Original Paper

Synergistic Inhibition of Delayed Rectifier K^+ and Voltage-Gated Na^+ Currents by Artemisinin in Pituitary Tumor (GH₃) CellsEdmund Cheung So^{a,b} Sheng-Nan Wu^c Ping-Ching Wu^{d,e,f} Hui-Zhen Chen^c
Chia-Jung Yang^c

^aDepartment of Anesthesia, An Nan Hospital, China Medical University, Tainan City, ^b Department of Anesthesia, College of Medicine, China Medical University, Taichung, ^cDepartment of Physiology, National Cheng Kung University Medical College, Tainan City, ^dDepartment of Biomedical Engineering, National Cheng Kung University, Tainan City, ^e Institute of Oral Medicine and Department of Stomatology, National Cheng Kung University Hospital, National Cheng Kung University Tainan City, ^fMedical Device Innovation Center, Taiwan Innovation Center of Medical Devices and Technology, National Cheng Kung University Hospital, National Cheng Kung University, Tainan City, Taiwan

Key Words

Artemisinin • Voltage-gated Na^+ current • Delayed-rectifier K^+ current • Current kinetics • Action potential • Pituitary cell

Abstract

Background: Artemisinin (ART) is an anti-malarial agent reported to influence endocrine function. **Methods:** Effects of ART on ionic currents and action potentials (APs) in pituitary tumor (GH₃) cells were evaluated by patch clamp techniques. **Results:** ART inhibited the amplitude of delayed-rectifier K^+ current ($I_{K(DR)}$) in response to membrane depolarization and accelerated the process of current inactivation. It exerted an inhibitory effect on $I_{K(DR)}$ with an IC_{50} value of 11.2 μM and enhanced $I_{K(DR)}$ inactivation with a K_D value of 14.7 μM . The steady-state inactivation curve of $I_{K(DR)}$ was shifted to hyperpolarization by 10 mV. Pretreatment of chlorotoxin (1 μM) or iloprost (100 nM) did not alter the magnitude of ART-induced inhibition of $I_{K(DR)}$ in GH₃ cells. ART also decreased the peak amplitude of voltage-gated Na^+ current (I_{Na}) with a concentration-dependent slowing in inactivation rate. Application of KMUP-1, an inhibitor of late I_{Na} , was effective at reversing ART-induced prolongation in inactivation time constant of I_{Na} . Under current-clamp recordings, ART alone reduced the amplitude of APs and prolonged the duration of APs. **Conclusion:** Under ART exposure, the inhibitory actions on both $I_{K(DR)}$ and I_{Na} could be a potential mechanisms through which this drug influences membrane excitability of endocrine or neuroendocrine cells appearing *in vivo*.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Artemisinin (ART, qinghaosu) has been well-recognized as an effective antimalarial agent for several centuries. ART is a natural product purified from the sweet woodworm

Ping-Ching Wu
and Sheng-NanDepartment of Biomedical Engineering, National Cheng Kung University, Tainan City, (Taiwan) and Department of Physiology, National Cheng Kung University Medical College, No. 1, University Road, Tainan City, (Taiwan)
E-Mail wbcxyz@gmail.com / snwu@mail.ncku.edu.tw

Artemisia annua L., is a sesquiterpenetrioxane lactone containing unusual peroxide bridge [1]. In addition to its antimalarial action, there are a variety of pharmacological effects including cytotoxicity against tumor cells as well as anti-viral and anti-parasitic actions [1, 2]. Specifically, the peroxide contained in ART and its semisynthetic derivatives is thought to be responsible for the drug's mechanism of action.

An early study shown that artesunate, an ART derivative, could suppress cell proliferation as well as decreasing the secretion of growth hormone in pituitary GH₃ cells [3]. Numerous reports demonstrated that ART was effective on suppressing tumor growth including pituitary macroadenoma [1-6]. A previous study reported the ability of ART in influencing the amplitude of ionic currents in neurons isolated from nodose ganglia [7]. ART-mediated sedative effects are also thought to be the consequence of the binding to central benzodiazepine receptors [8]. However, the detailed studies regarding ionic mechanism of ART actions on neurons or endocrine cells (e.g., pituitary cells) were not reported.

There are nine isoforms for voltage-gated sodium channel (Na_v1.1-1.9) found among mammalian excitable tissues, including central and peripheral nervous system, skeletal muscle and heart [9]. We have recently demonstrated that in pituitary GH₃ cells, KMUP-1 was able to decrease the peak amplitude of I_{Na} along with an increase in current inactivation [10]. Na_v1.7 was found to be a major subfamily of Na_v channels functionally expressed in these cells [11]. Regulation of these channels is indeed proven to be responsible for the actions on endocrine or neuroendocrine function occurring *in vivo* [12, 13]. The slowly inactivating delayed-rectifier K⁺ currents ($I_{K(DR)}$) have been reported in neurons and endocrine cells including GH₃ cells [14, 15]. The blockage by any compounds of $I_{K(DR)}$ becomes progressively significant when a train of action potentials (APs) occurs [15].

In this study we tried to explore the effect of ART on ionic currents (e.g., delayed rectifier K⁺ current [$I_{K(DR)}$], voltage-gated Na⁺ current [I_{Na}], and membrane potential in pituitary GH₃ cells. Our findings revealed that ART is able to suppress both $I_{K(DR)}$ and I_{Na} with different current kinetics, which will summate to affect the firing of APs. These inhibitory effects are not mediated by either changes in Cl⁻ channel activity or decreased intracellular cyclic AMP and they might conceivably contribute to pharmacological or toxicological actions of ART occurring *in vivo*.

Materials and Methods

Drugs and solutions

Artemisinin (ART, qinghaosu, C₁₅H₂₂O₅, (3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one), dopamine, tetraethylammonium chloride, tetrodotoxin, and trypan blue were purchased from Sigma-Aldrich (St Louis, MA). Iloprost (Ventavis®) was obtained from Bayer-Schering (Berlin, Germany). Chlorotoxin was kindly provided by Dr. Woei-Jer Chuang (Department of Biochemistry, National Cheng Kung University Medical College) and KMUP-1 (7-[2-[4-(2-chlorophenyl)piperazinyl]ethyl]-1,3-dimethylxanthine) was by Dr. Bin-Nan Wu (Department of Pharmacology, Kaohsiung Medical University, Kaohsiung, Taiwan). Unless stated otherwise, tissue culture media, horse serum, fetal calf serum, L-glutamine, penicillin-streptomycin, fungizone and trypsin were obtained from Invitrogen (Carlsbad, CA). All other chemicals including CdCl₂, CsCl and CsOH were commercially available and of reagent grade. Double-distilled water which was de-ionized through a Millipore-Q system (Bedford, MA) was used in all experiments.

The composition of normal Tyrode's solution was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.53 mM MgCl₂, 5.5 mM glucose, and 5.5 mM HEPES-NaOH buffer, pH 7.4. To record K⁺ currents or membrane potential, a patch pipette was filled with the following solution: 140 mM KCl, 1 mM MgCl₂, 3 mM Na₂ATP, 0.1 mM Na₂GTP, 0.1 mM EGTA, and 5 mM HEPES-KOH buffer, pH 7.2. To measure I_{Na} , KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7.2 with CsOH. To avoid possible contamination of Cl⁻ currents, Cl⁻ ions inside the pipette solution was replaced with aspartate. To measure *erg*-mediated K⁺ current ($I_{K(erg)}$), bath solution was replaced with a high-K⁺, Ca²⁺-free solution containing 130 mM KCl, 10 mM NaCl, 3 mM MgCl₂, and 5 mM HEPES-KOH, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl₂, and 5 mM HEPES-KOH, pH 7.2.

Cell preparation and differentiation

GH₃, a clonal cell line derived from rat prolactin-secreting pituitary tumor, was obtained from the Bioresources Collection and Research Center (BCRC-60015; Hsinchu, Taiwan). Detailed methodology has been prescribed previously [16]. Briefly, cells were cultured in Ham's F-12 medium (Invitrogen) supplemented with 15% heat-inactivated horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM L-glutamine in a humidified environment of 5% CO₂/95% air and they underwent passaged about three times a week. To promote cell differentiation, GH₃ cells were transferred to a serum-free, Ca²⁺-free medium. Cell viability was evaluated by the trypan blue dye-exclusion test. Under these conditions, cells remained 80-90% viable for at least 2 weeks. Our experiments were commonly performed 5 or 6 days after cells were cultured (60-80% confluence). In some experiments, cells were pre-incubated with chlorotoxin (1 μM) or iloprost (100 nM) at 37°C for 6 hours before electrophysiological recordings were performed.

Electrophysiological measurements

Shortly before each experiment, cells were dissociated and an aliquot of cell suspension was transferred to a home-made recording chamber mounted on the stage of an inverted DM-IL microscope (Leica, Wetzlar, Germany). Cells were immersed at room temperature (20-25°C) in normal Tyrode's solution, the composition of which is described above. Patch pipettes were fabricated from Kimax-51 glass capillaries (Kimble Glass, Vineland, NJ) on a PP-830 microelectrode puller (Narishige, Tokyo, Japan), and their tips were then fire-polished using an MF-83 microforge (Narishige). The resistances of patch pipettes used when filled with different internal solutions commonly ranged between 3 and 5 MΩ. Recordings of membrane potential or ion currents were measured with glass pipettes in the whole-cell configuration of the patch-clamp technique with an RK-400 patch-clamp amplifier (Biologic, Claix, France) [10, 16]. The liquid junction potentials were corrected when necessary.

Data recordings and analyses

The signals consisting of potential and current traces were displayed on a GW oscilloscope (GOS-622G; Good Will, Taipei, Taiwan) and on a liquid crystal projector (PJ550-2; ViewSonic Corp., Walnut, CA). The data achieved were digitized and stored online in a Slimnote VX₃ computer (Lemel, Taipei, Taiwan) at 10 kHz equipped with a Digidata-1320A or a Minidigi 1A interface (Molecular Devices, Sunnyvale, CA). Data were subsequently analyzed using either pCLAMP 10.2 (Molecular Devices), Origin 8.0 (OriginLab, Northampton, MA), and/or custom-made macros created in Excel 2013 under Windows 7 (Microsoft Corp., Redmond, WA). By digital-to-analog conversion, the pCLAMP-generated voltage-step protocols were computer driven to determine the *I-V* relations and either activation or inactivation curve for $I_{K(DR)}$ or I_{Na} obtained in the presence or absence of ART. Action potential (AP) durations (in msec) were measured at 90% of repolarization.

To evaluate the percentage inhibition of ART on $I_{K(DR)}$ elicited by membrane depolarization, GH₃ cells were bathed in nominally Ca²⁺-free Tyrode's solution and each cell was depolarized from -50 to +50 mV with a duration of 1 sec. The $I_{K(DR)}$ amplitude at the end of depolarizing pulse during cell exposure to different concentrations of ART (1-100 μM) was measured and compared. The concentration of ART required to suppress 50% of $I_{K(DR)}$ amplitude was then fitted to a Hill equation:

$$y = \frac{E_{\max} \times [C]^{n_h}}{IC_{50}^{n_h} + [C]^{n_h}}$$

where *y* is the percentage inhibition of ART on $I_{K(DR)}$; [*C*] is the ART concentration; IC₅₀ and *n_h* indicate the concentration required for a 50% inhibition and the Hill coefficient, respectively; and *E_{max}* is the ART-induced maximal inhibition of $I_{K(DR)}$.

The time constants of current inactivation (τ_{inact}) with or without addition of ART were determined by fitting the trajectory of each current trace with single or double exponential curve. To determine the ART effect on the steady-state inactivation of $I_{K(DR)}$, the normalized amplitude of $I_{K(DR)}$ (i.e., $I/I_{\max}$) was constructed against the conditioning potential and the smooth curve fitted by a Boltzmann function:

$$\frac{I}{I_{\max}} = \frac{1}{1 + \exp\left[\frac{(V - a)}{b}\right]}$$

where I_{max} is the maximal activated $I_{K(DR)}$, V the membrane potential in mV, a the membrane potential for half-maximal inactivation, and b the slope factor of the inactivation curve.

Statistical analyses

The Solver subroutine embedded in Excel (Microsoft) was commonly implemented to fit data set by a least-squares minimization procedure [17]. The averaged results are presented as the mean values $\pm$ SEM. The paired or unpaired t test and one-way analysis (ANOVA) with the least significance difference method for multiple comparisons were implemented for statistical evaluation of differences among the mean values. Non-parametric Kruskal-Wallis test was used, as the assumption of normality underlying ANOVA was violated. Statistical analyses were made using IBM SPSS version 22.0 (IBM Corp., Armonk, NY). Differences between values were considered significant when $P < 0.05$.

Kinetic study of ART effect on $I_{K(DR)}$

In this study, the inhibitory effect of ART on $I_{K(DR)}$ observed in GH₃ cells can be taken into account by a state-dependent blocker that binds to the open state of K_v channels according to a minimal kinetic scheme [18].

where α and β are the voltage-dependent rate constants for the opening and closing of the K_v channel; k_{+1} and k_{-1} those for blocking and unblocking by ART (i.e., the on- and off-rate constants for ART); and [ART] represents the ART concentration. Closed, open and inactivated shown in the scheme indicate the closed, open, and open-blocked states, respectively.

The blocking and unblocking rate constants (i.e., k_{+1} and k_{-1}) were appropriately determined from the inactivation rate constants (i.e., $1/\tau_{inact}$) of $I_{K(DR)}$ elicited by the voltage steps from -50 to +50 mV with a duration of 10 sec. Blocking and unblocking rate constants were estimated using the relation:

$$\frac{1}{\tau_{inact}} = k_{+1} \times [\text{ART}] + k_{-1}$$

where k_{+1} and k_{-1} are respectively derived from the slope and the y-axis intercept at [ART]=0 of the linear regression interpolating the reciprocal time constant versus different ART concentrations. Based on the values of k_{+1} and k_{-1} , the dissociated constant (K_D) (i.e., k_{-1}/k_{+1}) was obtained.

Results

Inhibitory effect of ART on $I_{K(DR)}$ in GH₃ cells

In the initial set of experiments, whole-cell current recordings were conducted to check whether ART has any effects on $I_{K(DR)}$ recorded from these cells. Cells were bathed in Ca²⁺-free Tyrode's solution containing 1 μ M tetrodotoxin and 0.5 mM CdCl₂, and the recording pipettes were filled with K⁺-containing solution. Tetrodotoxin and CdCl₂ were used to block Na⁺ and Ca²⁺ currents, respectively. As shown in Fig. 1A, the examined cell was held at -50 mV and the depolarizing step from -50 to +50 mV was applied to elicit $I_{K(DR)}$ with a slight inactivation. Under these experimental conditions, the biophysical and pharmacological properties of $I_{K(DR)}$ were noted to maintain the same as previous observations [14, 16]. Importantly, as GH₃ cells were exposed to ART, the amplitude of $I_{K(DR)}$ elicited by membrane depolarization decreased progressively. The appropriate ART concentration used in this study was primarily derived from earlier reports [1, 2]. For example, ART at a concentration of 10 μ M significantly decreased current amplitude measured at the end of depolarizing step by $47 \pm 4\%$ from 654 ± 17 to 348 ± 13 pA ($n = 11$, $P < 0.05$). After ART was removed, current amplitude was partially returned to 594 ± 14 pA ($n = 7$, $P < 0.05$, data not shown). However, addition of ART (10 μ M) produced minimal effects on $I_{K(erg)}$ in these cells (data not shown). The pharmacological and biophysical properties of $I_{K(erg)}$ in GH₃ cells were previously described [19]. Physiological roles of $I_{K(erg)}$ in different types of cells have been also reported

Fig. 1. Effect of ART on $I_{K(DR)}$ in GH₃ cells. Cells were bathed in Ca²⁺-free, Tyrode's solution and the recording pipette was filled with K⁺-containing solution. (A) Original current traces in response to membrane depolarization. 1: control; 2: 10 μ M ART; 3: 30 μ M ART; 4: 100 μ M ART. The upper part of (A) indicates the voltage protocol used. (B) Concentration-response curve for ART-induced inhibition of $I_{K(DR)}$ recorded from GH₃ cells. The amplitude of $I_{K(DR)}$ measured at the end of depolarizing pulse during cell exposure to ART was compared with the control value (mean $\pm$ SEM; n=7-14 for each point). The red smooth line represents the best fit to a Hill function. The values for IC₅₀, maximally inhibited percentage of $I_{K(DR)}$, and the Hill coefficient were 11.2 μ M, 99%, and 1.1, respectively. Note that addition of ART has a depressant action on $I_{K(DR)}$ amplitude in a concentration-dependent manner.

[20, 21]. However, at the level of -100 mV, the peak amplitude of deactivating $I_{K(erg)}$ did not differ significantly between the absence and presence of 10 μ M ART (787 ± 14 pA [control] versus 782 ± 13 pA [in the presence of ART], n = 7, $P > 0.05$).

The relationship between the ART concentration and the percentage inhibition of $I_{K(DR)}$ in GH₃ cells were determined and then constructed. In this set of experiments, each cell was depolarized from -50 to +50 mV with a duration of 1 sec and the amplitude of $I_{K(DR)}$ measured at the end of depolarizing pulse was obtained with or without addition of ART. As illustrated in Fig. 1B, ART at different concentrations (1-100 μ M) suppressed $I_{K(DR)}$ amplitude in a concentration-dependent manner. By virtue of a non-linear least-squares fit to the data, the IC₅₀ value required for the inhibitory effect of ART on $I_{K(DR)}$ in GH₃ cells was calculated to be 11.2 μ M. Our results reflected that ART can exert a concentration-dependent action on the inhibition of $I_{K(DR)}$ inherently in these cells.

Characterization of $I_{K(DR)}$ inactivation elicited by long-lasting depolarizing pulse

In addition to the reduction of current amplitude, when GH₃ cells were exposed to ART, the time course for the trajectory of $I_{K(DR)}$ inactivation tended to be reduced in time to a greater extent. The $I_{K(DR)}$ elicited in response to a 10-sec long depolarizing pulse was thus determined in the presence or absence of ART. Under these experimental conditions, as shown in Fig. 2A and 2B, when the $I_{K(DR)}$ in response to membrane depolarization to different potentials ranging between +10 and +50 mV was evoked, the presence of ART reduced $I_{K(DR)}$ amplitude together with the enhancement of current inactivation. In other words, there was a progressive rise in the rate of current inactivation during cell exposure to this drug. For example, after addition of 10 μ M ART, the inactivation time constant (τ_{inact}) of $I_{K(DR)}$ measured at the level of +50 mV was significantly decreased from 2.93 ± 0.22 to 2.71 ± 0.21 sec (n=12,

Fig. 2. Effect of ART on $I_{K(DR)}$ elicited by a 10-sec depolarizing pulse. Cells were bathed in Ca^{2+} -free Tyrode's solution and the examined cell was depolarized from -50 to different voltages ranging between +10 and +50 mV. (A) Original current traces obtained in the presence of 10 μM ART. The upper part in (A) indicates the voltage protocol used. (Ba) The time courses of $I_{K(DR)}$ inactivation in response to 10-sec long depolarization. The trajectory of each trace obtained at the level of +10, +30 and +50 mV was fitted by a single exponential with the τ_{inact} value of 2.7, 2.1, and 1.8 sec, respectively. (Bb) Summary of the data showing effect of ART on the inactivation time constant (τ_{inact}) of $I_{K(DR)}$ in GH₃ cells. (C) Averaged $I-V$ relationship of $I_{K(DR)}$ elicited by a 10-sec long depolarizing pulse in the absence (Ca, circles symbols) and presence (Cb, square symbols) of $I_{K(DR)}$. The $I_{K(DR)}$ amplitude was measured at the beginning (filled symbols) and end (open symbols) of voltage pulses. Each data point represents the mean $\pm$ SEM ($n=12-15$). (D) Kinetics of ART-induced block of $I_{K(DR)}$ in GH₃ cells. The $I_{K(DR)}$ taken during cell exposure to different ART concentrations was elicited by membrane depolarization from -50 to +50 mV with a duration of 10 sec. The reciprocal of τ_{inact} value obtained by exponential fit of the trajectory of $I_{K(DR)}$ inactivation was plotted against the ART concentration. Data points were fitted by a linear regression shown in gray straight line. Based on minimal reaction scheme (see text in details), inactivation (k_{+1}) and recovery from inactivation (k_{-1}) rate constants were calculated to be 0.0211 $\text{sec}^{-1}\mu\text{M}^{-1}$ and 0.3106 sec^{-1} , respectively. Each data point represents the mean $\pm$ SEM ($n=7-9$).

$P < 0.05$). Figure 2C showed averaged $I-V$ relationships of $I_{K(DR)}$ measured at the beginning and end of depolarizing pulses, which were obtained in the absence (Fig. 2Ca) and presence (Fig. 2Cb) of 10 μM ART. The magnitude of ART-mediated inhibitory effect on $I_{K(DR)}$ measured at the end of depolarizing pulses became greater than that at the beginning of voltage steps. For example, at the level of +50 mV, ART (10 μM) suppressed $I_{K(DR)}$ amplitude measured at the

Fig. 3. Effect of ART on the steady-state inactivation curve of $I_{K(DR)}$ in GH₃ cells. In this set of experiments, the conditioning voltage pulses with a duration of 10 sec to potentials ranging between -70 and +50 mV. After each conditioning pulse, a test pulse to +50 mV was applied to evoke $I_{K(DR)}$. The upper part of (A) indicates the voltage protocol used. An example of current traces obtained by such two-pulse protocol in the presence of 10 μ M ART is illustrated in (A). (B) Steady-state inactivation curve of $I_{K(DR)}$ in the absence ($\bullet$) and presence ($\circ$) of 10 μ M ART. The $I_{K(DR)}$ amplitudes were normalized and then constructed against the conditioning potentials. The smooth curve was appropriately fitted by the Boltzmann equation (see text for details). Note that the steady-state inactivation curve of $I_{K(DR)}$ in GH₃ cells was significantly shifted toward the hyperpolarizing voltage during the exposure to ART.

Fig. 4. Time course of excessive inactivation of $I_{K(DR)}$ in the absence and presence of ART. Current amplitudes of $I_{K(DR)}$ were measured during repetitive depolarizations from -50 to +50 mV with a duration of 50 msec at a rate of 10 Hz. a: control ($\bullet$); b: 3 μ M ART ($\circ$); c: 10 μ M ART ($\square$). Smooth curves in a, b and c show the single exponential fits with time constants of 2.86, 2.21, and 1.82 sec, respectively. Note that, apart from the inhibition of $I_{K(DR)}$ amplitude, ART can enhance the rate of excessive accumulative inactivation of $I_{K(DR)}$ evoked in response to repetitive stimuli. Each point represents the mean $\pm$ SEM (n=6-8).

end of voltage pulse by 49%, while it inhibited current amplitude at the beginning of voltage pulse only by 27%.

Kinetic studies of ART-induced increase in the rate of $I_{K(DR)}$ inactivation in GH₃ cells

When cells were exposed to ART, in addition to decrease amplitude of $I_{K(DR)}$, the inactivation rate of $I_{K(DR)}$ was noted to become faster. To provide evidence for ART-induced blocking of $I_{K(DR)}$, the time constants for $I_{K(DR)}$ inactivation in these cells were further examined. The inactivation time constants (τ_{inact}) of $I_{K(DR)}$ with or without addition of different ART concentrations were obtained by fitting inactivation trajectories to single exponential function. The concentration dependence of $I_{K(DR)}$ decay by ART is illustrated in Fig. 2D. The inhibitory effect of this drug was noted to be concentration-dependent increase in the rate of current inactivation accompanied by a drastic decrease in the residual, steady-state current. For example, when cells were depolarized from -50 to +50 mV with a duration of 10 sec, the inactivation time courses of $I_{K(DR)}$ obtained during the exposure to 1 and 10 μ M ART were fitted by a single exponential with the $1/\tau_{inact}$ values of $0.32 \pm 0.02 \text{ sec}^{-1}$ (n=8) and $0.52 \pm 0.03 \text{ sec}^{-1}$ (n=9), respectively.

Based on the first-order blocking scheme described in materials and methods, the relationship between $1/\tau_{inact}$ and $[ART]$ became linear with a correlation coefficient of 0.96 (Fig. 2D). The blocking and unblocking rate constant were calculated to be $0.0211 \text{ sec}^{-1}\mu\text{M}^{-1}$ and 0.3106 sec^{-1} , respectively. Hence, based on these rate constants, a value of $14.7 \mu\text{M}$ for the dissociation constant ($K_D = k_{-1}/k_{+1}$) could be generated. Notably, this value is similar to the IC_{50} value shown above.

Effect of ART on the steady-state inactivation of $I_{K(DR)}$

The effect of ART on the steady-state inactivation of $I_{K(DR)}$ in GH₃ cells was determined. In this set of experiments, cells were bathed in Ca^{2+} -free, Tyrode's solution and the steady-state inactivation parameters of $I_{K(DR)}$ were obtained in the presence or absence of $10 \mu\text{M}$ ART. As shown in Fig. 3A, the normalized amplitude of $I_{K(DR)}$ was constructed against the conditioning potential and the smooth curves were well fitted by the Boltzmann equation as described in materials and methods. In Fig. 3B, steady-state inactivation curve of $I_{K(DR)}$ was recorded in the absence (●) and presence (○) of $10 \mu\text{M}$ ART. The $I_{K(DR)}$ amplitudes were normalized and then constructed against the conditioning potentials. Results in the absence and presence of 10

μM ART were $a = -9.8 \pm 0.5 \text{ mV}$, $b = 7.8 \pm 0.3 \text{ mV}$ ($n = 10$) and $a = -20.5 \pm 1.1$, $b = 7.8 \pm 0.5 \text{ mV}$ ($n = 9$) respectively. Therefore, the presence of ART produced a clear shift in the midpoint of the inactivation curve toward hyperpolarizing voltage by approximately 10 mV , despite no significant change in the slope factor (i.e., b) was detected in the presence of this drug.

ART-induced increase in cumulative inhibition of $I_{K(DR)}$ inactivation

$I_{K(DR)}$ inactivation was previously described to accumulate during repetitive short pulses [22]. Therefore, in another set of experiments, we sought to evaluate whether ART exerts any changes in the inactivation time course of $I_{K(DR)}$ in response to repetitive depolarizing stimuli in GH₃ cells. Under control conditions, a single 10-sec depolarizing step to $+50 \text{ mV}$ from a holding potential of -50 mV produced an exponential decline with time constant of $3.02 \pm 0.14 \text{ sec}$ ($n = 9$). However, as the voltage step was repetitively applied from -50 to $+50 \text{ mV}$ with a duration of 50 msec at 10 Hz , the time constant for $I_{K(DR)}$ inactivation was significantly reduced to $2.86 \pm 0.12 \text{ sec}$ ($n = 8$, $P < 0.05$). Likewise, as shown in Fig. 4, addition of ART produced a progressive increase in the decline of $I_{K(DR)}$ in response to rapid depolarizing stimuli. ART at a concentration of 3 and $10 \mu\text{M}$ ART decreased the τ_{inact} value to $2.21 \pm 0.11 \text{ sec}$ ($n = 7$, $P < 0.05$) and $1.82 \pm 0.09 \text{ sec}$ ($n = 8$, $P < 0.05$), respectively. Therefore, it is clear from these experimental results that accumulative inactivation of $I_{K(DR)}$ recorded under these experimental protocol was enhanced in the presence of ART.

Effect of ART on $I_{K(DR)}$ in cells preincubated with chlorotoxin or iloprost

It has been shown that effects of ART could be associated with its effect on Cl^- channels or the inhibition of production in intracellular cyclic AMP [8, 23]. The effect of ART on $I_{K(DR)}$

Fig. 5. Effect of ART on averaged I - V relations of $I_{K(DR)}$ in GH₃ cells treated with chlorotoxin (upper) or iloprost (lower). Cells were preincubated with chlorotoxin ($1 \mu\text{M}$) or iloprost (100 nM) for 6 hours. In each cell tested, $I_{K(DR)}$ was elicited from -50 mV to different potentials ranging from -50 to $+50 \text{ mV}$ with 20-mV increments. Each point represents the mean $\pm$ SEM ($n = 7-9$). Filled symbols: controls; Open symbols: in the presence of $10 \mu\text{M}$ ART.

Fig. 6. Effect of ART on I_{Na} in GH₃ cells. In these experiments, cells were immersed in Ca²⁺-free, Tyrode's solution and the pipette was filled with a Cs⁺-containing solution. (A) Original I_{Na} traces elicited by membrane depolarization, the voltage protocol of which is indicated in the upper part. a: control; b: 3 μ M ART; c: 10 μ M ART. (B) Inactivation time courses of I_{Na} with or without addition of ART. The trajectories of I_{Na} traces in a, b and c were fitted by a two-exponential with the slow component in I_{Na} inactivation ($\tau_{inact(s)}$) of 9.0, 10.2, and 10.6 msec, respectively. a: control; b: 3 μ M ART; c: 10 μ M ART. Inset indicates an expanded record from dashed box. (C) Summary of data showing the effect of ART and ART plus KMUP-1 on the $\tau_{inact(s)}$ value (mean $\pm$ SEM; n=12-13 for each bar). In the experiments on ART plus KMUP-1, KMUP-1 was subsequently added in continued presence of ART. *Significantly different from control ($P < 0.05$) and #significant different from 10 μ M ART alone group ($P < 0.05$).

was thus scrutinized in cells treated with chlorotoxin (1 μ M) or iloprost (100 nM). Chlorotoxin is an inhibitor of Cl⁻ channels, whereas iloprost, a prostacyclin analog, can induce the production of cyclic AMP [24]. However, unexpectedly, for cells preincubated with either chlorotoxin or iloprost, the inhibitory effect of ART on the I - V relationship of $I_{K(DR)}$ remained unaltered (Fig. 5). For example, in cells pretreated with

chlorotoxin (1 μ M), addition of ART (10 μ M) significantly decreased the $I_{K(DR)}$ amplitude at the level of +50 mV from 723 ± 84 to 359 ± 68 pA ($n = 9$, $P < 0.05$). There was no significant difference in the magnitude of ART-mediated inhibitory effect on $I_{K(DR)}$ between control cells and cells treated with chlorotoxin or iloprost. Another earlier report showed the ability of ART to produce sedative effects through binding to dopamine D₂ receptors [25]. However, in GH₃ cells preincubated with dopamine (10 μ M), the inhibitory effect of ART on $I_{K(DR)}$ was unaffected. For example, the $I_{K(DR)}$ amplitude at the level of +50 mV was decreased from 723 ± 84 to 401 ± 12 pA ($n = 8$, $P < 0.05$) in control cells, while in cells treated with dopamine (10 μ M), ART (10 μ M) decreased current amplitude at the same level of potential from 721 ± 84 to 398 ± 21 pA ($n = 7$, $P < 0.05$). Therefore, these results prompted us to suggest that neither Cl⁻-channel activity, the level of intracellular AMP, nor binding to dopamine receptors is responsible for ART-mediated inhibition of $I_{K(DR)}$ in GH₃ cells.

Inhibitory effect of ART on I_{Na} in GH₃ cells

Previous reports have shown the ability of ART to suppress the amplitude of I_{Na} in neurons isolated from nodose ganglia [7]. We further investigated whether the I_{Na} in GH₃

Fig. 7. Effect of ART on the I - V relationship of I_{Na} in GH₃ cells. The I_{Na} was evoked by the depolarizing steps ranging from -70 to +40 mV with 10-mV increments. The peak amplitude of I_{Na} with or without addition of ART was measured at different levels of step potentials. ○: control; □: 3 μ M ART; △: 10 μ M ART. Notably, no modification in the overall I - V relationship of I_{Na} was seen in the presence of ART, although the peak I_{Na} was reduced during the exposure to this drug. Each point represents the mean $\pm$ SEM (n=8–12).

cells was subject to any changes by ART. In these experiments, cells were bathed in Ca²⁺-free, Tyrode's solution and the recording pipettes were filled with Cs⁺-containing solution. As shown in Fig. 6A, the presence of ART produced a progressive reduction of I_{Na} in these cells. For example, when cells were exposed to 10 μ M ART, the peak amplitude of I_{Na} was significantly decreased by $55 \pm 3\%$ to 204 ± 14 pA (n = 13, $P < 0.05$) from a control value of 451 ± 17 pA (n = 13). After washout of this drug, peak I_{Na} amplitude was partially returned to 409 ± 16 pA (n = 8).

However, it is also noted that the inactivation of I_{Na} slowed down in the presence of ART. The inactivation time course of I_{Na} in response to membrane depolarization was fitted to a two-exponential function. As cells were exposed to ART, the slow component of I_{Na} inactivation (τ_{inact}) was increased (Fig. 6B). For example, addition of ART at a concentration of 10 μ M significantly increased the (τ_{inact}) value from 9.01 ± 0.06 to 10.83 ± 0.07 msec (n = 13, $P < 0.05$). In continued presence of 10 μ M ART, subsequent addition of 10 μ M KMUP-1 reversed the τ_{inact} value, as evidenced by a significant shortening of τ_{inact} to 9.41 ± 0.06 msec (n = 12, $P < 0.05$). KMUP-1 was recently reported to suppress peak I_{Na} and to increase inactivation rate of I_{Na} [10]. However, the fast component of inactivation time constant for I_{Na} did not differ significantly between the absence and presence of ART. Therefore, distinguished from ART-induced increase of $I_{K(DR)}$ inactivation, the inactivation rate of peak I_{Na} in response to depolarizing step became depressed in the presence of this drug. The ART-induced slowing of I_{Na} inactivation was reversed by subsequent addition of KMUP-1. Additionally, the effect of ART on I_{Na} measured at different levels of voltage steps ranging between -70 and +40 mV was further evaluated. As shown in Fig. 7, the presence of ART at a concentration of 3 and 10 μ M did not exert clear effect on the modification in the overall I - V relationship of peak I_{Na} , although it can decrease peak I_{Na} amplitude concomitantly with the attenuation of current inactivation elicited by rapid membrane depolarization.

Effect of ART on the AP firing recorded from GH₃ cells

In a final set of experiments, ART-induced changes in membrane potential was evaluated. The cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl₂, the recording pipettes were filled with K⁺-containing solution, and current-clamp condition was made. As illustrated in Fig. 8, as cells were exposed to ART, the amplitude of APs was decreased and the duration of APs became longer. For example, ART (10 μ M) significantly decreased AP amplitude from 68 ± 3 to 54 ± 2 mV (n = 9, $P < 0.05$) and caused a significant lengthening in the duration of APs from 87 ± 5 to 104 ± 7 msec (n = 9, $P < 0.05$). Additionally, ART (10 μ M) depolarized the resting membrane potential from -72 ± 1 to -61 ± 2 mV (n = 9, $P < 0.05$). However, the firing frequency did not differ significantly between the absence and presence of 10 μ M ART (0.92 ± 0.07 Hz, [control] versus 0.94 ± 0.06 Hz [in the presence of ART], n = 8, $P > 0.05$). Therefore, ART-induced changes in AP firing can be partly explained by its inhibition of $I_{K(DR)}$ and I_{Na} in these cells.

Fig. 8. Effects of ART on the firing of APs recorded from GH₃ cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl₂, the patch pipettes used were filled with K⁺-containing solution, and current-clamp recordings were performed. (A) Original potential traces obtained in control (a) and during cell exposure to 3 μ M ART (b) and 10 μ M ART (c). The right part shown in (A) indicates expanded records from dashed box. In (B) and (C), summary of the data respectively shows effects of ART on the amplitude and duration of APs recorded from GH₃ cells. Each bar indicates the mean $\pm$ SEM (n=11–13). *Significantly different from controls ($P < 0.05$).

Discussion

The pharmacological effects of ART on ionic currents (i.e., $I_{K(DR)}$ and I_{Na}) seen in GH₃ cells were described in this study. Application of ART did not simply reduce the amplitude of $I_{K(DR)}$. It caused a significant increase in the rate of $I_{K(DR)}$ inactivation elicited by a 10-sec long depolarizing pulse. In other words, as the time course of current decay in response to such depolarizing step was fitted to a single exponential function, the τ_{inact} value of $I_{K(DR)}$ became notably lower. Our findings reflect that ART is capable of blocking $I_{K(DR)}$ channels in a concentration- and state-dependent manner. It is most likely that binding speed and affinity of the ART molecule for a particular state determine the kinetic behavior of the $I_{K(DR)}$ in the presence of ART. In particular, a leftward shift in the midpoint of the steady-state inactivation curve for $I_{K(DR)}$ was detected during cell exposure to ART. It is thus possible that ART has a greater affinity toward the open-inactivated $I_{K(DR)}$ channels than toward the closed (or resting) channels in GH₃ cells, although the gating conformational changes of these channels in the presence of ART need to be further delineated.

A notable feature on the block of $I_{K(DR)}$ by ART in GH₃ cells was that the initial rising phase of the current (i.e., activation time course) was unaffected. At the beginning of the voltage pulse, dI/dt should be proportional to the number of channels available for activation. The results showed that dI/dt was unchanged during cell exposure to ART suggesting that, prior to channel activation, there should be no significant resting block for $I_{K(DR)}$. Unlike previous observations made in neurons isolated from nodose ganglia [7], another pertinent finding in this study was that ART tended to accelerate $I_{K(DR)}$ inactivation, suggesting that the ART molecule appears to reach the blocking site only when the channel is in the open state.

Previous observations reported the ability of ART and structurally similar compounds to inhibit neurite outgrowth in Nb2a neuronal cells differentiated with dibutyryl cyclic AMP [26]. ART-mediated sedative effect is probably linked to benzodiazepine receptors

[8]. However, in GH₃ cells treated with iloprost, a prostacyclin analog, or chlorotoxin, ART-induced block of $I_{K(DR)}$ or I_{Na} remained unaffected. Iloprost is known to increase the level of intracellular cyclic AMP through its bindings to prostacyclin receptors [24]. Chlorotoxin can block the activity of Cl⁻ channels. Therefore, ART-induced inhibition of ion currents in GH₃ cells is unlikely to arise from any change in either intracellular cyclic AMP and/or Cl⁻-channel activity.

An earlier report showed that ART-induced sedative effects in rodent could be mediated through its binding to dopamine D₂ receptors [25]. ART may interfere with the actions of dopamine as a neurotransmitter at D₂ receptors existing in GH₃ cells. However, in our study, dopamine did not reverse the inhibition by ART of I_{Na} and $I_{K(DR)}$ in these cells. In GH₃ cells preincubated with dopamine (10 μM), the inhibitory effect of ART on $I_{K(DR)}$ was unaffected (data not shown). Hence, the present results showed that ability of ART to suppress the amplitude of I_{Na} , $I_{K(DR)}$, or both is likely to be independent of the mechanism linked to its blockade of dopamine D₂ receptors.

Our results showed that the IC₅₀ value for the ART-induced inhibition of $I_{K(DR)}$ was 11.2 μM, a value that is similar to the K_D value (14.7 μM) derived from the first-order reaction scheme. ART at a concentration of 10 μM exerted little or no effects on $I_{K(erg)}$ in GH₃ cells. The IC₅₀ value required for the inhibitory effect of ART on $I_{K(DR)}$ in GH₃ cells appears to be higher. However, in addition to decreased $I_{K(DR)}$ amplitude, the time course for the trajectory of $I_{K(DR)}$ inactivation was reduced in time to a greater extent. Moreover, the presence of ART shifted the midpoint of the inactivation curve of $I_{K(DR)}$ toward hyperpolarizing voltage as well as produced an increase in cumulative inhibition of $I_{K(DR)}$ inactivation. Therefore, the IC₅₀ value required for ART-induced block of $I_{K(DR)}$ in this study appears to match its therapeutic concentrations [1, 2], lending credence to a link between anti-oxidative property and its inhibitory effect on $I_{K(DR)}$.

Another pertinent finding is that, unlike its action on $I_{K(DR)}$, the presence of ART can interact with Na_v channels to decrease the peak amplitude of I_{Na} and slow down the inactivation rate of this current in a concentration-dependent manner. In other words, although ART was effective at suppressing the amplitude of both $I_{K(DR)}$ and I_{Na} , instead of being decreased, the $\tau_{inact(s)}$ value for I_{Na} became enhanced in the presence of ART. The reason for this discrepancy is currently unknown; however, the ART action seems to be cell type related and dependent on the specific types of Na_v or K_v channels present. This probably reflects changes in the location of the binding site produced by differences in the channel sequence of Na_v and K_v channels. In addition, ART and its derivatives were recently reported to induce iron-dependent cell death in different types of tumor cells [6]. It thus remains to be determined whether cytoplasmic factors or free radicals may mediate the differential effects of ART on $I_{K(DR)}$ and I_{Na} in GH₃ cells. Regardless of the detailed mechanism on its actions, this study served as a platform for us to propose that ART is a slow on-off blocker of $I_{K(DR)}$ and a fast on-off blocker of I_{Na} . The inhibition of $I_{K(DR)}$ caused by ART is able to retard AP repolarization and slow down the recovery of I_{Na} inactivation, which may pose excitable cells to decrease rhythmic firing of APs [27]. Therefore, the observed inhibition of I_{Na} and $I_{K(DR)}$ in the presence of ART may synergistically act to affect the functional activities of pituitary cells, if similar findings occur *in vivo*.

The stimulus-secretion coupling occurring in endocrine or neuroendocrine cells is closely linked to Ca²⁺ signaling. The relationship between the AP amplitude and Ca²⁺ signaling is not simple. For example, spiking of smaller amplitude but longer duration may drive higher amplitude of Ca²⁺ transients in different types of endocrine cells. Moreover, pituitary lactotrophs (e.g., GH₃ cells) can secrete in a Ca²⁺ regulated manner and hormonal secretion occurs mainly through constitutive exocytosis [28]. Therefore, to what extent ART-mediated changes in ion-channel activity effect the inhibition of growth hormone or prolactin in pituitary cells occurring *in vivo* still need our further investigation.

Previous studies have demonstrated the ability of ART to suppress cell proliferation and hormonal secretion in different cell types including GH₃ cells [3, 4, 29, 30]. It is important to determine to what extent ART-induced neurological or ototoxic actions is linked to its

effects on ionic currents presented herein. Because of the ability of KMUP-1 to counteract ART-induced slowing in I_{Na} inactivation, it is also worthy to investigate whether KMUP-1 can ameliorate neurological adverse effects to any extent.

Abbreviations

ART (artemisinin); AP (action potential); $I_{K(DR)}$ (delayed-rectifier K⁺ current); *erg* (*ether-à-go-go*-related gene); IC_{50} (the concentration required for 50% inhibition); $I_{K(erg)}$ (*erg*-mediated K⁺ current); I_{Na} (voltage-gated Na⁺ current); *I-V* relationship (current-voltage relationship); K_D (dissociation constant); K_v channel (voltage-gated K⁺ channel); Na_v channel (voltage-gated Na⁺ channel); τ_{inact} (inactivation time constant); $\tau_{inact(s)}$ (slow component of inactivation time constant).

Acknowledgments

The authors thank Yen-Ming Huang for technical assistance and Kevin Su for helping with the English proof reading.

Funding

This work was supported by National Cheng Kung University (grant numbers D104-35A16) to Dr. Sheng-Nan Wu and Tainan Municipal An Nan Hospital (grant number ANHRF-104-1) to Dr. Edmund Cheung So and Ministry of Science and Technology (grant number 105-2221-E-006 -088) to Dr. Ping-Ching Wu.

Disclosure Statements

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

References

- 1 Li Y: Qinghaosu (artemisinin): chemistry and pharmacology. *Acta Pharmacol Sin* 2012;33:1141-1146.
- 2 Ho WE, Peh HY, Chan TK, Wong WS: Artemisinins: pharmacological actions beyond anti-malarial. *Pharmacol Ther* 2014;142:126-139.
- 3 Mao ZG, Zhou J, Wang H, He DS, Xiao WW, Liao GZ, Qiu LB, Zhu YH, Wang HJ: Artesunate inhibits cell proliferation and decreases growth hormone synthesis and secretion in GH3 cells. *Mol Biol Rep* 2012;39:6227-6234.
- 4 Singh NP, Panwar VK: Case report of a pituitary macroadenoma treated with artemether. *Integr Cancer Ther* 2006;5:391-394.
- 5 Noori S, Hassan ZM, Farsam V: Artemisinin as a Chinese medicine, selectively induces apoptosis in pancreatic tumor cell line. *Chin J Integr Med* 2014;20:618-623.
- 6 Ooko E, Saeed ME, Kadioglu O, Sarvi S, Colak M, Elmasaoudi K, Janah R, Greten HJ, Efferth T: Artemisinin derivatives induce iron-dependent cell death (ferroptosis) in tumor cells. *Phytomedicine* 2015;22:1045-1054.
- 7 Qiao G, Li S, Yang B, Li B: Inhibitory effects of artemisinin on voltage-gated ion channels in intact nodose ganglion neurones of adult rats. *Basic Clin Pharmacol Toxicol* 2007;100:217-224.
- 8 Emadi F, Yassa N, Hadjiakhoondi A, Beyer C, Sharifzadeh M: Sedative effects of Iranian *Artemisia annua* in mice: possible benzodiazepine receptors involvement. *Pharm Biol* 2011;49:784-788.
- 9 Catterall WA, Goldin AL, Waxman SG: International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev* 2005;57:397-409.
- 10 Lo YC, Tseng YT, Liu CM, Wu BN, Wu SN: Actions of KMUP-1, a xanthine and piperazine derivative, on voltage-gated Na(+) and Ca(2+) -activated K(+) currents in GH3 pituitary tumour cells. *Br J Pharmacol* 2015;172:5110-5122.

- 11 Morinville A, Fundin B, Meury L, Jureus A, Sandberg K, Krupp J, Ahmad S, O'Donnell D: Distribution of the voltage-gated sodium channel Na(v)1.7 in the rat: expression in the autonomic and endocrine systems. *J Comp Neurol* 2007;504:680-689.
- 12 Noda M, Hiyama TY: The Na(x) Channel: What It Is and What It Does. *Neuroscientist* 2015;21:399-412.
- 13 Liang Z, Chen L, McClafferty H, Lukowski R, MacGregor D, King JT, Rizzi S, Sausbier M, McCobb DP, Knaus HG, Ruth P, Shipston MJ: Control of hypothalamic-pituitary-adrenal stress axis activity by the intermediate conductance calcium-activated potassium channel, SK4. *J Physiol* 2011;589:5965-5986.
- 14 Wang YJ, Lin MW, Lin AA, Peng H, Wu SN: Evidence for state-dependent block of DPI 201-106, a synthetic inhibitor of Na⁺ channel inactivation, on delayed-rectifier K⁺ current in pituitary tumor (GH3) cells. *J Physiol Pharmacol* 2008;59:409-423.
- 15 O'Leary T, van Rossum MC, Wyllie DJ: Homeostasis of intrinsic excitability in hippocampal neurones: dynamics and mechanism of the response to chronic depolarization. *J Physiol* 2010;588:157-170.
- 16 Wu SN, Hwang T, Teng CM, Li HF, Jan CR: The mechanism of actions of 3-(5'-(hydroxymethyl)-2'-furyl)-1-benzyl indazole (YC-1) on Ca(2+)-activated K(+) currents in GH(3) lactotrophs. *Neuropharmacology* 2000;39:1788-1799.
- 17 Zhu S, Chen G: Numerical solution of a microbial growth model applied to dynamic environments. *J Microbiol Methods* 2015;112:76-82.
- 18 Lin MW, Wang YJ, Liu SI, Lin AA, Lo YC, Wu SN: Characterization of aconitine-induced block of delayed rectifier K⁺ current in differentiated NG108-15 neuronal cells. *Neuropharmacology* 2008;54:912-923.
- 19 Huang MH, Shen AY, Wang TS, Wu HM, Kang YF, Chen CT, Hsu TI, Chen BS, Wu SN: Inhibitory action of methadone and its metabolites on erg-mediated K⁺ current in GH(3) pituitary tumor cells. *Toxicology* 2011;280:1-9.
- 20 Urrutia J, Alday A, Gallego M, Malagueta-Vieira LL, Arechiga-Figueroa IA, Casis O, Sanchez-Chapula JA: Mechanisms of IhERG/IKr Modulation by alpha1-Adrenoceptors in HEK293 cells and cardiac myocytes. *Cell Physiol Biochem* 2016;40:1261-1273.
- 21 Shi YQ, Yan M, Liu LR, Zhang X, Wang X, Geng HZ, Zhao X, Li BX: High glucose represses hERG K⁺ channel expression through trafficking inhibition. *Cell Physiol Biochem* 2015;37:284-296.
- 22 Wu SN, Chen BS, Lin MW, Liu YC: Contribution of slowly inactivating potassium current to delayed firing of action potentials in NG108-15 neuronal cells: experimental and theoretical studies. *J Theor Biol* 2008;252:711-721.
- 23 Lee J, Lee J, Jung E, Cho JY, Park D: Artemisinic acid inhibits melanogenesis through downregulation of C/EBP alpha-dependent expression of HMG-CoA reductase gene. *Food Chem Toxicol* 2013;51:225-230.
- 24 Uchida T, Hazekawa M, Yoshida M, Matsumoto K, Sakai Y: Novel long-acting prostacyclin agonist (ONO-1301) with an angiogenic effect: promoting synthesis of hepatocyte growth factor and increasing cyclic AMP concentration via IP-receptor signaling. *J Pharmacol Sci* 2013;123:392-401.
- 25 Amos S, Chindo BA, Abbah J, Vongtau HO, Edmond I, Binda L, Akah PA, Wambebe C, Gamaniel KS: Postsynaptic dopamine (D(2))-mediated behavioural effects of high acute doses of artemisinin in rodents. *Brain Res Bull* 2003;62:255-260.
- 26 Fishwick J, McLean WG, Edwards G, Ward SA: The toxicity of artemisinin and related compounds on neuronal and glial cells in culture. *Chem Biol Interact* 1995;96:263-271.
- 27 Huang CW, Hung TY, Liao YK, Hsu MC, Wu SN: Underlying mechanism of regulatory actions of diclofenac, a nonsteroidal anti-inflammatory agent, on neuronal potassium channels and firing: an experimental and theoretical study. *J Physiol Pharmacol* 2013;64:269-280.
- 28 Stojilkovic SS, Tabak J, Bertram R: Ion channels and signaling in the pituitary gland. *Endocr Rev* 2010;31:845-915.
- 29 Farombi EO, Adedara IA, Abolaji AO, Anamelechi JP, Sangodele JO: Sperm characteristics, antioxidant status and hormonal profile in rats treated with artemisinin. *Andrologia* 2014;46:893-901.
- 30 Farombi EO, Abolaji AO, Adedara IA, Maduako I, Omodanisi I: Artemisinin induces hormonal imbalance and oxidative damage in the erythrocytes and uterus but not in the ovary of rats. *Hum Exp Toxicol* 2015;34:83-92.