Original Paper

The Inhibition by Oxaliplatin, a Platinum-Based Anti-Neoplastic Agent, of the Activity of Intermediate-Conductance Ca^{2+} -Activated K^+ Channels in Human Glioma Cells

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Key Words

Oxaliplatin • Glioma cell • K^+ current • Intermediate-conductance Ca^{2+} -activated K^+ channel

Abstract

Oxaliplatin (OXAL) is a third-generation organoplatinum which is effective against advanced cancer cells including glioma cells. How this agent and other related compounds interacts with ion channels in glioma cells is poorly understood. OXAL (100 μM) suppressed the amplitude of whole-cell K^+ currents (I_{K}); and, either DCEBIO or ionomycin significantly reversed OXAL-mediated inhibition of I_{K} in human 13-06-MG glioma cells. In OXAL-treated cells, TRAM-34 did not suppress I_{K} amplitude in these cells. The intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels subject to activation by DCEBIO and to inhibition by TRAM-34 or clotrimazole were functionally expressed in these cells. Unlike cisplatin, OXAL decreased the probability of IK_{Ca} -channel openings in a concentration-dependent manner with an IC_{50} value of 67 μM . No significant change in single-channel conductance of IK_{Ca} channels in the presence of OXAL was demonstrated. Neither large-conductance Ca^{2+} -activated K^+ channels nor inwardly rectifying K^+ currents in these cells were affected in the presence of OXAL. OXAL also suppressed the proliferation and migration of 13-06-MG cells in a concentration- and time-dependent manner. OXAL reduced IK_{Ca} -channel activity in LoVo colorectal cancer cells. Taken together, the inhibition by OXAL of IK_{Ca} channels would conceivably be an important mechanism through which it acts on the functional activities of glioma cells occurring *in vivo*.

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Introduction

Glioblastoma multiforme is a cancer with dismal prognosis and the relative short life expectancy for its victims was disappointing despite recent extensive treatment [1]. Ion currents inherent in malignant glioma cells have been increasingly reported to influence the behavior of these cells [2-10]. Specifically, numerous reports demonstrated that any perturbations of functional expression in intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels enriched in glioma cells or other types of neoplastic cells are capable of interfering with invasiveness or progression of malignant tumors [4, 5, 11-16]. Alternatively, oxaliplatin (OXAL) belongs to a family of platinum-based chemotherapeutic compounds and is one of the most active drugs in many types of solid tumors encompassing gliomas [17-19]. However, whether OXAL or other similar structurally-related compounds can interact with ion currents and to alter malignant behaviors in these cells is incompletely understood, in spite of its ability to produce multiple pharmacological activities [17].

The IK_{Ca} channels (also known as K_{Ca} 3.1, SK4, IK_{Ca1} or *KCNN4*) which are encoded by the *KCNN4* gene have been increasingly investigated in many non-excitable or neoplastic cells concerning their contributory roles in cell behaviors including hormonal secretion, cell migration, cell proliferation, and regulation of Ca^{2+} influx and/or K^+ efflux [2, 8, 9, 13-15, 20, 21]. More importantly, the pharmacological modulators of IK_{Ca} (K_{Ca} 3.1) channels may represent an attractive therapeutic approach to a variety of tumors including gliomas [2, 4, 9, 15, 22]. However, whether OXAL or other related drugs exert any effects on the activity of IK_{Ca} channels is not thoroughly understood, despite the fact that it can block K^+ currents [23].

Therefore, in this study, we attempted to investigate whether, in human 13-06-MG glioma cells, OXAL exerts any effects on ion currents including K^+ currents (I_K) and IK_{Ca} channels, to determine how this drug interacts with IK_{Ca} channel to change I_K amplitude, and to address the issue of how this drug has any effects on the growth of these cells. The OXAL action thus is not exclusively connected to the formation of platinum-DNA adducts [16, 24, 25]. The results presented herein lead us to propose that the perturbation by OXAL of IK_{Ca} channels is another intriguing mechanism through ability of this drug and its structurally related compounds to interfere with the proliferative activity of glioma cells, if similar findings occur *in vivo*. The data also raise the possibility of the OXAL actions on other types of neoplastic cells in which IK_{Ca} (or K_{Ca} 3.1) channels are functionally expressed [5, 8, 13-15, 20].

Materials and Methods

Drugs and Solutions

Oxaliplatin (OXAL; *trans*-1-diaminocyclohexane oxaliplatin; $C_8H_{12}N_2O_4Pt$) was obtained from Sanofi-Aventis (New York, NY). Ionomycin, lipopolysaccharide (LPS), 12-o-tetradecanoylphorbol 13-acetate (TPA; phorbol 12-myristate 13-acetate), tetraethylammonium chloride, triptolide and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO), cisplatin (cis-diaminodichloroplatinum), DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one), PF573228 and TRAM-34 (1-((2-chlorophenyl)(diphenyl)methyl)-1H-pyrazole) were from Tocirs Cookson Ltd. (Bristol, UK), and tetrodotoxin was from Alomone Labs. (Jerusalem, Israel). All culture media, fetal bovine serum (FBS), L-glutamine, trypsin/EDTA, fungizone and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). All other chemicals were commercially and of reagent grade. Deionized water used throughout the experiments was created from a Milli-Q water purification system (Millipore, Bedford, MA).

The composition of bath solution (i.e., normal Tyrode's solution) was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.5 mM HEPES-NaOH buffer, pH 7.4. To record whole-cell K^+ currents (I_K), the recording pipettes were backfilled with a solution consisting of 130 mM K-aspartate, 20 mM KCl, 1 mM KH_2PO_4 , 1 mM $MgCl_2$, 3 mM Na_2ATP , 0.1 mM Na_2GTP , 0.1 mM EGTA and 5 mM HEPES-KOH buffer, pH 7.2. To measure single K^+ -channel (IK_{Ca} or BK_{Ca}) activity, pipette solution contained 145 mM KCl, 2 mM $MgCl_2$ and 5 mM HEPES-KOH buffer, pH 7.2. To avoid possible contamination of Cl^- currents, Cl^- ions inside the pipette solution was replaced with aspartate.

Cell Preparations

The glioblastoma multiforme cell lines (13-06-MG and CNS-1) were kindly provided by Professor Dr. Carol A. Kruse, Department of Neurosurgery, Ronald Reagan UCLA Medical Center, LA, U.S.A.. 13-06-MG cells were routinely grown at a density of 10^6 /ml in high-glucose (4 g/l) Dulbecco's modified Eagle media (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 10 μ g/ml streptomycin, and CNS-1 astrocytoma cells were in RPMI-1640 media (Invitrogen) supplemented with 10% FBS and 200 mM L-glutamine [26]. The LoVo cell line, a colorectal cancer cell line, obtained from American Type Culture Collection ([CCL 229], Manassas, VA), was maintained in RPMI 1640 culture medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a 5% CO₂ incubator as monolayer cultures and sub-cultured weekly and fresh media were generally added every 2-3 days to maintain a healthy cell population. Glial cells were verified by identifying glial fibrillary acidic protein, a cytoskeletal protein. To observe cell growth, a Nikon Eclipse Ti-E inverted microscope (Li Trading Co., Taipei, Taiwan) equipped with a 5-megapixel cooled digital camera was commonly used. The camera was connected to a personal computer controlled by NIS-Elements BR 3.0 software (Nikon, Kanagawa, Japan). In some set of experiments, 13-06-MG cells were treated with 100 μ M OXAL for 1-3 days.

Assay of Cell Proliferation

13-06-MG cells (2×10^4 per ml) were cultured at 37 °C in a 96-well microplate and treated with different concentrations of OXAL. After the exposure to OXAL for 24 hours, 20 μ l of methyl tetrazolium (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and cells were incubated for another 4 hours. The formazan crystals were dissolved in 150 μ l dimethylsulfoxide and the absorbance of samples was thereafter measured at 490 nm by a microplate reader (model 3550; Bio-Rad Laboratories, CA) [12, 14].

Trypan blue exclusion assay

13-06-MG cells were maintained in culture medium for 1-3 days. Trypan blue exclusion assay was performed on these cells to determine the number of cells in the absence and presence of OXAL (100 μ M). This assay was determined by distinguishing viable (white) from dead cells (blue). The cell number was counted using a hemocytometer (Cambridge Instrument Inc., Somerville, MA) under microscope with a magnification of $100 \times$ [10].

Migration Assay

Chemotactic migration ability of 13-06-MG cells was analyzed by a 64-well Boyden chamber (AP48; Neuro Prob Inc., Gaithersburg, MD). Cells were cultured in serum-free medium for 12 hours. Subsequently, 1×10^7 cells/well were suspended in serum-free DMEM with indicated drugs and placed into the upper chamber of Boyden chamber with a polycarbonate filter (8 μ m pore size; GE osmonics labstore, MN). The lower chamber was filled with a medium containing 10% FBS (as a chemoattractant) and indicated drugs. The indicated drugs loaded into both upper and lower chambers are LPS, TPA, or OXAL. After being incubated for indicated times, cells in the upper chamber were cleaned off with cotton swab. The cells present on the lower surface of the filters were fixed with methanol for 10 min. Filters were stained with hematoxylin (Vector Laboratories, CA) for 30 min. Subsequently, the nuclei stained in brown color cells in three different fields of each well were imaged at $20 \times$, counted and averaged as a representative number of migrated cells per field.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To detect the expression of *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) in 13-06-MG cells, a semi-quantitative RT-PCR assay was made. Total RNA samples were extracted from these cells according to TRIzol reagent (Invitrogen) and reverse-transcribed into complementary DNA using Superscript II reverse-transcriptase (Invitrogen). The sequences of forward and reverse primers used for *KCNMA1* and *KCNN4* are illustrated in Table 1. In general, the PCR cycling conditions were 35 cycles of 95 °C for 2 min, 95 °C for 20 sec, 60 °C for 20 sec, and 72 °C for 10 min. The PCR products were resolved on 2% agarose gels, analyzed on 1.5% (w/v) agarose gel containing ethidium bromide and then visualized by ultraviolet trans-illumination. The optical densities of DNA bands were then scanned and quantified using Alphamager 2200 (ProteinSimple; Santa Clara, CA).

Table 1. Primer sequences of target genes and expected sizes of RT-PCR products for *KCNMA1* (K_{Ca} 1.1) and *KCNN4* (K_{Ca} 3.1)

Target Gene	Primer Sequence	Expected Size, bp	GenBank Accession No.
<i>KCNMA1</i> (K_{Ca} 1.1)		201	NM_001014797.2
Forward	5'- CTG ATT TAA GGG CTG TCA AC- 3'		
Reverse	5'- ATC TAT CCA TTC CTG GAG GT- 3'		
<i>KCNN4</i> (K_{Ca} 3.1)		180	NM_002250.2
Forward	5'-ACC TTT CAG ACA CAC TTT GG-3'		
Reverse	5'-TCT CTG CCT TGT TAA ACT CC-3'		

Electrophysiological Measurements

CNS-1, 13-06-MG or LoVo cells were harvested with 1% trypsin/EDTA solution prior to the experiments and a small aliquot of cell suspension was immediately transferred to a recording chamber mounted on the mechanical stage of an inverted fluorescent microscope (CKX-41; Olympus, Tokyo, Japan) coupled to a digital video system (DCR-TRV30; Sony, Tokyo, Japan). Cells were immersed at room temperature (20-25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch electrodes used were pulled from Kimax-51 capillary tubes (#34500; Kimble Glass, Vineland, NJ) using either a PP-83 (Narishige, Tokyo, Japan) or a P-97 Flaming/Brown (Sutter, Novato, CA) puller. Their electrodes had a resistance of 3-5 M Ω when filled with the different pipette solution described above. Ion currents were measured in whole-cell, cell-attached or inside-out configuration of the standard patch-clamp technique using an RK-400 patch amplifier (Bio-Logic, Claix, France) [14, 15, 27, 28]. Junctional potentials between the pipette solution and extracellular medium became nulled prior to seal formation.

The signals, consisting of potential and current traces, were stored online on a TravelMate-6253 laptop computer (Acer, Taipei, Taiwan) at 10 kHz through a Digidata-1440A interface device (Molecular Devices, Sunnyvale, CA), which was controlled by pCLAMP 10.2 software (Molecular Devices). Current signals were low-pass filtered at 3 kHz. Through digital-to-analog conversion, the voltage-step profiles of rectangular or ramp pulses created from pCLAMP 10.2 were used to evaluate the current-voltage (I - V) relationships for different types of ion currents (e.g., I_K) obtained with or with addition of OXAL. Some signals digitally stored through either wired USB or wireless Bluetooth were further analyzed using different tools which include Origin 8.0 (OriginLab, Northampton, MA), LabChart 7.0 program (AD Instruments; Gerin, Tainan, Taiwan), and custom-made macros built in an Excel 2013 spreadsheet under Windows 7 (Microsoft).

Single-Channel Analyses

Single amplitudes of BK_{Ca} - or IK_{Ca} -channel currents were commonly analyzed with pCLAMP 10.2 (Molecular Devices). Multigaussian adjustments of the amplitude distributions among channels were used to determine single-channel events. The number of active channels in a patch was taken as the maximum number of channels simultaneously open under conditions of maximum open probability. The probabilities of channel openings were evaluated using an iterative process to minimize χ^2 values, calculated with an adequately large number of independent observations.

To evaluate concentration-dependent inhibition of OXAL on the probability of IK_{Ca} -channel openings, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and each cell examined was held at -80 mV relative to the bath. The channel open probabilities were measured in the control and during cell exposure to different concentrations (3 μ M-1 mM) of OXAL, and they were then compared with those measured after subsequent addition of TRAM-34 (3 μ M). TRAM-34 is known to be a selective blocker of IK_{Ca} channels [22, 29, 30]. The concentration required to suppress 50% of channel activity was determined by means of a Hill function [28]:

$$\text{Percentage inhibition} = \frac{E_{\max} \times [C]^{n_H}}{IC_{50}^{n_H} + [C]^{n_H}},$$

where $[C]$ represents the OXAL concentration; IC_{50} and n_H are the concentration required for a 50% inhibition and the Hill coefficient, respectively; and $E_{\max}$ indicates the maximal reduction in channel open probability (i.e., TRAM-34-sensitive channel activity) caused by OXAL.

Calculation of energies among different complexes

With the aid of Chem3D and ChemDraw programs embedded in ChemBio3D Ultra version 14 (PerkinElmer; Waltham, MA), the different parameters for OXAL/Na, OXAL/Ca, cisplatin/Na and cisplatin/Ca complexes including torsion energy, 1,4-Van der Waals force, and bend, stretch and stretch-bend energies, were calculated and compared [31].

Statistical Analyses

Data are presented as the mean $\pm$ standard error of the mean (SEM), with sample sizes (n) indicating the number of cells examined. Paired or unpaired Student's *t*-test, or one-way analysis of variance with the least-significance difference method for multiple-group comparisons, were used for the statistical evaluation of differences among means. Statistical analyses were performed using IBM SPSS version 20.0 (IBM Corp., Armonk, NY). The non-linear least-squares fitting was generally made using the Solver add-in bundled with Excel 2013 (Microsoft, Redmond, WA). Differences were considered significant at $P < 0.05$ unless otherwise indicated.

Results

The mRNA Expression for *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) in 13-06-MG Cells

We initially examined the mRNA levels of *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) on 13-06-MG cells. The mRNA expression of these genes was made with a semi-quantitative RT-PCR assay. Our RT-PCR analysis clearly presented the mRNA expression of *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) in these cells (Fig. 1). Thus, the results indicate that under our experimental conditions, the mRNA expression of *KCNMA1* and *KCNN4* channels inherently in these cells can be detected and apparently is not lost in culture.

Effect of OXAL on Whole-Cell K^+ Current (I_K) in 13-06-MG Cells

In an initial set of experiments, we evaluated whether OXAL exerts any effects on I_K in these cells. As cells were bathed in normal Tyrode's solution which contained 1.8 mM $CaCl_2$, a family of whole-cell I_K were readily elicited (Fig. 2A). When cells were exposed to OXAL (100 μ M), the amplitudes of I_K throughout the entire voltage-clamp examined were progressively diminished (Fig. 2B). The whole-cell conductance of I_K measured between -70 and 0 mV was drastically decreased to 0.57 ± 0.03 nS from a control value of 0.95 ± 0.11 nS ($n=11$, $P < 0.05$). After washout of OXAL, the I_K conductance was returned to 0.61 ± 0.04 nS ($n=8$). As the difference in current amplitudes at the same level of voltage between the absence and presence of OXAL was taken, the *I-V* relationship of OXAL-sensitive current in these cells was derived and is depicted in Fig. 2C. However, unexpectedly, as cells were constantly exposed to OXAL, subsequent addition of either DCEBIO (10 μ M) or ionomycin (10 μ M) was effective in reversing OXAL-mediated inhibition of I_K in these cells (Fig. 2D). DCEBIO is an activator of IK_{Ca} channels, while ionomycin is a Ca^{2+} ionophore [22, 30, 32]. These results led us to suggest that the component of K^+ currents suppressed by OXAL in 13-06-MG cells should be linked to the activity of IK_{Ca} channels [14, 30].

Fig. 1. The expression levels of *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) mRNAs isolated from 13-06-MG glioma cells. Total RNA was extracted from cells and RT-PCR analysis was performed. Amplified RT-PCR products for *KCNMA1* ($K_{Ca}1.1$) and *KCNN4* ($K_{Ca}3.1$) were shown. The marked lane indicating DNA molecular size was shown in leftmost lane (MW). The sequences of target genes are illustrated in Table 1.

Fig. 2. Inhibitory effect of OXAL on whole-cell K^+ current (I_K) in 13-06-MG cells. In these whole-cell recording experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The cells were held at -50 mV and different voltage pulses ranging from -70 to +70 mV with 10-mV increments were applied. (A) Original I_K traces obtained in the absence (a) and presence (b) of 100 μ M OXAL. The upper-most part in (A) indicates the voltage protocol examined. (B) I - V relationships of I_K obtained with or

without exposure to 100 μ M OXAL (mean $\pm$ SEM; n=10-12 for each point). $\square$: control; $\circ$: in the presence of 100 μ M OXAL. (C) Averaged I - V relationship of OXAL-sensitive current. (D) Summary of the data depicting effects of OXAL on I_K amplitude in the absence and presence of OXAL, OXAL plus DCEBIO, and OXAL plus ionomycin. OXAL: 100 μ M OXAL; DCEBIO: 10 μ M DCEBIO; Iono: 10 μ M ionomycin (mean $\pm$ SEM; n=9-12 for each bar). The I_K amplitude was measured at the end of depolarizing pulse when the cells were depolarized from -50 to +50 mV. *Significantly different from control ($P<0.05$). **Significantly different from OXAL alone group ($P<0.05$). Note that further application of either DCEBIO or ionomycin can reverse OXAL-induced inhibition of I_K .

Fig. 3. Effect of TRAM-34 and TRAM-34 plus OXAL on I_K in response to the ramp pulse in 13-06-MG cells. In this set of experiments, cells were bathed in normal Tyrode's solution. The examined cell was held at -50 mV and the ramp pulse from -90 to +50 mV as indicated in inset was applied. In the experiments of TRAM-34 plus OXAL, OXAL was subsequently applied during cell exposure to TRAM-34. a: control, b: 1 μ M TRAM-34; c: 1 μ M TRAM-34 plus 100 μ M OXAL.

I_K Amplitude Obtained in the Presence of TRAM-34 and TRAM-34 plus OXAL in 13-06-MG Cells

One would expect that the glioma cells exposed to OXAL could alter the effect of TRAM-34, an inhibitor of IK_{Ca} channels, on I_K amplitude in 13-06-MG cells. As illustrated in Fig. 3, the ramp pulse-elicited I_K in these cells can be significantly suppressed by TRAM-34 (1 μ M). However, in continued presence of TRAM-34, further addition of 100 μ M OXAL was not found to decrease I_K further. For example, the I_K amplitude measured at the level of +40

Fig. 4. Inhibitory effect of TRAM-34 on averaged I - V relationship of I_K in OXAL-treated 13-06-MG cells. In these experiments, 13-06-MG cells were preincubated with OXAL (100 μ M) for 24 hours. In each cell examined, I_K was elicited from -50 mV to different voltages ranging from -80 to +40 mV with 10-mV increments. $\square$: control; $\circ$: in the presence of 1 μ M TRAM-34. Each point indicates the mean $\pm$ SEM ($n=8-11$). Addition of TRAM-34 was not found to have any effects on I - V relationship of I_K in OXAL-treated 13-06-MG cells.

Fig. 5. Effects of DCEBIO, TRAM-34 and clotrimazole on the activity of intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels in 13-06-MG cells. Cells were immersed in normal Tyrode's solution which contained 1.8 mM $CaCl_2$. Cell-attached current recordings were made and the cell examined was held at -80 mV relative to the bath. (A) Single IK_{Ca} -channel currents recorded from 13-06-MG cells. IK_{Ca} -channel opening shown in this and the following figures gives a downward deflection in current. a: control; b: 10 μ M DCEBIO; c: 1 μ M TRAM-34; d: 3 μ M clotrimazole. CTZ: 3 μ M clotrimazole. (B) Summary of the data showing effects of DCEBIO, TRAM-34 and clotrimazole on the open probability of IK_{Ca} channels (mean $\pm$ SEM; $n=8-10$ for each bar). The probabilities of IK_{Ca} channels were measured at -80 mV relative to the bath. *Significantly different from control ($P<0.05$). Notably, addition of DCEBIO increases the open probability of IK_{Ca} channels effectively, while that of either TRAM-34 or clotrimazole diminishes it.

mV did not significantly differ between TRAM-34 alone group and TRAM-34 plus OXAL group (78 ± 6 pA [in the presence of TRAM-34, $n=7$] versus 77 ± 7 pA [in the presence of TRAM-34 plus OXAL, $n=7$], $P>0.05$). The data suggest that OXAL can block an IK_{Ca} -channel-mediated current in these cells.

Inability of TRAM-34 to Suppress I_K in 13-06-MG Cells Preincubated with OXAL

In this study, effect of TRAM-34 on I_K was also evaluated in OXAL-treated cells. As depicted in Fig. 4, in 13-06-MG cells preincubated with 100 μ M OXAL for 24 hours, inhibitory effect of TRAM-34 (1 μ M) on I - V relationships of I_K became abolished. For example, in cells pretreated with OXAL (100 μ M), after addition of TRAM-34 at a concentration of 1 μ M, the I_K amplitude measured at the level of +40 mV was not changed significantly (115 ± 13 pA [control, $n=11$] versus 110 ± 12 pA [in the presence of TRAM-34, $n=9$], $P>0.05$). Therefore,

Fig. 6. Concentration-dependent inhibition of OXAL on IK_{Ca} -channel activity in 13-06-MG cells. Cell-attached recordings of single channels were performed in these experiments and the probabilities of channel openings were measured at -80 mV relative to the bath when cells were exposed to different OXAL concentrations. (A) Original current traces of IK_{Ca} channels obtained with or without addition of OXAL. a: control; b: 30 μ M OXAL; c: 100 μ M OXAL. (B) Concentration-response curve for OXAL-induced suppression of IK_{Ca} channels recorded from 13-06-MG cells (mean $\pm$ SEM; n=12-15 for each point). OXAL at various concentrations (3 μ M-1 mM) was added to the bath as the activity of IK_{Ca} channels was clearly detected at -80 mV relative to the bath. Smooth curve was well fitted with a least-squares procedure.

in OXAL-treated 13-06-MG cells, addition of TRAM-34 was unable to suppress I_K amplitude effectively.

Properties of IK_{Ca} Channels in 13-06-MG Cells

Because the OXAL-mediated inhibition of I_K described above may arise from its effects on IK_{Ca} channels, we next examined the electrophysiological and pharmacological properties of single IK_{Ca} -channel currents in 13-06-MG cells. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, cell-attached current recordings were performed, and the recording pipette was filled with K^+ -containing solution described above. As shown in Fig. 5A, in cell-attached patches, the activity of IK_{Ca} channels with spontaneous open and closed transition was readily detected when the cell was held at -80 mV relative to the bath. Under our experimental conditions, the activity of IK_{Ca} channels can be functionally expressed as reported previously in different types of cells [2, 4, 13-15]. In cell-attached configuration, immediately following addition of 2 mM $CaCl_2$ or 10 μ M ionomycin, IK_{Ca} channels greatly opened with no change in single-channel amplitude. Further addition of 2 mM $CaCl_2$ and that of 10 μ M ionomycin significantly raised channel activity to 0.093 ± 0.021 (n=6, $P<0.05$) and 0.104 ± 0.0025 (n=7, $P<0.05$) from a control of 0.031 ± 0.011 (n=9), respectively. These channels are thus sensitive to the level of cytosolic Ca^{2+} ions. Additionally, cell exposure to DCEBIO (10 μ M) increased the probability of IK_{Ca} -channel openings by 3 folds, while TRAM-34 (1 μ M) or clotrimazole (3 μ M) was effective at suppressing channel activity by 20% (Fig. 5). Similar results were also obtained in rat CNS-1 astrocytoma cells. In addition, in continued presence of OXAL (100 μ M), subsequent addition of TRAM-34 (1 μ M) did not suppress channel activity further (0.006 ± 0.004 pA [in the presence of OXAL, n=8] versus 0.006 ± 0.003 pA [in the presence of OXAL plus TRAM-34, n=8], $P>0.05$). It is thus clear from these data that the IK_{Ca} channels were functionally expressed in 13-06-MG cells and

Fig. 7. The activity of IK_{Ca} channels measured at different potentials during cell exposure to various concentrations of OXAL. Panels (A) and (B) illustrate original current traces of single IK_{Ca} channels at different membrane voltages in the absence and presence of 100 μ M OXAL, respectively. The number shown in each current trace indicates the potential held. (C) The relationships of single-channel amplitude versus potential (i.e., Δ voltage) in the absence and presence of 30 and 100 μ M OXAL (mean $\pm$ SEM; $n=9-11$ for each point). Single-channel amplitudes at the potentials ranging from -80 to 0 mV with 10-mV increments were measured. ■: control; □: in the presence of 30 μ M OXAL; ○: in the presence of 100 μ M OXAL. Note that the single-channel conductance (i.e., slope factor) of IK_{Ca} channels with or without addition of OXAL did not differ significantly in 13-06-MG cells.

that their biophysical and pharmacological profiles tend to be indistinguishable from those previously reflected [2, 4, 11-13].

Inhibitory effect of OXAL on the activity of IK_{Ca} channels in 13-06-MG cells

The relationship between the OXAL concentration and the percentage inhibition of IK_{Ca} channel activity was derived and then constructed. In these experiments, each cell was held at -80 mV relative to the bath and the probabilities of channel openings in the absence and presence of different OXAL concentrations were measured. As illustrated in Fig. 6, OXAL (3

Fig. 8. Effect of OXAL on the activity of large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels in 13-06-MG cells. Cell-attached current recordings were conducted when cells were bathed in high K^+ solution containing 1.8 mM $CaCl_2$. (A) Single BK_{Ca} -channel traces obtained as the cells were held at +60 mV. Upward deflection shown in each trace indicates the opening event of the channel. a: control; b: 100 μ M OXAL; c: 100 μ M OXAL plus 1 μ M paxilline. (B) Summary of the data showing effects of OXAL, OXAL plus paxilline and OXAL plus PF573228. OXAL: 100 μ M OXAL; Pax: 1 μ M paxilline; PF: 3 μ M PF573228. Each bar indicates the mean $\pm$ SEM (n=10-13). *Significantly different from control ($P < 0.05$). **Significantly different from OXAL (100 μ M) alone group ($P < 0.05$).

μ M-1 mM) suppressed the activity of IK_{Ca} channels in a concentration-dependent manner. The IC_{50} value required for inhibitory effect of OXAL on IK_{Ca} -channel activity in 13-06-MG cells was calculated to be 67 μ M, and this drug at a concentration of 1 mM nearly abolished channel activity. Findings from these observations thus indicate that OXAL is capable of exerting a depressant action on the probability of IK_{Ca} -channel openings in these cells.

Effects of OXAL on Single-Channel Conductance of IK_{Ca} Channels

The effect of OXAL on the activity of IK_{Ca} channels at different levels of membrane potentials was also determined. The single-channel amplitudes were increased with greater hyperpolarization. It was also noted that OXAL suppressed the probability of IK_{Ca} -channel openings at different levels of membrane potential (Fig. 7A and 7B). The single-channel conductance calculated from a linear I - V relationship between the absence and presence of OXAL (30 and 100 μ M) was not found to differ significantly (32.3 ± 0.4 pS [in the absence of OXAL], n=9; 31.9 ± 0.4 pS [in the presence of 30 μ M OXAL], n=9; and 32.0 ± 0.4 pS [in the presence of 100 μ M OXAL], n=10; $P > 0.05$) (Fig. 7C). These data clearly indicated that despite its inhibitory effects on the activity of IK_{Ca} channels, OXAL exerted little or no effects on single-channel conductance of these channels in 13-06-MG cells.

Effect of OXAL, OXAL plus Paxilline, and OXAL plus PF573228 on Large-Conductance Ca^{2+} -Activated K^+ (BK_{Ca}) Channels in 13-06-MG Cells

We further evaluated whether another types of Ca^{2+} -activated K^+ channels (e.g., BK_{Ca} channels) can be affected by OXAL in these cells, since the mRNA expression of *KCNMA1* was detected (Fig. 1). To detect the activity of BK_{Ca} channels, cells were bathed in high- K^+ solution which contained 1.8 mM $CaCl_2$. Consistent with previous observations [7, 33], as the cells were held at +60 mV, BK_{Ca} -channel activity can be readily detected (Fig. 8). After

Fig. 9. Effect of OXAL on inwardly rectifying K^+ current ($I_{K(IR)}$) in 13-06-MG cells. In these experiments, cells were immersed in Ca^{2+} -free high- K^+ solution. (A) Original current traces in response to the ramp pulse. The cells were held at -50 mV and the ramp pulses from -80 to $+80$ mV with a duration of 1 sec were applied. a: control; b: $100 \mu M$ OXAL, c: $100 \mu M$ OXAL plus 1 mM $BaCl_2$. (B) Summary of the data showing effects of OXAL ($100 \mu M$), $BaCl_2$ (1 mM) and triptolide ($3 \mu M$) on $I_{K(IR)}$ amplitude in 13-06-MG cells (mean $\pm$ SEM; $n=8-9$ for each bar). *Significantly different from control ($P<0.05$).

addition of OXAL at a concentration of $100 \mu M$, the probability of channel openings was not changed significantly (0.126 ± 0.009 [control, $n=13$] versus 0.125 ± 0.011 [in the presence of OXAL, $n=11$], $P>0.05$). However, in continued presence of $100 \mu M$ OXAL, subsequent addition of paxilline ($1 \mu M$) suppressed channel activity significantly to 0.010 ± 0.005 ($n=12$), while that of PF573228 ($3 \mu M$) effectively raised it to 0.243 ± 0.023 ($P<0.05$). PF573228 was previously reported

to activate BK_{Ca} channels, while paxilline was effective at inhibiting them [31]. Hence, by comparison, BK_{Ca} channels inherent in these cells is not sensitive to inhibition by OXAL.

Lack of OXAL to Influence the Inwardly Rectifying K^+ Current ($I_{K(IR)}$) in 13-06-MG Cells

Earlier reports have clearly shown the presence of $I_{K(IR)}$ in glioma cells [6]. We also investigated whether OXAL can exert any effects on $I_{K(IR)}$ functionally expressed in these cells. This set of experiments was conducted in cells which were bathed in Ca^{2+} -free high- K^+ solution. Each cell was held at -50 mV and the up-sloping ramp pulses from -80 to $+80$ mV were applied. We failed to detect that OXAL at a concentration of $100 \mu M$ exerts any effect on the I - V relationship of $I_{K(IR)}$ in these cells (Fig. 9). On the other hand, addition of $BaCl_2$ (1 mM) or triptolide ($3 \mu M$) alone was effective at suppressing the amplitude of $I_{K(IR)}$ elicited by the ramp pulse. Triptolide was recently reported to suppress $I_{K(IR)}$ effectively in glioma cells [6]. These results reflect that, unlike IK_{Ca} channels, the $I_{K(IR)}$ in these cells is not vulnerable to block by OXAL.

OXAL-Mediated Inhibition of Proliferation and Migration in 13-06-MG Cells

In final set of experiments, the effect of OXAL on the growth in these cells was evaluated. As depicted in Fig. 10A, as OXAL was added to the culture medium of proliferating glioma cells, the rate of cell growth became decreased in a concentration-dependent manner. Moreover, as total cell number was suppressed by OXAL ($100 \mu M$), further addition of TRAM-34 ($1 \mu M$) did not decrease the proliferation of 13-06-MG cells. At a concentration of 1 mM, OXAL almost fully inhibited cell proliferation. The OXAL concentration required for effective inhibition of IK_{Ca} channels appears to be comparable to that used to suppress cell proliferation in 13-06-MG cells, suggesting that this drug exerts cytotoxic effects in these cells.

We further examined the effect of OXAL on cell viability by trypan blue exclusion assay. As shown in Fig. 10B, after 13-06-MG cells were exposed to OXAL ($100 \mu M$) for 1, 2 and 3 days, a significant inhibition in cell growth was observed. However, cell treatment with OXAL ($100 \mu M$) for 12 hours did not suppress the proliferation of these cells significantly. The results suggest that OXAL can exert an inhibition of cell growth in a time-dependent fashion.

Fig. 10. OXAL-mediated suppression of cell proliferation (A and B) and migration ability (C) in 13-06-MG cells. In (A), the percentage inhibition of cell growth was calculated in the absence and presence of different concentrations of OXAL. Cell number in the absence and presence of OXAL was measured using colorimetric method described in Materials and Methods. Each bar indicates the mean $\pm$ SEM derived from four independent experiments with triplicate samples. In (B), the number of viable cells was counted at 1, 2 and 3 days after being incubated with or without 100 μ M OXAL. Each point represents the mean $\pm$ SEM derived from four independent experiments. *Significantly different from controls (i.e., in the absence of OXAL) ($P < 0.05$). In (C), Boyden chamber cell migration assay was used. The cells were induced to migrate by means of the exposure to LPS (0.5 μ g/ml) and TPA (400 ng/ml) for 6 hours. The migration ability during exposure to LPS and TPA was taken to be 100% and those in the presence of LPS and TPA plus different OXAL concentrations were measured and compared. Each bar shown in each panel indicates the mean $\pm$ SEM derived from four independent experiments with triplicate samples. *Significantly different from control ($P < 0.05$).

By use of Boyden chamber migration assay, treatment of cells with LPS (0.5 μ g/ml) plus TPA (400 ng/ml) for 6 hours was noted to increase the migration ability significantly. However, after cells were treated with different OXAL concentrations for 6 hours, cell migration ability was drastically impaired. Incubation of cells with LPS, TPA and OXAL for 6 hours significantly decreased the migration ability, as compared with LPS and TPA (Fig. 10C). The results showed that OXAL was able to depress the migration ability induced by LPS plus TPA in 13-06-MG cells.

Ability of TRAM-34 and OXAL to Suppress the Activity of IK_{Ca} Channels in LoVo Colorectal Carcinoma Cells

OXAL may not be the standard first or second line therapy in glioma. Because OXAL has been used in adjuvant treatment of advanced colorectal cancers, we further attempted

Fig. 11. Inhibitory effects of TRAM-34 and OXAL on IK_{Ca} -channel activities in LoVo colorectal carcinoma cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and cell-attached current recordings were made in these experiments. In (A), single IK_{Ca} -channel currents were recorded in the control and in the presence of TRAM-34 (1 μ M) or OXAL (100 μ M), as the cells were held at -80 mV relative to the bath. Downward deflection shown in each trace indicates the opening event of the channel. TRAM-34: 1 μ M TRAM-34; OXAL: 100 μ M OXAL. (B) Summary of the data showing effects of TRAM-34 (1 μ M) or OXAL (100 μ M) on the probability of IK_{Ca} -channel openings in 13-06-MG cells (mean $\pm$ SEM; n=11 for each bar). *Significantly different from control ($P<0.05$).

to explore whether the activity of IK_{Ca} channels present in LoVo cells is sensitive to block by OXAL or TRAM-34. Consistent with the observations seen in 13-06-MG glioma cells, OXAL was effective in decreasing the probability of IK_{Ca} -channel openings in LoVo cells (Fig. 11). For example, when the cells were held at -80 mV relative to the bath, OXAL at a concentration of 100 μ M significantly decreased channel activity to 0.022 ± 0.005 (n=11, $P<0.05$) from a control of 0.092 ± 0.009 (n=11). Therefore, OXAL-induced proliferation of colorectal cancer cells appears to be linked to its perturbation of IK_{Ca} -channel activity.

Discussion

Findings of this study show that (a) in human 13-06-MG glioma cells, OXAL decreases the amplitude of I_K in human 13-06-MG glioma cells; (b) DCEBIO or ionomycin can reverse OXAL-induced inhibition of I_K ; (c) OXAL diminishes the activity of IK_{Ca} channels in a dose-dependent manner, while it did not change single-channel conductance of these channels significantly; (d) neither the activity of BK_{Ca} channels nor $I_{K(IR)}$ amplitude was affected by OXAL; (e) OXAL suppressed cell growth and migration in a concentration- and time-dependent manner in these cells; and (f) this compound decreased the activity of IK_{Ca} channels in LoVo colorectal cancer cells. Therefore, the OXAL-mediated effect on ion channels presented herein may potentially influence the malignant behaviors of glioma cells, if similar *in vivo* actions exist.

Consistent with previous observations [2], the activity of IK_{Ca} channels was clearly detected in 13-06-MG cells. The probability of IK_{Ca} -channel openings in these cells can be decreased by TRAM-34 or clotrimazole and be subject to stimulation by DCEBIO. Additionally,

in the present study, OXAL-mediated inhibition of I_K observed in 13-06-MG cells is primarily attributed to its suppression of IK_{Ca} channels. Moreover, the IC_{50} value of OXAL required for the inhibition of IK_{Ca} -channel activity observed in this study was approximately 67 μ M, a value that is comparable to the concentrations of this drug used to suppress cell growth. This value is slightly higher than that used to open gap junction channels [34]. Single-channel recordings clearly showed the functional expression of IK_{Ca} in 13-06-MG and LoVo cells. The inhibitory effect of OXAL on IK_{Ca} channels in glial or glioma cells may thus occur at a concentration achievable in humans. Whether the effects of OXAL on ion currents in this *in vitro* study occur in humans remains to be further delineated.

It needs to be noted that the OXAL concentration used in our study tends to be greater than that achieved in the plasma of treated patients (3.6-5.6 μ M) [35, 36]. Any changes of IK_{Ca} -channel activity suppressed by OXAL depend on not only the OXAL concentration, but also membrane potential, intracellular Ca^{2+} concentration and cell volume. Moreover, the OXAL concentrations may not be similar to those present in tissues, because there are areas outside the circulation where OXAL can accumulate at much higher concentrations [36]. For example, heated intraperitoneal chemotherapy was previously demonstrated to produce high peritoneal and tumor OXAL concentrations with limited systemic absorption [37]. One of the reasons could arise from the possibility that OXAL can react with endogenous sulfur compounds (e.g., glutathione) [36, 37]. It has also been shown that in patients heavily pre-treated with OXAL, a redistribution of the pool of intra-erythrocytic OXAL biotransformation products into the plasma may aggravate neurotoxic effect of OXAL [38]. Notably, a current study showed that, in heat intraperitoneal chemotherapy with OXAL which is not standard chemotherapy, the OXAL concentrations in tissue can be around 47-85 mg/Kg (118-214 μ M) [39]. Additionally, recent investigations have focused on the utilization of different drug-delivery vehicles in facilitating specific delivery of OXAL to tumor tissues [40, 41]. The observed effects by this compound in this study may occur at the range of clinically achievable concentrations, as such maneuvers are implemented to reduce the systemic toxicity.

It needs to be mentioned that cisplatin (100 μ M), another platinum-based agent, was not found to have any effects on the activity of IK_{Ca} channels in 13-06-MG cells. OXAL-induced inhibition of IK_{Ca} -channel activity may be attributed to an interaction of the oxalate group embedded in this molecule with free Ca^{2+} ions [42]. OXAL and its structurally related compounds would be intriguing pharmacological or toxicological tools used to characterize the IK_{Ca} channels.

With the aid of ChemBio3D analysis, the minimal energies for OXAL/Na complex and OXAL/Ca were calculated and compared in our study. Of particular, as Ca^{2+} ions are exposed to OXAL, the total energy was greatly changed to -0.0601 kcal/mol. However, the total energies for OXAL/Na complex or cisplatin/Ca complex were 6.3399 and -37.2413 kcal/mol, respectively. The data led us to indicate that, as compared with that of OXAL/Na or cisplatin/Ca complex, the degree of freedom in OXAL/Ca complex becomes strongly restricted. In OXAL-preincubated cells, addition of TRAM-34 was unable to suppress I_K amplitude in 13-06-MG cells. Therefore, the mode of inhibitory action on IK_{Ca} channels and cell proliferation is necessarily linked to an interaction of the OXAL molecule with free Ca^{2+} ions [42], although it can adduct with DNAs that lead to cell death and intracellular generation of reactive oxygen species following the crosslinking with DNAs [18, 24, 25].

It is also noteworthy that the observed decrease in whole-cell I_K by OXAL did not appear to match OXAL-induced decrease in IK_{Ca} -channel activity. The OXAL-induced decrease in I_K amplitude was found to be less than its inhibition of IK_{Ca} -channel activity. The reason for this discrepancy is currently unclear. It is possible that the observed I_K amplitude was contaminated with other K^+ currents under whole-cell current recordings. Because the probability of IK_{Ca} -channel openings can be regulated by calmodulin activity [29], to what extent changes in the level of either intracellular Ca^{2+} or calmodulin affect the effects of OXAL on I_K remains to be determined.

This study prompts us to propose that, assuming that similar findings observed in this study occur in glial or glioma cells *in vivo*, any changes in the magnitude of functional

expression in IK_{Ca} channels may underlie another important elements through which OXAL modulates their functional activities of these cells. Cytotoxic actions of OXAL are also likely to be enhanced under states as the activity of IK_{Ca} channels in neurons or glial cells are abnormally increased [13, 42]. Whether the activators of IK_{Ca} channels or/and suppression of gap junction channels are effective at alleviating OXAL-induced neurotoxicity [23, 34, 41, 43] necessitates further investigation.

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the author(s).

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