FULL PAPER *Pharmacology*

Anticholinergic Effects of Artemisinin, an Antimalarial Drug, in Isolated Guinea Pig Heart Preparations

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(Received 13 October 2006/Accepted 13 March 2007)

ABSTRACT. Concern has been growing about the cardiac toxicity of antimalarial drugs. Artemisinin, a unique type of antimalarial drug originating from a Chinese medicinal plant, has minimal adverse effects, but it has been reported to inhibit delayed rectifier potassium current, a voltage-gated potassium current. However, no studies have been published concerning the effect of artemisinin on ligand-gated potassium currents. Therefore, in the present study, we examined the influence of artemisinin on the acetylcholine receptor-operated potassium current ($I_{K.ACh}$), a ligand-gated potassium current, in guinea pig atrial myocytes using a patch clamp technique. Artemisinin (1 to 300 μM) inhibited $I_{K.ACh}$ induced by extracellular application of both carbachol (1 μM) and adenosine (10 μM) and that induced by intracellular loading of GTPγS (100 μM) in a concentration-dependent manner. Artemisinin inhibited carbachol-induced, adenosine-induced, and GTPγS-activated $I_{K.ACh}$ within almost the same concentration range. In left atria, artemisinin (1 to 100 μM) partially reversed the shortening of action potential duration induced by carbachol in a concentration-dependent manner. Carbachol-induced negative inotropic action in left atria was also inhibited by artemisinin (10 to 300 μM). In conclusion, we suggest that the anticholinergic action of artemisinin is mediated through inhibition of $I_{K.ACh}$ via inhibition of the muscarinic potassium channel and/or associated GTP-binding proteins.

KEY WORDS: acetylcholine receptor-operated potassium current, anticholinergic effect, artemisinin, guinea pig, heart muscle.

J. Vet. Med. Sci. 69(7): 697–702, 2007

Malaria is a severe, life threatening infectious disease in many tropical and subtropical countries. Because of the increasing resistance of malaria parasites to conventional drugs, new approaches have been developed, particularly the artemisinin-based combination therapy [7]. Artemisinin is a unique type of antimalarial drug originating from a Chinese medicinal plant and has minimal adverse effects in humans [2]. According to many clinical reports, artemisinin and its derivatives are very effective against severe or complicated malarias and produce no major side effects [8, 20, 22, 23]. Nevertheless, there is growing concern regarding the cardiac toxicity of antimalarial drugs [3, 6, 19, 21]. For example, we previously reported that some antimalarial drugs, including chloroquine, primaquine, and pyrimethamine, produce anticholinergic effects through inhibition of the acetylcholine receptor-operated potassium current ($I_{K.ACh}$), a ligand-gated potassium current, in guinea pig atrial myocytes [9]. $I_{K.ACh}$ is known to play an important role in repolarization of action potential and maintenance of resting membrane potential in atrial cells [14]. Yang *et al.* [25] reported that artemisinin decreased the delayed rectifier potassium current (I_K), a voltage-gated potassium current, in guinea pig ventricular cells in a concentration-dependent manner. However, the influence of artemisinin on the ligand-gated potassium current has not been reported. Therefore, the purpose of the present study was to evaluate the influence of artemisinin on $I_{K.ACh}$ in guinea pig atrial myocytes by the patch clamp method. Furthermore, the anticholinergic actions of artemisinin were investigated the functional studies using isolated atrial preparations.

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MATERIALS AND METHODS

This study was performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society.

Male Hartley guinea pigs weighing 250–450 g were used in our experiments.

Cell preparations: Single atrial cells were isolated by enzymatic dispersion, as described previously [16]. Briefly, the heart was removed from the anesthetized guinea pig and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation with a nominally Ca^{2+}-free N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid] (HEPES)-Tyrode's solution containing 0.02%w/v of collagenase. The isolated cells were stored in modified Kraft-Brühe (KB) solution [10] at 4°C for later use.

Whole-cell current recordings: Whole-cell membrane currents were recorded by the patch clamp method. Single atrial cells were placed in a recording chamber (1-m*l* volume) and superfused with the HEPES-Tyrode's solution at a rate of 3 m*l*/min at 35°C. Glass patch pipettes filled with a pipette solution were used to produce the whole-cell voltage-clamp mode. The electrode was connected to a patch

clamp amplifier (CEZ-2300, Nihon Kohden, Tokyo, Japan) and controlled with the pCLAMP software (Axon Instruments, Inc., Foster City, CA, U.S.A.). Current signals were recorded on a pen recorder (SR-6511, Graphthec, Yokohama, Japan).

Action potential recordings in left atrial preparations: Transmembrane action potentials were recorded by the standard microelectrode method. The left atrium was placed in a tissue bath (3-m*l* volume) and superfused with Krebs-Henseleit solution (95% O_2 + 5% CO_2) at a rate of 5 m*l*/min at 35°C. The preparations were stimulated at 0.5 Hz for 5-ms in duration at 1.5 times the diastolic threshold. The glass microelectrode filled with 3 M KCl was coupled to an amplifier (MEZ-7101, Nihon Kohden). The action potential was stored in a computer-supported data acquisition system (PowerLab, Bioresearch Center, Nagoya, Japan). The atrial preparations were exposed to a solution containing 0.3 μM carbachol. Fifteen minutes after superfusion of carbachol, various concentrations of artemisinin were applied for 15 min.

Isometric contraction of left atrial preparations: The left atrium was placed in a 20-m*l* tissue bath filled with Krebs-Henseleit solution (95% O_2 + 5% CO_2) at 35°C. The preparations were stimulated by electrical field pulses at 2 Hz for 5-ms in duration at 1.5 times the diastolic threshold using bipolar platinum electrodes for an equilibration period of at least 90 min. Isometric contraction was recorded with a force-displacement transducer (TB-651T, Nihon Kohden) and monitored with PowerLab.

Drugs: The drugs used in this study were as follows: artemisinin (Aldrich Chem. Co., Milwaukee, WI, U.S.A.), HEPES, GTP-Na_3, GTPγS-Li_4 (Sigma Chemical Co., St. Louis, MO, U.S.A.), ATP-Na_2, carbachol chloride, adenosine, and collagenase (Wako Pure Chemical Co., Osaka, Japan). Other chemicals used were of reagent grade. Artemisinin was dissolved in ethanol. The final concentration of ethanol used throughout the present experiment was 0.3%. This concentration of ethanol produced no apparent change in the $I_{K.ACh}$ of atrial myocytes, or in action potential or developed tension of atrial preparations.

Solutions: The compositions of the solutions used were as follows. Normal HEPES-Tyrode's solution was comprised of 143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5.5 mM glucose, and 5.0 mM HEPES (pH 7.4). Modified KB solution was comprised of 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4, 3 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4). Standard pipette solution was comprised of 110 mM K-aspartate, 20 mM KCl, 1.0 mM $MgCl_2$, 5.0 mM ATP-K_2, 10 mM EGTA, and 5.0 mM HEPES (pH 7.4). Krebs-Henseleit solution was comprised of 119 mM NaCl, 4.8 mM KCl, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, and glucose 10 mM.

Data analysis: All values are presented as means ± standard error (SEM). IC_{50} values, which were the concentrations required to produce 50% of the maximal inhibitory effect, were calculated from concentration-response curves using Math Curve Fitter (SigmaPlot, Jandel Scientific, CA, U.S.A.) to solve nonlinear equations. Statistical significance was evaluated using Student's *t*-test; $P<0.05$ was considered significant.

RESULTS

Patch clamp studies: The effects of artemisinin on carbachol-induced $I_{K.ACh}$ in GTP-loaded cells were examined. Carbachol binds to muscarinic M_2 receptors and induces $I_{K.ACh}$ through activation of pertussis toxin-sensitive GTP-binding proteins in atrial cells. On application of carbachol (1 μM) to the bath solution, an outward potassium current was rapidly activated at a holding potential of –40 mV. Artemisinin (1 to 300 μM) was then added to the bath solution. The concentration was increased incrementally every 3 min. Artemisinin effectively decreased the carbachol-induced $I_{K.ACh}$ in a concentration-dependent manner (Fig. 1A). The outward current reappeared following washout of artemisinin. The IC_{50} value of artemisinin for depression of carbachol-induced $I_{K.ACh}$ was 30.0 ± 6.9 μM (Fig. 2, n=5 to 10).

The effect of artemisinin on the potassium channel current induced by adenosine in the GTP-loaded cells was also examined. Adenosine binds to adenosine A_1 receptors, inducing $I_{K.ACh}$ through activation of pertussis toxin-sensitive GTP-binding proteins in atrial cells [15]. Extracellular application of adenosine (10 μM) produced an outward current at a holding potential of –40 mV. After activation of the adenosine-induced outward current, artemisinin was applied to the bath solution. Artemisinin (1 to 300 μM) also decreased the adenosine-induced $I_{K.ACh}$ in a concentration-dependent manner (Figs. 1B, 2). The influence of artemisinin was partially reversed by washout. The IC_{50} value of artemisinin for depression of adenosine-induced $I_{K.ACh}$ was 31.0 ± 9.4 μM (n=5 to 10).

Pertussis toxin-sensitive GTP-binding proteins couple muscarinic receptors with a specific potassium channel in atrial cells [12]. Intracellular application of GTPγS, a non-hydrolysable GTP analogue, can directly activate GTP-binding proteins and evoke antagonist-resistant, persistent activation of the muscarinic potassium channels [4, 18]. The potassium current was activated gradually in a time-dependent fashion following intracellular loading of GTPγS (100 μM) at a holding potential of –40 mV, even in the absence of any agonists. Artemisinin (1 to 300 μM) inhibited the GTPγS-induced $I_{K.ACh}$ in a concentration-dependent manner (Figs. 1C, 2). The IC_{50} value of artemisinin for depression of GTPγS-activated $I_{K.ACh}$ was 30.2 ± 6.9 μM (n=5 to 10).

No significant differences were apparent among these three IC_{50} values.

Functional studies: The action potentials of guinea pig left atrial preparations constantly stimulated at 0.5 Hz were recorded using a standard microelectrode technique. Table 1 summarizes the action potential variables for each treat-

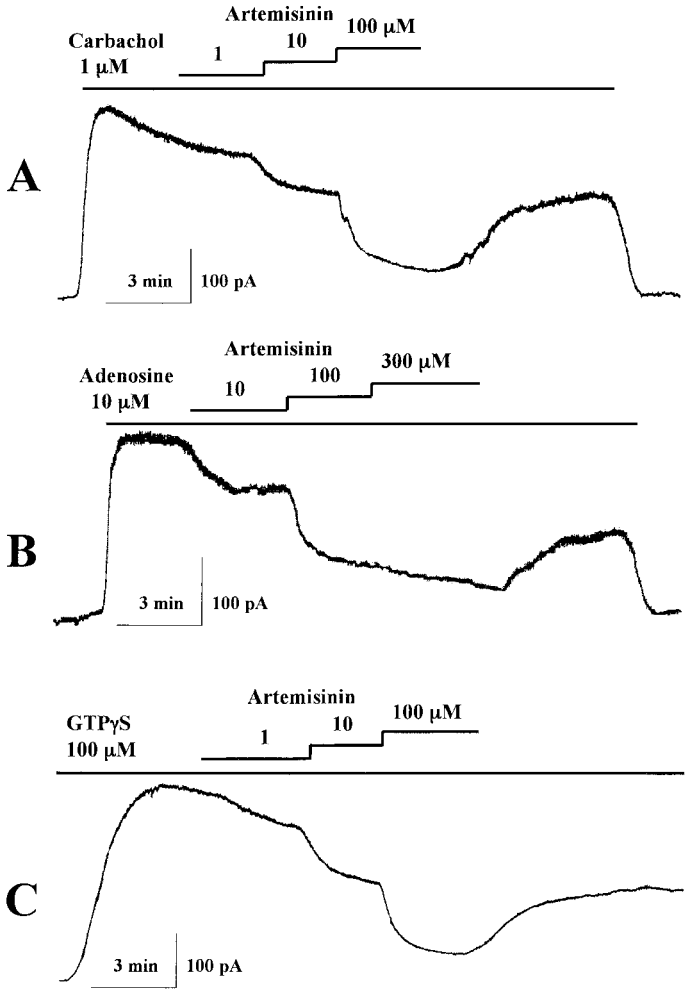

Fig. 1. Representative recordings for the effects of artemisinin on the muscarinic acetylcholine receptor-operated potassium current ($I_{K.ACh}$) induced by extracellular application of carbachol (1 μM, A), adenosine (10 μM, B), or intracellular loading of GTPγS (100 μM, C) in guinea pig single atrial myocytes. The patch clamp method was used with whole cells in this experiment; a single atrial myocyte was clamped at a holding potential of –40 mV. Extracellular application of carbachol, adenosine, and artemisinin and intracellular loading of GTPγS are shown by the bars above each original current trace. Artemisinin inhibited the outward currents in a concentration-dependent manner.

ment. After superfusion of 0.3 μM carbachol for 15 min, the action potential durations (APD) at the 20% (APD_{20}), 50% (APD_{50}), and 90% repolarization levels (APD_{90}) were shortened significantly. However, carbachol failed to influence action potential amplitude (APA), resting membrane potential (RMP), and maximum rate of rise of action potential upstroke (V_{max}). Artemisinin (1 to 100 μM) partially reversed the shortening of APD_{20}, APD_{50}, and APD_{90} induced by carbachol in a concentration-dependent manner. Artemisinin had no influence on APA, RMP, or V_{max}.

The influence of artemisinin on the negative inotropic effects of carbachol were also examined in left atrial preparations. Concentration-response curves for carbachol were generated before and after treatment of the preparations with artemisinin (10 to 300 μM). Artemisinin caused a rightward shift of the concentration-response curves for the negative inotropic effects of carbachol. Moreover, the maximal negative inotropic effect observed with a high concentration of carbachol (10 μM) was significantly attenuated by a higher concentration of artemisinin (12.1 ± 1.1% vs. 36.9 ± 4.9% in the control [n=17] and 300 μM artemisinin groups [n=7], respectively, $p<0.05$; Fig. 3). Developed tensions observed after 30 min of treatment with artemisinin at concentrations of 10, 30, 100, and 300 μM were 42.2%, 37.4%, 32.6%, and

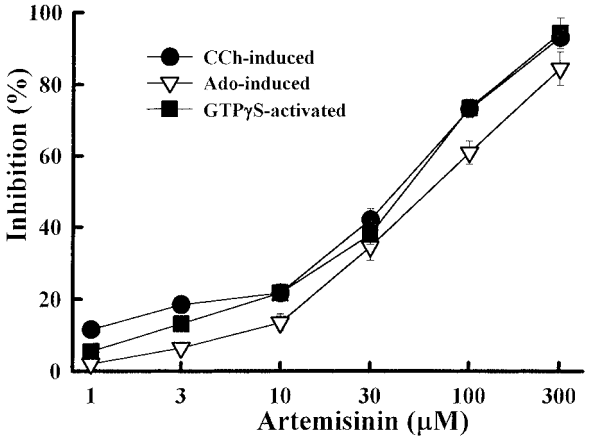

Fig. 2. Concentration-response curves for the inhibitory effect of artemisinin on the carbachol-induced $I_{K.ACh}$ (closed circles), adenosine-induced $I_{K.ACh}$ (triangles), and GTPγS-activated $I_{K.ACh}$ (closed squares) in guinea pig single atrial myocytes. Values are expressed as means ± SEM of five to ten myocytes for each point.

32.2% of the initial values, respectively. Thus, artemisinin per se decreased the developed tension of the atrial preparations.

In summary, the data from both our functional studies demonstrated that artemisinin had a concentration-dependent anticholinergic action.

DISCUSSION

The results of our study demonstrated that artemisinin inhibited the carbachol-induced $I_{K.ACh}$ in a concentration-dependent manner, thereby suggesting that the antimalarial drug might evoke anticholinergic-mediated cardiotoxicities, such as tachycardia and electrocardiographic abnormality. Clinically, it has been suggested that enhancement of vagal tone can result in atrial fibrillation [5], although the contribution of increased vagal tone may be variable. Moreover, induction of atrial fibrillation is assumed to stem from muscarinic receptor-mediated shortening of action potential durations and refractory periods. It is well known that some antiarrhythmic drugs inhibit $I_{K.ACh}$ in guinea pig atrial cells [12, 13, 17, 24]. Two mechanisms for this inhibition of $I_{K.ACh}$ have been proposed: 1) blockade of muscarinic receptors and 2) inhibition of the muscarinic potassium channel itself and/or associated GTP-binding proteins [10, 12, 13, 16, 17, 24].

Artemisinin produced inhibitory effects on the GTPγS-activated $I_{K.ACh}$ in a concentration-dependent manner. To elucidate the predominance of molecular mechanisms for this effect of artemisinin, the ratio of the IC_{50} values for inhibition of GTPγS-activated $I_{K.ACh}$ and carbachol-induced $I_{K.ACh}$ were calculated using the following equation [9]:

$$IC_{50}\ \text{Ratio} = \frac{IC_{50}\ \text{for GTP}\gamma\text{S-activated current}}{IC_{50}\ \text{for carbachol-induced current}}$$

The ratio of the IC_{50} values for artemisinin was 1. Hence, artemisinin might elicit its anticholinergic action through interaction with the common pathway for induction of $I_{K.ACh}$, i.e., potassium channels and/or associated GTP-binding proteins, rather than by blocking the muscarinic receptors. Yang *et al.* [25] reported that artemisinin at concentrations of 5 and 50 μM, which are similar to the concentration range used in our present experiment, inhibit the delayed rectifier potassium current in guinea pig ventricular myocytes. Thus, it appears that this antimalarial drug interacts with both voltage-gated and ligand-gated potassium channels. Therefore, there is a need for further studies in order to clarify the exact mechanisms of artemisinin in relation to the potassium channels. The maximum plasma concentration of artemisinin has been reported to be approximately 2.1 μM after oral administration of 500 mg artemisinin in the clinical setting [1]. This concentration is 15 times lower than the IC_{50} (30 μM) obtained in our present experiment. Hence, the influence of artemisinin on the heart may appear at higher doses than those used in normal clinical practice (around 500 mg) in humans.

Carbachol and adenosine cause action potential shortening in atrial cells via the same molecular mechanism; i.e., activation of $I_{K.ACh}$ through pertussis toxin-sensitive GTP-

Table 1. Effects of artemisinin on carbachol (0.3 μM)-induced action potential variables in isolated guinea pig left atrial preparations

	Control	Carbachol	0.3 μM Carbachol + Artemisinin		
		0.3 μM	1 μM	10 μM	100 μM
APA (mV)	124.6 ± 2.7	120.2 ± 3.0	120.6 ± 3.8	121.0 ± 3.4	122.0 ± 3.2
RMP (mV)	–91.5 ± 1.0	–91.0 ± 1.1	–90.5 ± 1.5	–91.0 ± 1.2	–90.9 ± 1.3
V_{max} (V/s)	104.0 ± 4.2	105.1 ± 5.8	100.7 ± 6.8	104.2 ± 8.5	98.4 ± 8.5
APD_{20} (ms)	15.0 ± 1.0	6.3 ± 0.4	7.3 ± 0.6	8.3 ± 0.7	10.5 ± 0.8*
APD_{50} (ms)	30.8 ± 1.3	12.2 ± 0.8	14.1 ± 1.0	15.8 ± 0.7*	20.2 ± 0.6*
APD_{90} (ms)	76.5 ± 1.6	25.9 ± 1.6	29.9 ± 1.3	34.0 ± 0.6*	47.2 ± 1.1*

APA: action potential amplitude. RMP: resting membrane potential. V_{max}: maximum rate of rise of action potential upstroke. APD_{20}: action potential duration (APD) at 20% repolarization. APD_{50}: APD at 50% repolarization. APD_{90}: APD at 90% repolarization.

Values are expressed as means ± SEM of five experiments. Asterisks indicate significant differences ($p<0.05$) compared to carbachol alone. Artemisinin partially reversed the shortening of APD in a concentration-dependent manner.

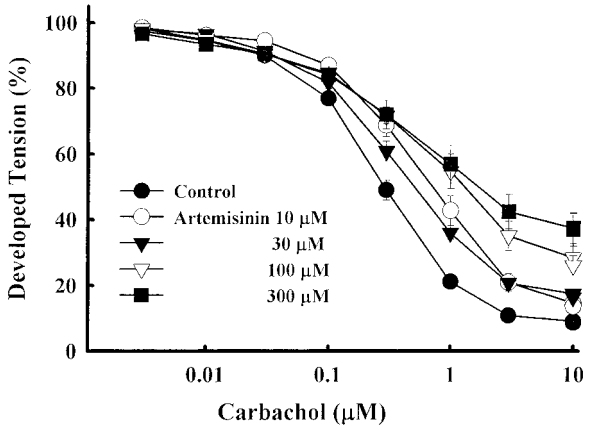

Fig. 3. Influence of artemisinin on the carbachol-induced negative inotropic effect in isolated guinea pig left atrial muscles. The preparations were incubated at 35°C and stimulated at 2 Hz. After 90 min equilibration, the preparations were incubated for 30 min in the absence or presence of artemisinin. Concentration-response curves of carbachol were then generated. Each point represents the mean ± SEM of six to eight experiments. Concentration-response curves for the negative inotropic effect of carbachol were shifted rightward by pretreatment with artemisinin.

binding proteins [15] that couple to a specific K^+ channel in atrial cells [18]. $I_{K.ACh}$ plays an important role in repolarization of action potential and maintenance of resting potential in atrial cells [14]. Artemisinin partially reversed the shortening of APD induced by carbachol in a concentration-dependent manner, and inhibition of $I_{K.ACh}$ by artemisinin could induce the phenomenon observed in the present functional studies.

Artemisinin reversed the carbachol-induced negative inotropic effect in the isolated left atrial muscle preparations. The rightward shift of the concentration-response curves for carbachol might be explained by an antagonistic action for artemisinin on $I_{K.ACh}$. The negative inotropic reactions of left atrial preparations induced by muscarinic agonists are mediated by induction of $I_{K.ACh}$ through the muscarinic M_2 receptor via G_i proteins. Experiments that have directly measured $I_{K.ACh}$ revealed that artemisinin might inhibit the current acting on the muscarinic potassium channel and/or associated GTP-binding proteins. In the present experiments, however, artemisinin reduced the maximal negative inotropic effect of carbachol, and artemisinin per se inhibited the developed tension of the left atrial preparations. Although a previous study reported inhibitory effects for artemisinin derivatives on voltage-gated sodium current in a cultured cell system [11], artemisinin had no significant influence on V_{max}, an indirect parameter of sodium current, in our action potential study. Many possible inhibitory mechanisms, such as other ionic currents, ion exchangers, ion pumps, calcium handling, and the contractile machinery of heart preparations, were considered in regard to the negative inotropic action of artemisinin; however, further experiments were needed to determine the exact effects of artemisinin on the heart.

In conclusion, we demonstrated that artemisinin produced its anticholinergic action in guinea pig atrial cells through inhibition of $I_{K.ACh}$. We postulate that this resulted from suppression of the muscarinic potassium channel itself and/or associated GTP-binding proteins. The anticholinergic action of artemisinin in heart cells was further confirmed by functional studies, i.e., change in action potential duration and inotropic responses of atrial preparations. In summary, we determined that artemisinin, an important antimalarial drug, has a potential cardiac influence.

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