 264.7 cells [20]. MEM was recognized to be efficacious at blocking NMDA-receptor currents [10, 11]. In our study, L-glutamate alone (1 mM) had little or no effect on either $I_{K(IR)}$ amplitude or Kir-channel activity. In continued presence of L-glutamate (1 mM), MEM also suppressed hyperpolarization-induced $I_{K(IR)}$ effectively in RAW 264.7 cells. Therefore, MEM-induced perturbation of $I_{K(IR)}$ observed in this study apparently is not because of its antagonism of NMDA receptors. The participation of its antagonizing actions of endogenous glutamate which could be released from cells was excluded as well. However, whether MEM can interact directly with the postsynaptic density protein (i.e., PSD-95) to influence both NMDA receptors and Kir-channel activity simultaneously remains to be further determined, given the ability of this family of proteins to anchor NMDA receptors and to regulate K^+ -channel activity in neurons [3, 35].

With the aid of Blastx program (<http://blast.ncbi.nlm.nih.gov/>), we have further examined similarity of mRNA sequence between Kir2.1 channel α -subunit (NM_011807.3) and PSD-95 (NM_011807.3). Notably, there are eight regions for Kir2.1 channel α -subunit which were noted to share the similarity (80-100 % identity) to those for PSD-95. Therefore, it appears likely that MEM or other structurally similar compounds can interact with some of these regions to influence the activity of Kir channels and/or PSD-95.

While neither RAW 264.7 nor BV2 cells were found to display voltage-gated or Na^+ or Ca^{2+} currents, our electrophysiological studies have clearly demonstrated that both cell types functionally express classical Kir channels [22, 23]. Through Kir channels, currents flow more readily in the inward direction than outward. As a result, functional expression of classical Kir channels in these cells is able to set the V_{rest} of cells to negative potentials, which provides the electrochemical driving force for inwardly transported Ca^{2+} via different types of Ca^{2+} -permeable channels inherently in macrophages or microglial cells [4-6].

As MEM is lipid soluble to some extent, it is possible that this compound may reach the target site by partitioning into the lipid phase of the membrane [34, 36]. The MEM molecules are unlikely to interact within central pore cavity of the Kir2.x channels because of their inability to alter the single-channel conductance. The site of action cannot be precisely determined from the data presented here; however, it becomes clear that this compound can bind to the membrane preferentially at the intracellular leaflet of the channel. Moreover, MEM and polyamines may interact with at least two negatively charged amino-acid residues located in the M2 domain (R1) and in the C-terminus (R2) of Kir-channel subunits, because these two sites are both negative charged and important for inward rectification [3].

Although the detailed mechanism of inhibitory actions of MEM has yet been unraveled, our study clearly demonstrated that under current-clamp conditions, this agent at clinically relevant concentrations could depolarize the membrane in RAW 264.7 and BV2 cells. MEM at the concentrations used in this study neither changed cell viability nor the proliferation rate of RAW 264.7 or BV2 cells (data not shown). MEM-mediated inhibition of $I_{K(IR)}$ can result in membrane depolarization, which decreases the inwardly directed driving force of Ca^{2+} and subsequently diminishes the amplitude of Ca^{2+} -activated K^+ currents over time. It is anticipated that such positive feedback of membrane depolarization on Kir channels in these cells is unique and may provide an intriguing tool for fine tuning of Kir-channel activity [3, 5, 29, 31]. Assuming that similar findings in this study take place in macrophages or microglial cells *in vivo*, any changes in Kir-channel activity may underlie the potential mechanisms through which MEM or other structurally similar compounds influence their functional

activities. Moreover, MEM may share many features with quaternary amine compounds that can be utilized to explore K^+ structure-function relationships [16].

Conflict of Interest

We declare that there is no conflict of interest that would prejudice its impartiality.

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