Acta Pharmacol Sin 2008 Sep; 29 (9): 1006–1012

Full-length article

Wuweizisu C from *Schisandra chinensis* decreases membrane potential in C6 glioma cells[1]

Young-whan CHOI[2,4], Kyeok KIM[3,4], Ji-yeong JO[3], Hyo-lim KIM[3], You-jin LEE[2], Woo-jung SHIN[2], Santosh J SACKET[3], Mijin HAN[3], Dong-soon IM[3,5]

[2]*Department of Horticultural Bioscience Pusan National University, Miryang-si 627-706, Republic of Korea;* [3]*Laboratory of Pharmacology, College of Pharmacy (BK21 project) and Longevity Life Science and Technology Institutes, Pusan National University, Busan 609-735, Republic of Korea*

Key words

Schisandra chinensis; lignan; wuweizisu C; membrane potential; glioma; dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate

[1]Project supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Korea.
[4]These two authors contributed equally to this article.
[5]Correspondence to Dr Dong-soon IM.
Phn 82-51-510-2817.
Fax 82-51-513-6754.
E-mail imds@pusan.ac.kr

Received 2008-03-10
Accepted 2008-06-02

doi: 10.1111/j.1745-7254.2008.00838.x

Abstract

Aim: To study the effects of dibenzocyclooctadiene lignans isolated from *Schisandra chinensis*, such as wuweizisu C, gomisin N, gomisin A, and schisandrin, on the membrane potential in C6 glioma cells. **Methods:** The membrane potential was estimated by measuring the fluorescence change in DiBAC-loaded glioma cells. **Results:** Wuweizisu C decreased the membrane potential in a concentration-dependent manner. Gomisin N and gomisin A, however, showed differential modulation and no change was induced by schisandrin or dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate, a synthetic drug derived from dibenzocyclooctadiene lignans. We found no involvement of $G_{i/o}$ proteins, phospholipase C, and extracellular Na^+ on the wuweizisu C-induced decrease of the membrane potential. Wuweizisu C by itself did not change the intracellular Ca^{2+} $[Ca^{2+}]_i$ concentration, but decreased the ATP-induced Ca^{2+} increase in C6 glioma cells. The 4 lignans at all concentrations used in this study did not induce any effect on cell viability. Furthermore, we found a similar decrease of the membrane potential by wuweizisu C in PC12 neuronal cells. **Conclusion:** Our results suggest that the decrease in the membrane potential and the modulation of $[Ca^{2+}]_i$ concentration by wuweizisu C could be important action mechanisms of wuweizisu C.

Introduction

Schisandra chinensis Baill grows wild in Russia, China, Korea, and Japan. In Oriental countries, ripe Schisandra fruits have been used as a tonic[1]. The fruit of *Schisandra chinensis* has been found to be effective in viral and chemically-induced hepatitis[2]. It is enriched in lignans, and more than 30 lignans have been isolated from this fruit[3,4]. Several lignans, including wuweizisu C and gomisin A, have been reported to protect the liver from hepatotoxic chemicals[5–7]. Pharmacological studies of lignans have also revealed anti-inflammatory[8] and anti-HIV effects[9]. Gomisin A also suppresses liver carcinogenesis by tumor-promoting agents[10–12]. Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB), a synthetic drug derived from dibenzocyclooctadiene lignans, was developed as a supplement for acute and chronic hepatitis patients[13–17].

In a previous study, wuweizisu C, gomisin N, and deoxyschisandrin exhibited significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells[18]. Gomisin A improved scopolamine-induced memory impairment via the enhancement of the cholinergic nervous system in mice[19]. In addition, the beneficial effect of *Fructus schisandrae* on cycloheximide-induced amnesia was found to be enhanced by treatment with serotonergic 5-HT_2 receptor antagonists, but was attenuated by serotonergic 5-HT_{1A} receptor agonists and cholinergic antagonists[20,21]. *Schisandra chinensis* fruit was accepted as a main herbal prescription for the improvement of learning performance in senescence-accelerated mice and for scopolamine-induced memory impairment in mice[22,23].

Ion channels for K^+ and Cl^- are involved in regulating the proliferation rate through the modulation of the membrane potential and cell volume[24]. Chlorotoxin, a putative Cl^- channel-specific inhibitor, has been shown to interfere with glioma cell invasion and is now in phase II of clinical trials for the treatment of gliomas[25]. The modulation of the membrane potential plays an important role in neuronal and smooth muscle cells. However, it has been poorly investigated in glioma cells, although voltage-gated ion channels expressed in glia cells function to modulate the membrane potential and cell cycle[26]. Thus in this study, we tested the effect of 4 dibenzocyclooctadiene lignans, wuweizisu C, gomisin N, gomisin A, and schisandrin isolated and identified from *Schisandra chinensis* on the membrane potential in C6 glioma and PC12 neuronal cells (Figure 1) and further characterized the response with specific pharmacological inhibitors.

Materials and methods

Extraction and isolation Fruits of *Schisandra chinensis* were collected in September 2002 from Mujoo, Korea. A voucher specimen (Accession No SC-NCNPR-1) was deposited at the herbarium of The University of Mississippi (USA). The plant was identified by one of the authors (Young-whan CHOI). The compounds wuweizisu C, gomisin N, gomisin A, and schisandrin (Figure 1) were isolated from the fruits of *Schisandra chinensis* [27], and the purity checked with HPLC was 92%–96%[28].

Chemical agents $DiBAC_4(3)$ was acquired from Biotium (Hayway, CA, USA). U73122 was from Biomol (Plymouth Meeting, PA, USA), and DDB was kindly provided by PharmaKing (Seoul, Korea).

Cell culture Rat C6 glioma cells were maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% (*v*/*v*) fetal bovine serum, 100 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate at 37 °C in a humidified 5% CO_2 incubator[29]. PC12 cells were maintained in RPMI-1640 containing 5% (*v*/*v*) fetal bovine serum, 15% (*v*/*v*) horse serum, 100 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate at 37 °C in a humidified 5% CO_2 incubator.

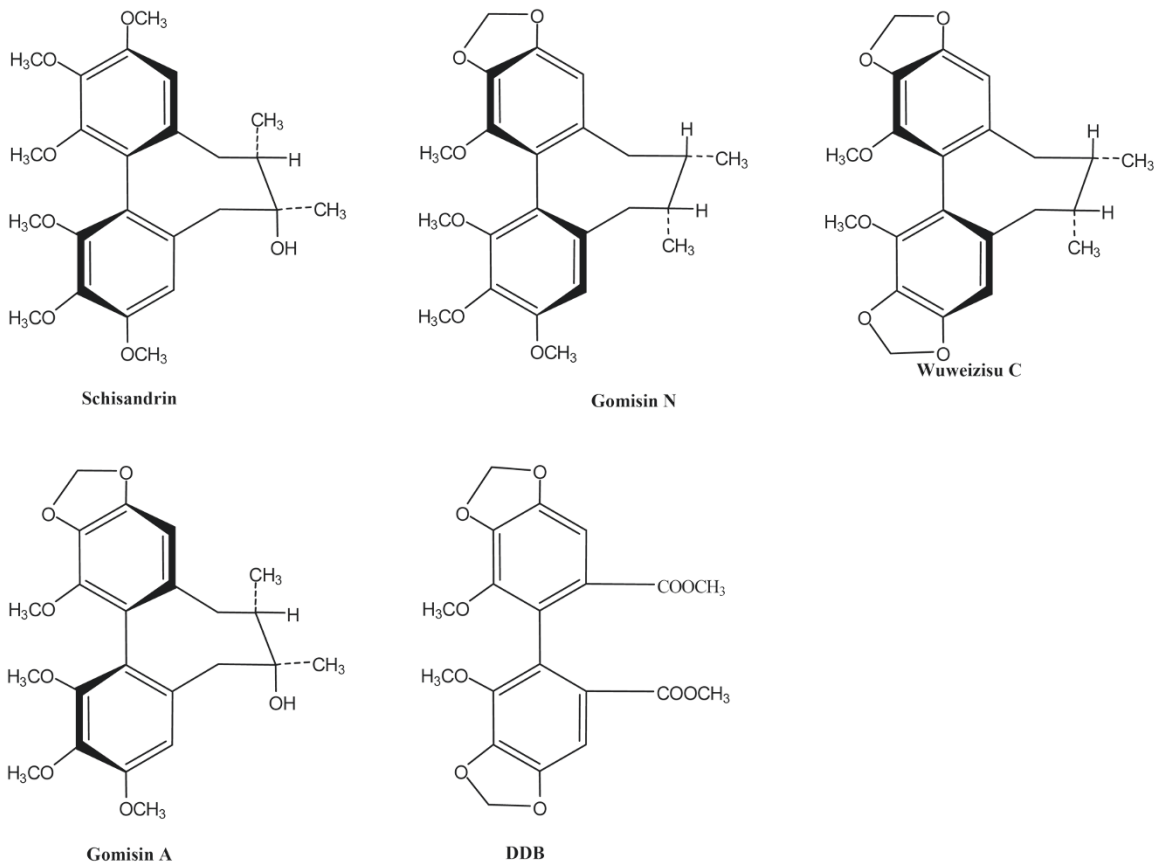

Figure 1. Chemical structures of the 4 lignans isolated from *Schisandra chinensis* and DDB.

Measurement of membrane potential The cells were trypsin digested, sedimented, and resuspended in HEPES-buffered medium consisting of 20 mmol/L HEPES (pH 7.4), 103 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 0.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 15 mmol/L glucose, and then incubated for 30 min with 5 μmol/L $DiBAC_4(3)$. Fluorescence emission from the excitation wavelength (488 nm) was measured at 530 nm every 0.1 s by a F4500 fluorescence spectrophotometer (Hitachi, Japan). The membrane potential was estimated from measurements of the fluorescence change of DiBAC-loaded cells[29].

Measurements of intracellular Ca^{2+} concentration Cells were trypsin digested, sedimented, resuspended in HEPES-buffered medium (20 mmol/L HEPES [pH 7.4], 103 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 0.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, 15 mmol/L glucose, and 0.1% bovine serum albumin [fatty acid free]), and incubated for 40 min with 5 μmol/L Fura-2/AM for the Ca^{2+} measurement. Intracellular Ca^{2+} $[Ca^{2+}]_i$ was estimated from the change in fluorescence of the Fura 2-loaded cells[30]. The fluorescence emissions at 510 nm from excitation wavelengths of 340 and 380 nm were measured every 0.1 s, and the ratio of fluorescence intensities from the 2 wavelengths (340/380) was monitored to estimate $[Ca^{2+}]_i$[31].

Data presentation Representative traces for the membrane potential were chosen from 3 independent experiments and are shown in Figures 2–5. The results from 3 independent experiments, shown as the percentage of the control level, are shown in Figures 2 and 5.

Results

Lignans from *Schisandra chinensis* induce changes of membrane potential in C6 glioma cells In C6 glioma cells, wuweizisu C decreased the membrane potential in a concentration-dependent manner (Figure 2A, 2E). Gomisin N and gomisin A decreased the membrane potential by 100 μmol/L (Figure 2C, 2D, 2G, 2H). Schisandrin, however, did not change the membrane potential up to 100 μmol/L (Figure 2B, 2F). Similarly, DDB did not change the membrane potential, even at 100 μmol/L (data not shown).

Involvement of G proteins and phospholipase C in wuweizisu C-induced membrane potential Pertussis toxin has been used to elucidate the involvement of $G_{i/o}$-type G proteins in various signaling pathways[29,32]. Thus we treated C6 glioma cells with pertussis toxin (100 ng/mL, 24 h). However, the wuweizisu C-induced decrease in the membrane potential was not blunted, suggesting no involvement of G-protein-coupled receptors coupled to $G_{i/o}$-type G proteins (Figure 3A). U73122 is a pharmacological tool to test the involvement of phospholipase C located in the plasma membrane and activated through $G_{q/11}$-protein-coupled receptors[32]. We treated C6 glioma cells with U73122 (5 μmol/L, 10 min) and found no change in the wuweizisu C-induced decrease in the membrane potential (Figure 3B).

Effects of 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA) and Na^+-free media on the wuweizisu C-induced membrane potential We next tested the effects of EIPA and Na^+-free media on the wuweizisu C-induced change in the membrane potential. EIPA is an inhibitor of the Na^+/H^+ exchanger (NHE). By itself, EIPA treatment dramatically decreased the membrane potential and this decrease lasted for 30 min (Figure 3C). After 30 min of EIPA treatment, it was impossible to say whether NHE was a component of the wuweizisu C-induced decrease in the membrane potential because the resting membrane potential was decreased by the EIPA treatment. However, EIPA-sensitive NHE was definitely a regulator of the membrane potential in C6 cells[33].

When the effect of wuweizisu C on the membrane potential was measured in Na^+-free medium, the wuweizisu C-induced decrease in the membrane potential was not affected by the depletion of Na^+ in the extracellular medium (Figure 3D), suggesting that extracellular Na^+ is not important for the membrane potential decrease by wuweizisu C in C6 glioma cells.

Effect of wuweizisu C on $[Ca^{2+}]_i$ concentration We also measured changes in the $[Ca^{2+}]_i$ concentration. By themselves, the 4 lignans did not change $[Ca^{2+}]_i$, with the exception of gomisin N (Figure 4C). Gomisin N slowly increased $[Ca^{2+}]_i$; however, the increase did not affect the following the ATP-induced $[Ca^{2+}]_i$ increase. Wuweizisu C inhibited the ATP-induced increase of $[Ca^{2+}]_i$, but the increase was not inhibited by other lignans (Figure 4E-4H). The wuweizisu C-induced inhibition and gomisin N-induced increase of $[Ca^{2+}]_i$ implied the modulation of Ca^{2+} homeostasis by the lignans from *Schisandra chinesis*.

Wuweizisu C induces a decrease in membrane potential in PC12 neuronal cells To see the effects of these 4 lignans on the membrane potential in neurons, changes in the membrane potential induced by the lignans were measured in PC12 neuronal cells. Similarly, wuweizisu C decreased the membrane potential in a concentration-dependent manner (Figure 5A, 5E). Gomisin N decreased the membrane potential only at 100 μmol/L (Figure 5C). Schisandrin and gomisin A, however, did not change the

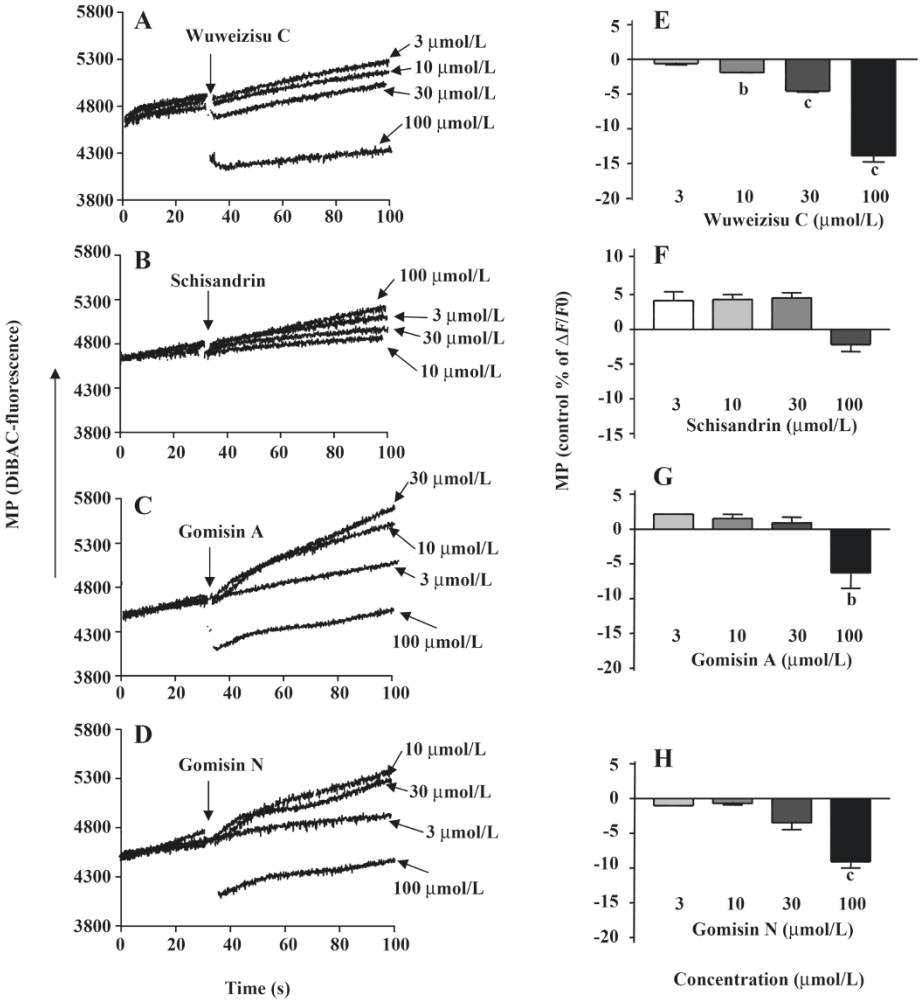

Figure 2. Effect of lignans from *Schisandra chinensis* on the membrane potential in C6 glioma cells. Representative traces of the membrane potential with various concentrations of wuweizisu C (A), schisandrin (B), gomisin A (C), and gomisin N (D) in DiBAC-loaded C6 glioma cells are shown. Lignans were added as indicated by the arrow (30 s). Concentration dependence of wuweizisu C- (E), schisandrin- (F), gomisin A-(G), and gomisin N (H)-induced decrease in the membrane potential (E). MP, membrane potential; F0, fluorescence value before addition of wuweizisu C; deltaF, maximum fluorescence change after the wuweizisu C addition. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

membrane potential even at 100 μmol/L (Figure 5B, 5D), suggesting differential sensitivity of PC12 neuronal cells to each lignan.

Discussion

In this study, the effects of wuweizisu C, gomisin A, gomisin N, and schisandrin, which are dibenzooctadiene lignans of *Schisandra* fruits, on the membrane potential were investigated in C6 glioma cells. We previously observed an increase of the membrane potential with sarcotride A and bio-active lysophospholipids, such as lysophosphatidic acid and lysophosphatidylserine in C6 glioma cells[29,33,34]. In the present study, we showed decreases of the membrane potential by wuweizisu C, gomisin N, and gomisin A. Although the precise mechanism for the decreases was not elucidated, we found independence of pertussis-sensitive G proteins, U73122-sensitive phospholipase C, and extracellular Na^+. Furthermore, we found that wuweizisu C decreased the membrane potential in PC12 neuronal cells. We also found that wuweizisu C inhibits the ATP-induced increase of the $[Ca^{2+}]_i$ concentration, and by itself, gomisin N increases the basal Ca^{2+} concentration. In primary-cultured rat cortical cells, the kainic acid-induced Ca^{2+} influx was significantly inhibited by wuweizisu C and gomisin N, supporting our observation[18].

Because schisandrin, gomisin A, and gomisin N have been shown to reverse cancer drug resistance by targeting P-glycoprotein (P-gp, ABCB1) or multidrug resistance-associated protein 1 (ABCC1)[35–41], we tested the effect of probenecid on the membrane potential in C6 glioma cells. However, probenecid (50 and 500 μmol/L) did not change the membrane potential in C6 glioma cells, excluding the possibility that wuweizisu C decreases the membrane potential through the modulation of P-gp in C6 glioma cells (data not shown).

The modulation of the membrane potential was induced

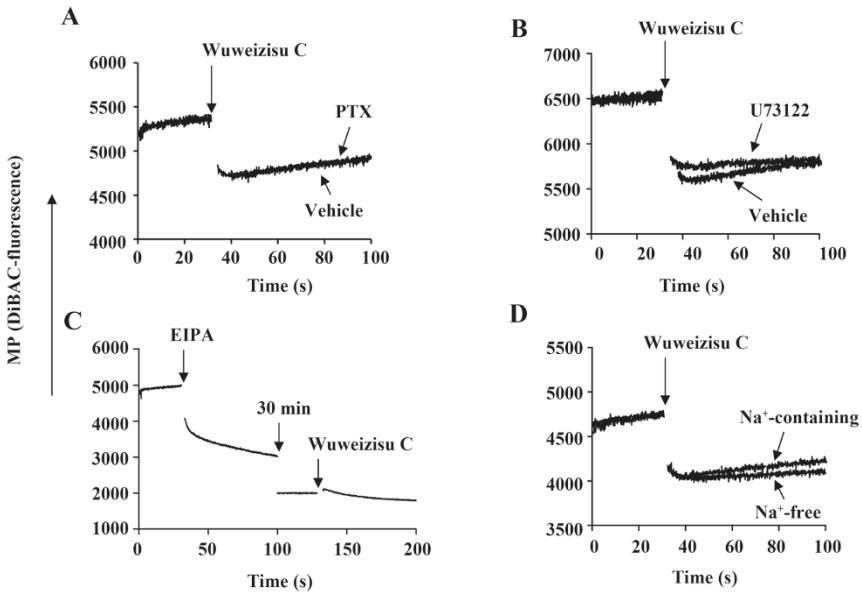

Figure 3. Effect of pharmacological inhibitors on wuweizisu C-induced decrease of the membrane potential. Representative traces of the membrane potential with 100 μmol/L wuweizisu C in DiBAC-loaded C6 glioma cells treated with pertussis toxin (A, 100 ng/mL, 24 h), U73122 (B, 5 μmol/L, 10 min), or vehicle are shown. Representative traces of the membrane potential with 100 μmol/L wuweizisu C in EIPA-treated cells (C, 100 μmol/L, 30 min) or Na^+-free media (D) in DiBAC-loaded C6 glioma cells are shown.

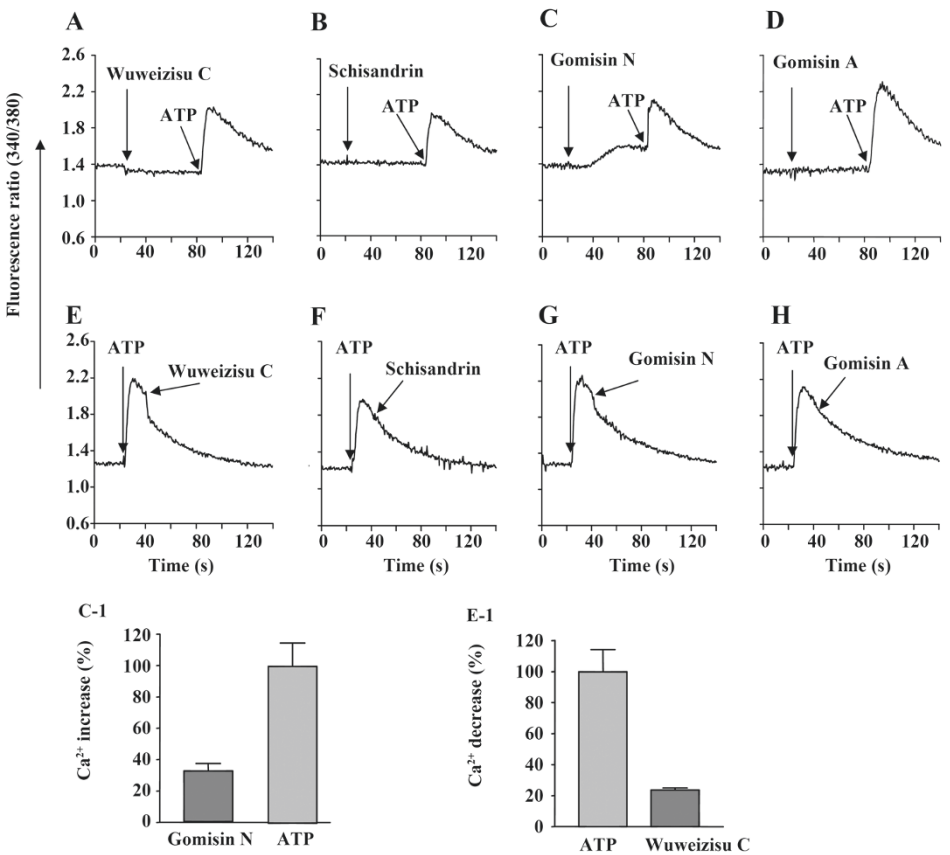

Figure 4. Effect of wuweizisu C on $[Ca^{2+}]_i$ concentration in C6 glioma cells. Representative traces of $[Ca^{2+}]_i$ concentration with 100 μmol/L wuweizisu C (A,E), schisandrin (B,F), gomisin N (C,G), and gomisin A (D,H) in Fura-2-loaded C6 glioma cells are shown. Each lignan (100 μmol/L) was added as indicated by the first arrow (30 s), and ATP (10 mmol/L) was added as indicated by the second arrow (90 s) in the upper panels (A-D). ATP (10 mmol/L) was added as indicated by the first arrow (30 s) and each lignan (100 μmol/L) was added as indicated by the second arrow (45 s) in the lower panels (E-H). Histograms show mean±SEM of $[Ca^{2+}]_i$ change from 3 independent experiments (C-1 and E-1). C-1 shows increase of $[Ca^{2+}]_i$ by gomisin N (100 μmol/L) and ATP (10 mmol/L). E-1 shows decrease of $[Ca^{2+}]_i$ by ATP (10 mmol/L) and wuweizisu C (100 μmol/L).

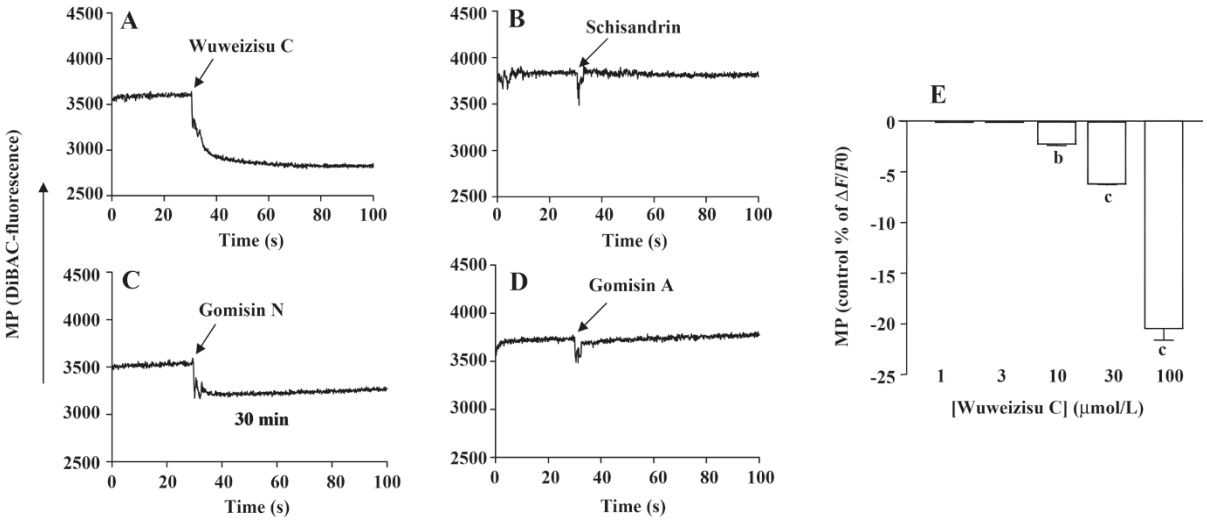

Figure 5. Effect of lignans from *Schisandra chinensis* on the membrane potential in PC12 neuronal cells. Representative traces of the membrane potential with 100 μmol/L of wuweizisu C, schisandrin, gomisin N, and gomisin A in DiBAC-loaded PC12 neuronal cells are shown. Lignan was added as indicated by the (30 s). Concentration dependence of wuweizisu C-induced decrease of the membrane potential (E). MP, membrane potential; F0, fluorescence value before addition of wuweizisu C; ΔF, maximum fluorescence change after addition of wuweizisu C. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

by wuweizisu C, gomisin N, and gomisin A, but not by schisandrin. Three modulatory lignans have 1–2 methylenedioxy groups on dibenzocyclooctadiene, but not in the schisandrin structure. Furthermore, gomisin N and gomisin A, which have just 1 methylenedioxy group, showed a decrease of the membrane potential at a 100 μmol/L concentration. Wuweizisu C, which has 2 methylenedioxy groups, showed a concentration-dependently decrease of the membrane potential. Thus we presumed that the 2 methylenedioxy groups are important for the modulation of the membrane potential and tested DDB, which has 2 methylenedioxy groups, but not the cyclooctadiene ring. DDB did not change the membrane potential like schisandrin in C6 glioma cells (data not shown), suggesting the importance of the cyclooctadiene ring as well as the methylenedioxy groups. This idea is supported by the changes in the membrane potential in PC12 neuronal cells because gomisin N decreased the membrane potential, but gomisin A did not, showing differential responses in glioma and neuronal cells to lignans containing 1 methylenedioxy group.

In summary, we showed the decrease in the membrane potential by wuweizisu C in C6 glioma and PC12 neuronal cells and its inhibition of the ATP-induced $[Ca^{2+}]_i$ increase. Although the precise mechanism for this decrease was not elucidated, the present study provides useful information for the elucidation of the action mechanisms of *Schisandra chinensis*, especially the lignans and drug development using *Schisandra chinensis* (fruits) or active lignans.

Author contribution

Young-whan CHOI and Dong-soon IM designed research; Kyeok KIM, Ji-yeong JO, Hyo-lim KIM, You-jin LEE, Woo-jung SHIN, Santosh J SACKET, and Mijin HAN performed research; Young-whan CHOI, Kyeok KIM analyzed data; Dong-soon IM wrote the paper.

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