Effects of artemisinin on action potentials from C-type nodose ganglion neurons

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ABSTRACT

AIM: To investigate the effects of artemisinin (Art) on the action potentials (AP) recorded from identified C-type nodose neurons and study its anti-arrhythmic and anesthetic mechanisms. **METHODS:** Neonatal and adult rats were selected for the preparation of isolated nodose ganglia neurons (NGN) and nodose ganglion-vagus slice preparation. Somatic AP were recorded from both isolated and slice NGN using whole-cell patch technique. Conduction velocity (CV) was measured using slice preparation. The effects of Art on AP were evaluated with the reference to ketamine. **RESULTS:** Effects of Art on AP were that: (1) AP depolarizing profiles were inhibited without changing resting membrane potential (RMP). The peak of AP (AP_{peak}) and upstroke velocity (UV_{APD50} and UV_{max}) decreased markedly ($P<0.01$). (2) The duration of AP at the point of half repolarization (APD_{50}) was obviously prolonged ($P<0.01$). (3) Art also slowed down AP repolarization profiles (downstroke velocity, $DV_{APD50,}$ and DV_{max}) and the peak of after-hyperpolarization (AHP_{peak}) was less negative. (4) Total inward and outward currents over the course of AP were significantly reduced in the presence of Art. (5) CV did not changed by Art. (6) The effects of Art on AP were concentration-dependent and resembled with those of ketamine except for CV. **CONCLUSION**: Art inhibited both depolarization and repolarization of AP, suggesting that the effects of Art were probably, due to the blockade of Na^+ and K^+ ion channels.

INTRODUCTION

Artemisinin (Art), an endoproxide-containing sesquiterpene isolated from Chinese herbal plant, *Artemisia annua* L, or sweet wormwood[1], has long been used for centuries as an active intergradient responsible for the antimalarial[2]. Since 1992, Art has also demonstrated the cytotoxicity against tumor cells[3-6]. Art might be an effective candidate for anti-arrhythmia[7]. Art also possessed the effect on intracellular calcium mobilization[8], and Art could change ion channel activities[9], which probably associated with its local anesthetic action[10]. Nodose ganglion neurons (NGN) provide the primary visceral sensory inputs, including pain and baroreceptor inputs. Unmyelinated C-fibers (conduction velocity <2.5 m/s) within the vagus are mainly associated with pain. The majority of the sensory terminals (baroreceptors) of the cardiac afferents are distributed throughout the heart (atria, ventricles, and coronary arteries) and large vessels (aortic and carotid arteries). Application of intact nodose ganglion-

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vagus preparation[11] may provide comprehensive insight into the ionic mechanisms of anti-arrhythmic and local anesthetic actions of Art. The main purpose of the present research is focused on the effects of Art on the action potentials (AP) recorded from NGN to provide the useful preliminary data for the further studies of the effects of Art on ion channel properties.

MATERIALS AND METHODS

Chemicals and reagents Art was provided by Shengyang Pharmaceutical University, (Shengyang, China) and the stock solution of Art was prepared to 10 mmol/L with AP recording solution[7] and diluted respectively into 10, 30, and 100 μmol/L with recording solution just prior to use. Ketamine HCl (100 μmol/L, Ketaset®, Fort Dodge, USA) was used as the reference drug. The reagents for preparation of the isolated NGN were exactly the same as the previous study[11]. Collagenase type II (308-358 kU/g) and Trypsin-3X (195-205 kU/g) (Worthington Biochem Corp, NJ) were applied for the treatment of slice and cell surface cleaning. Na-GTP and Na-ATP (Sigma, MO) were added in the pipette solution before recording. Earle's balance salt solution (Sigma, MO) was used for dissolved enzymes. Other chemicals used for recording solution were from Sigma and Fisher.

Experimental animals Neonatal (3-8 d, 5-12 g) and adult (250-350 g) Sprague-Dawley rats with either gender were selected for isolated NGN and nodose ganglion-vagus nerve slice preparation. All experimental animals were ordered directly from Experimental Animal Center of Harbin Medical University and housed in medical school animal facility at least 3 d before use. Harbin Medical University had previously approved all experimental protocols.

Preparation of isolated NGN and nodose ganglion-vagus nerve slice The methods for these preparations exactly followed the procedures in our published paper[11,12]. According to our pilot experiments and published data, the effect of Art was irreversible. For the economic reason, all the experiments were performed on isolated NGN except for the effects of Art on conduction velocity (CV), which will be carried out on the slice preparation.

Recording solutions The extracellular solution (in mmol/L) contained NaCl (l37.0), KCl (5.4), $MgCl_2$ (1.0), $CaCl_2$ (2.0), glucose (10.0), and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES, 10.0), in which pH was adjusted to 7.3 using 1 mol/L NaOH. The pipette solution (in mmol/L) contained NaCl 10.0, KCl 50.0, K_2SO_4 50.0, $MgCl_2$ 5.0, and HEPES 10.0. $CaCl_2$ (0.25 mmol/L), 1,2-*bis* (2-aminophenoxy) ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid tetrapotassium salt (4.0 mmol/L, Bapta-K, Sigma), Na-ATP and Na-GTP (2.0 mmol/L) were added to the pipette solution just before the experiments, which buffered $[Ca^{2+}]_i$ to about 10 nmol/L. The pH was adjusted to 7.2 with KOH 1 mol/L. The osmolarity of extracellular and intracellular solution was adjusted to about 310 and 290 using *D*-manitol (Sigma, MO), respectively. The solution exchanges (about 1 mL/min) were performed using 6-channel perfusion system (VC-6 channel valve controller, Warner Instrument Corp, CT).

Electrophysiological techniques[7] Briefly, whole-cell patch was conducted by patch pipette (7052, Corning) pulled (P-87, Sutter) and polished (MF-830, Narishigi) down to a resistance of 1.0-3.0 MΩ. Whole-cell current clamp (Axoclamp 2B amplifier, Axon Instruments, CA) was employed for AP recordings on NGN by stimulating the soma or vagus. Brief constant current pulse of sufficient magnitude (≤500 μs & ≥400 pA) was applied to elicit somatic AP through patch electrode. Monophasic constant current pulse with short duration (≤200 μs) was applied to stimulate vagus through the platinum (90 %)-iridium (10 %) bipolar stimulating electrode. The cathode was positioned a measured distance (1-1.5 cm) from the center of the nodose ganglia. As the stimulus artifact could generally be kept to less than 0.5 ms CV approaching 30 m/s could, theoretically, be resolved with 1.5 cm length of nerve[12]. The entire experimental protocol, data acquisition, storage, displaying, and preliminary waveform analysis were accomplished using the p-CLAMP (V8.2, Axon Instruments, CA) software package operating on a PC platform. All experiments were conducted at room temperature.

Data analysis and statistics All data were analyzed using Clampfit software (Axon Instruments) and Excel (Microsoft). The average data were presented as mean±SD and statistical significance was evaluated using a two-tail Student's *t*-test.

RESULTS

For better understanding the insight into the mechanisms of anti-arrhythmic and local anesthetic action of Art, firstly, the effects of Art on electrophysiological features were investigated on the isolated C-type NGN identified by the existence of the hump over the course

of repolarization, combining with afferent fiber CV in slice recording (Tab 1).

Effects of Art on AP depolarization All the AP parameters measured including UV_{APD50}, UV_{max}, and $Peak_{AP}$ represented the AP depolarization properties. Art (30 and 100 μmol/L) significantly slowed down UV_{APD50} and UV_{max}, dramatically reduced $Peak_{AP}$ (P<0.05 or P<0.01, Tab 1 and Fig 1) without the effect on RMP.

Effects of Art on AP repolarization The inhibitory effects of Art on AP repolarization were also observed. Art significantly reduced DV_{APD50}, DV_{max}, and $Peak_{AHP}$ (P<0.05 and/or P<0.01, Tab 1 and Fig 1). There was no significant effect on 80 % recovery of after-hyperpolarization (AHP_{80}).

Effects of Art on APD Action potential duration (APD) is directly associated with the AP depolarization and especially AP repolarization. Due to the significantly inhibitory effects of Art on AP up- and down-phase, APD at half repolarization (APD_{50}) was increased by more than 100 % (P<0.01, Tab 1 and Fig 1) in the presence of Art.

Effects of Art on total inward and outward currents The displacement current of AP was calculated using Axon Clampfit software package and plotted as the function of membrane voltage[12]. With phase plot, total inward current (TIC) and outward current (TOI) were observed. The peaks of both TIC and TOC were significantly reduced by Art 100 μmol/L but the hump over the course of repolarization remained (Fig 1 and inset).

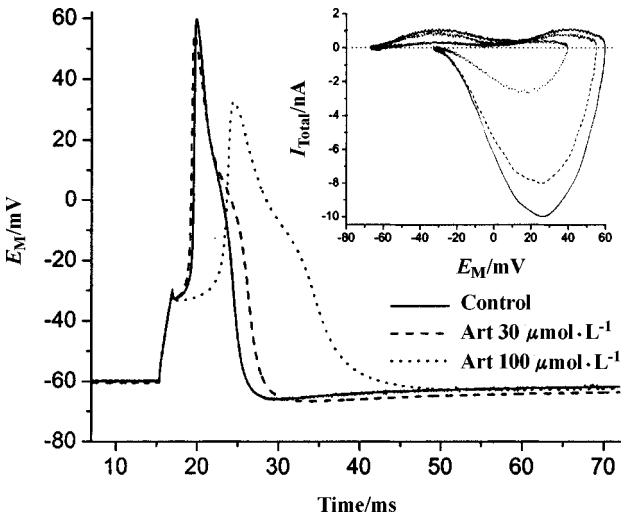

Fig 1. Inhibitory effects of Art 30 and 100 μmol/L on AP recorded on identified C-type isolated NGN of neonatal rat. Inset: the displacement currents phase plot.

Effects of Art on CV To know if there was the effect of Art on CV, AP was also recorded from NGN in nodose ganglion-vagus slice preparation and CV was calculated. The effects of Art were similar to those

Tab 1. Effects of artemisinin and ketamine on somatic AP recorded on identified C-type isolated NGN of neonatal rats. n=8-12. Mean ±SD. ^bP<0.05, ^cP<0.01 *vs* control.

Parameters	Control	Artemisinin/μmol·L^{-1} 10	30	100	Ketamine/μmol·L^{-1} 100
RMP/mV	-65±5	-63.8±2.4	-65±5	-62±4	-64±3
APD_{50}/ms	2.8±2.6	2.8±1.7	4.6±1.0^c	7.7±1.4^c	6.6±1.8^c
$Peak_{AP}$/mV	50±6	46±9	45±4	36±5^c	31±3^c
$Peak_{AHP}$/mV	-69.2±2.2	-67±4	-66.3±1.9^b	-63±0.9^c	-65.2±1.1^c
AHP_{80}/ms	68±21	64±23	66±25	68±19	62±27
UV_{APD50}/V·s^{-1}	83±42	74±37	69±22^b	26±8^c	28±7^c
DV_{APD50}/V·s^{-1}	-37±12	-28±6^b	-18±5^c	-13.3±2.7^c	-16±7^c
UV_{max}/V·s^{-1}	140±36	145±40	103±16^b	73±13^c	57±12^c
DV_{max}/V·s^{-1}	-50±14	-42±8	-27±11^c	-14±7^c	-18±5^c
$Peak_{TIC}$ /nA	-6.7±2.5	-6.3±2.1	-4.7±1.0^b	-2.6±1.2^c	-2.9±1.4^c
$Peak_{TOC}$ /nA	1.7±0.4	1.6±0.3	0.8±0.4^c	0.45±0.29^c	0.54±0.23

RMP: Resting membrane potential. APD_{50}: Action potential duration at the point of 50 % height of AP. $Peak_{AP}$: Peak of AP. $Peak_{AHP}$: Peak of after-hyperpolarization. AHP_{80}: Time of 80 % recovery of after-hyperpolarization. UV_{APD50}: Upstroke velocity at the point of APD_{50}. DV_{APD50}: Downstroke velocity at the point of APD_{50}. UV_{max}: Maximal upstroke velocity. DV_{max}: Maximal downstroke velocity. $Peak_{TIC}$: Peak of total inward current measured from phase plot. $Peak_{TOC}$: Peak of total outward current measured from phase plot.

recorded from isolated NGN without the effect on CV (P> 0.05, Fig 2 and Tab 2).

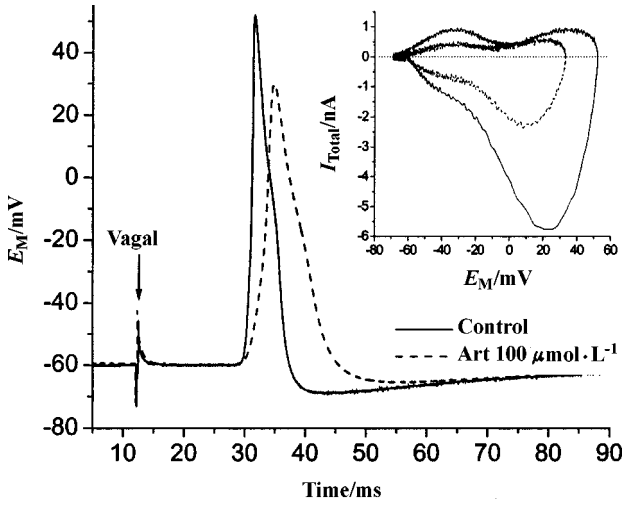

Fig 2. Inhibitory effects of Art 100 μmol/L on AP recorded on C-type NGN identified by CV from nodose ganglia slice of adult rat. The CV from present recording was 0.71 m/s. The arrow indicates the vagal stimulation. Insets: the displacement currents phase plots.

Tab 2. Effects of Art 100 μmol/L and ketamine 100 μmol/L on AP and CV measured from nodose ganglion-vagus slice preparation of adult rats. n=4. Mean±D. ^bP<0.05, ^cP<0.01 *vs* control.

Parameters	Control	Artemisinin	Ketamine
CV/m·s^{-1}	0.64±0.18	0.62±0.14	0.36±0.11^c
RMP/mV	-65±7	-66±5	-67±8
APD_{50}/ms	2.4±0.9	6.8±1.9^c	7.3±2.4^c
$Peak_{AP}$/mV	59±8	34±8^c	40±5^c
$Peak_{AHP}$/mV	-71±3	-65±0.8^b	-66.2±0.7^b
AHP_{80}/ms	66±18	64±21	65±23
UV_{APD50}/V·s^{-1}	89±23	42±12^c	47±7^c
DV_{APD50}/V·s^{-1}	-46±13	-19.0±5.1^c	-22±8^c
UV_{max}/V·s^{-1}	177±41	97±18^c	82±20^c
DV_{max}/V·s^{-1}	-57±11	-21.3±6^c	-19.6±9^c
$Peak_{TIC}$/nA	-7.3±2.3	-3.6±0.7^c	-3.1±0.8^c
$Peak_{TOC}$ /nA	1.4±0.6	0.37±0.21^c	0.3±0.3^c

Effects of ketamine on AP and CV Ketamine as the reference drug inhibited both AP depolarization and repolarization features significantly (P<0.05) at a concentration of 100 μmol/L (Tab 2 and Fig 3), and TIC and TOC measured from phase plot (Tab 2, Fig 3 and inset) were also reduced (P<0.01) without significant change of repolarization hump. There was only exception that ketamine also reduced the CV dramatically (P<0.01, Tab 2 and Fig 4).

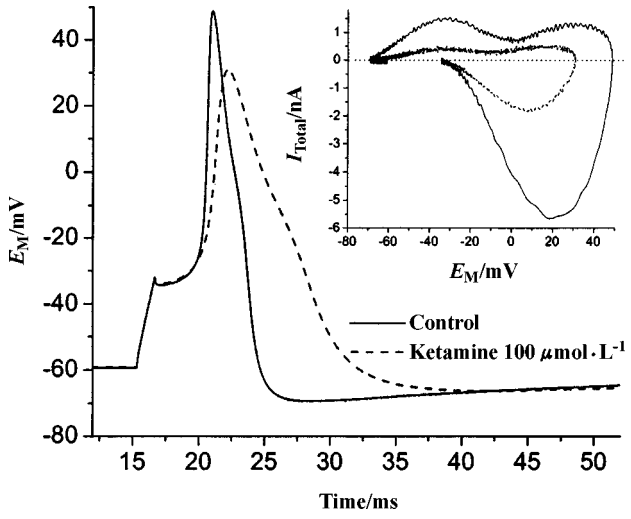

Fig 3. Inhibitory effects of ketamine 100 μmol/L on AP recorded on identified C-type isolated NGN of neonatal rat. Inset: the displacement currents phase plot.

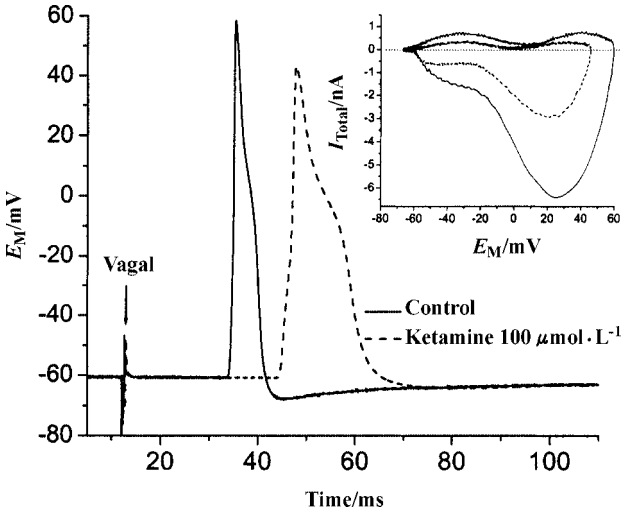

Fig 4. Inhibitory effects of ketamine 100 μmol/L on AP recorded on C-type NGN identified by CV from nodose ganglia slice of adult rat. The CV from present recording was 0.68 m/s. The arrow indicates the vagal stimulation. Insets: the displacement currents phase plots.

Effects of ω-conotoxin on AP In order to understand if Art affected the Ca^{2+} ion channel ω-conotoxin (1.0 μmol/L), a specific N-type Ca^{2+} channel blocker, was applied to isolated NGN (Fig 5). The AP depolarization was absolutely unaffected. While, the repolarization slowed down significantly. APD became much

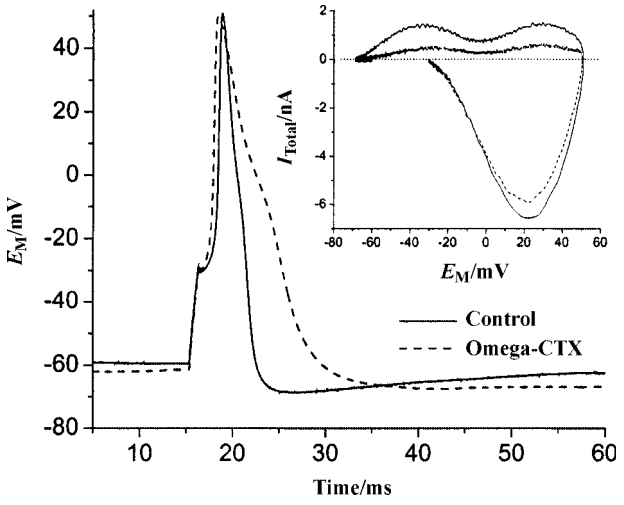

Fig 5. Inhibitory effects of ω-conotoxin 1.0 μmol/L on AP recorded on identified C-type isolated NGN of neonatal rat. Insets: the displacement current phase plots.

wider and the hump over the course of repolarization was remained. Data from the phase analysis (Fig 5 inset) also indicated that TIC was slightly reduced without influence on the AP depolarization, while TOC was obviously inhibited.

DISCUSSION

Although the present research was carried out on isolated NGN and nodose ganglia slice not cardiomyocyte, the data indirectly showed the mechanisms of drug that was studied here, because almost all major ion channels in the membrane of cardiomyocyte are expressed on the membrane of NGN. In addition, all the afferent baroreceptor fibers from heart are travelled to CNS through vagus. It has also been known that the pain-related and baroreceptor fibers within the vagus all belong to C-type fibers which are sensitive to capsaicin with slower afferent fiber CV. Therefore, the most common features of anti-arrhythmic and anesthetic agents could be investigated using C-type NGN. In the present study, the anti-arrhythmic and anesthetic effects of Art were observed on identified C-type NGN using whole-cell patch technique.

As anti-arrhythmic and anesthetic agents, they shared many common properties, for example, the effects on the APD, Na^+, and/or K^+ ion channel activities, and CV as well. Ketamine has been long used as anesthetics clinically[13]. It was found that ketamine also possessed the anti-arrhythmic action[14,15]. Our data indicated that ketamine significantly changed the waveform of AP recorded from NGN. So, ketamine as the reference drug perfectly matched the aim of this research.

It has been long cleared that several kinds of ion channels are involved over the course of AP. In the nodose neurons, Na^+ channel, especially TTX-sensitive Na^+ channel, is the most important in the development of AP depolarization, while TTX-resistive Na^+, Ca^{2+} channels, including 70 % of N-type and 30 % of T-, L-, P-type, and K^+ channels, containing DTX-sensitive, 4-AP-sensitive (transient), TEA-sensitive K^+ (delay rectified) channels, and Ca^{2+} activated K^+ channel, are all contributed to AP repolarization. Any of those ion channel property modified by the drug will change the waveform of AP. The present results showed that both depolarization and repolarization trajectory were changed by Art in a concentration-dependent manner without changing the RMP. TIC and TOC displayed in the AP phase plots were significantly reduced, suggesting that at least Na^+ and K^+ channels were inhibited by Art, this observation was strongly supported by the previous studies [7,9]. TTX-resistive Na^+ channels play a critical role in forming the hump but Ca^{2+} channels (our unpublished data). The hump over the course of repolarization remained in the presence of higher concentration (100 μmol/L) of Art, indicating that TTX-resistive Na^+ was not affected at this concentration because TTX-resistive Na^+ channel was much less sensitive to Art[9].

Recent study showed that Art increased intracellular Ca^{2+} mobilization in cardiomyocyte [8], the conclusion from the present investigation could not be drawn clearly that whether or not Art influence the Ca^{2+} channels, but the inhibitory effect of Art on Ca^{2+} channel could not be excluded since the similarity of Art and ω-conotoxin on AP repolarization was observed. So, if the effect of Art on AP repolarization induced by inhibition of Ca^{2+} channel or by blockade of Ca^{2+} and K^+ channels was still not quite clear. After exposure to Art, NGN tended to become repetitive discharge (data not shown) probably due to the decrease in the peak of after-hyperpolarization. With nodose slice recording, the afferent fiber CV was not changed by Art. All the effects of Art observed in the present investigation were very much similar to those of ketamine except for the afferent fiber CV. For better and further understanding the effects of Art based on the ion channel level the voltage-clamp studies of Art on all major ion channel

will need using cardiomyocyte and nodose neurons in the future.

Art inhibited AP depolarization and repolarization, prolonged the APD with no effects on RMP and CV and the actions of Art resembled with those of ketamine except for CV, suggesting that the inhibitory effects of Art on AP were probably, at least, due to the blockade of Na^+ and K^+ ion channels, but the inhibitory effect on Ca^{2+} channel could not be excluded under this experiment. APD prolongation and Na^+ ion channel inhibition might be the possible basic mechanisms of Art in its anti-arrhythmic and anesthetic effects.

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