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Induction of contracture and extracellular Ca^{2+} influx in cardiac muscle by sanguinarine: a study on cardiotoxicity of sanguinarine

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Summary

In this study, the toxic effect of sanguinarine (SANG) on heart was studied with isolated cardiac muscle strip isolated from Wistar rat. SANG induced positive inotropic action followed by contracture on the left ventricle and both atria strips. In addition, SANG dose-dependently inhibited spontaneous beat of the right atrium. SANG-induced contracture was completely suppressed by pretreatment with La^{3+} or in a Ca^{2+} free Tyrode solution containing 2.5 mM EGTA. Incubating isolated cardiomyocytes with SANG enhanced the $^{45}Ca^{2+}$ influx, which could be inhibited by pretreatment with La^{3+}. However, the SANG-induced $^{45}Ca^{2+}$ influx could not be inhibited by pretreatment with other Ca^{2+} channel blockers, such as nifedipine, verapamil, diltiazem, nickel and manganese, and amiloride. Although antioxidants can inhibit the SANG-induced lipid peroxidation, they could not prevent the SANG-induced contracture. *N*-acetylcysteine and dithiothreitol, the sulfhydryl reducing agents, were shown to be effective in preventing the SANG-induced contracture. These data suggested that the SANG-induced contracture is caused by the influx of extracellular Ca^{2+} through a La^{3+}-sensitive Ca^{2+} channel.

Abbreviations: DTT – dithiothreitol; LA – left atria; NAC – *N*-acetylcysteine; RA – right atria; RV – right ventricle; SANG – sanguinarine.

Introduction

The effects of sanguinarine (SANG), a benzophenanthridine alkaloid, on various biological activities have been reported by Sarkar [1] and others [2–6]. Recent reports have indicated that SANG might be useful as anticancer drug [7] or dental medicine, such as for decreasing gingival inflammation and supragingival plaque [8]. Sanguinaria extract or sanguinarine has been used in many over-the-counter products including toothpaste, mouthwash, cough and cold remedies, and homeopathic preparations. On the other hand, the toxic effect of sanguinarine has also been reported [9–11]. The Epidemic Dropsy prevalence caused by the contamination of argemone oil in mustard oil has been attributed to toxicity of sanguinarine found in argemone oil [12–14]. The clinical syndromes of argemone oil poisoning include tachycardia, gallop rhythm, hepatomegaly, diarrhea,

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nausea, erythema, pitting edema, and breathlessness, etc. In some extreme cases, sanguinarine poisoning can cause glaucoma and death due to cardiac arrest [15]. The mechanism leading to cardiac arrest has not been resolved.

Previously, we have demonstrated SANG-induced contracture and twitch depression of mouse diaphragm [16] and inhibited phenylephrine-induced contraction of rat thoracic aorta [17] in a Ca^{2+} dependent manner. In the present study, the effect of SANG on cardiac muscle was studied. We found SANG-induced contracture of cardiac muscle due to the increase of Ca^{2+} permeability of myocytes.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Preparation of heart tissues and tension recording

The method was according to Su et al. [18]. Male Wistar rats (weighing 250–350 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. Rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) and injected intraperitoneally with heparin (10,000 U/kg), and the hearts were quickly dissected and removed in Tyrode solution of the following composition (mM): NaCl 137.0; KCl 5.4; $MgCl_2$ 1.1; $NaHCO_3$ 11.9; NaH_2PO_4 0.33; glucose 11.1; and $CaCl_2$ 2.0; pH 7.4. Right atria, left atria and right ventricular strips (4 × 6 mm) were dissected from the heart and placed in an organ bath containing 10 ml of Tyrode solution gasses with 95% O_2 + 5% CO_2 at 37 ± 0.5 °C. Contractions of electrically driven right ventricular strip and left atria and spontaneously beating right atria were measured by connecting one end of the preparation to a force–displacement transducer (Grass FT.03) by a fine silk thread, and the tension was recorded on a MacLab/8e recorder (ADInstruments Pty Ltd., Australia). Preparations were equilibrated in the medium and maintained under an optimal tension of 1 g for 40–60 min before experiments started. Both right ventricular strips and left atria were stimulated at a frequency of 2 Hz through bipolar platinum electrodes with rectangular pulses of 1-ms duration, twice threshold strength. For the Ca^{2+} free experiment, the ventricular strips were washed three times in Ca^{2+} free Tyrode solution containing 2.5 mM EGTA. For individual twitch, contractile force (in g) was measured from the lowest tension (bottom) to the highest tension (top). For contracture, the contracture force (in g) was measured as the difference between the baseline tension and the maximal tension. All drugs were added from the respective stock solution with at least 1000 times the final concentration used in each experiment except for divalent cationic solutions, in which 500 mM stock solutions were used. If DMSO was used as solvent, the final DMSO concentration in medium was kept at $< 0.5\%$. This concentration of DMSO has no effect on muscular contraction.

Measurement of lipid peroxidation

Lipid peroxidation was measured according to Wills [19]. About 0.2–0.5 g of right ventricular strips were incubated in 2 ml Tyrode solution with 95% O_2 + 5% CO_2 at 37 ± 0.5 °C. Various concentrations of sanguinarine were added and incubated for 40 min. The treated right ventricular strips were placed in 4 parts of 1.15% KCl solution and homogenized quickly at 4 °C. Reactions were initiated by adding 40 μl homogeneous right ventricular strip, 40 μl 8.1% SDS, 300 μl 20% acetic acid, and 420 μl 0.8% thiobarbituric acid and incubated at 95 °C for 1 h. After cooling, 200 μl H_2O was added to each sample tube and mixed well. The samples were vortexed to disperse with 1 ml butanol/pyridine (15:1) and centrifuged. The organic layer was measured for absorbance at 532 nm. The absorbance was standardized against the acid hydrolyzed malonaldehyde bis (dimethylacetyl) over the range 0.1–2.0 nmol to give malondialdehyde equivalence in a similar reaction mixture.

Cardiomyocyte culture

Culture and isolation of cardiomyocytes from rat heart was carried out according to procedures modified from several published methods [20–22]. Basically, the neonatal Wistar rats (1–2-day old)

were given heparin, 100 units, ip, and hearts were quickly removed into chilled DMEM (containing 10% FBS). The ventricles were cut into fragments by scissors and washed with DMEM twice, and changed with pancreatin buffer (pH 7.4), containing (in mM): NaCl, 137; KCl, 2.7; $NaH_2PO_4 \cdot H_2O$, 0.42; $NaHCO_3$, 12; and glucose, 10; plus phenol red 20 mg/l; penicillin 50 U/ml and streptomycin 50 μg/ml. The ventricular fragments were dissociated by alternating treatments at 37 ± 0.5 °C with pancreatin buffer, and stirred for 20 min at 100 rpm (50–125 ml flask). Cells from the first two treatments were discarded, and the sequence was repeated about 8 times until tissue almost dissociated. Cells were collected in cold culture medium with 10% FBS and centrifuged (0 °C, 433 × *g* for 3 min). After removing the supernatant, the cells were resuspended with culture medium and filtered through 80 μm mesh filter. The cells were then plated in culture dish for about 45 min; the non-myocardial cells will attach more rapidly to the dish surface leaving the myocardial cells in suspension. An aliquot of the non-attached cells was counted in 0.4% trypan blue. After 6 h of incubation, nonattached cells were discarded. Bromodeoxyuridine (0.1 mM) was added in the culture medium in the beginning of the experiment but was omitted after 3 days. The cardiomyocytes reached confluence by day 3 and contained about 90% beating myocytes at rates varying from 180 to 220 beats/min.

Measurement of $^{45}Ca^{2+}$ influx

Calcium influx was studied in cardiomyocytes according to Saito et al. [23] and Graier et al. [24]. Briefly, myocytes were cultured in 6-well plastic plates until reaching confluence and spontaneously beat. After 72 h of cultivation, $^{45}Ca^{2+}$ was added to the culture medium (3 μCi/ml) at 37 °C for 5 min. Continuously, various concentrations of sanguinarine and high K^+ (60 mM) were added for 20 min. After the time indicated, myocytes were washed 3 times with 2 ml of cold Ca^{2+}-free Tyrode solution (containing 2.5 mM EGTA and 10 mM $LaCl_3$) and denatured by adding 1 ml 0.01 N HCl. After 1.5 h, the radioactivity of the supernatant was determined by adding 3 ml scintillation solution and counting on a liquid scintillation counter (Back Model 2200 CA). All drugs were added from the respective stock solution with at least 1000 times the final concentration used in each experiment except for divalent cationic solutions, in which 500 mM stock solutions were used. If DMSO was used as solvent, the final DMSO concentration in medium was kept at $< 0.5\%$. This concentration of DMSO has no effect on muscular contraction.

Statistics

The statistical significance of difference between control and drug effects was evaluated by Student's *t*-test. A *p*-value of 0.05 or less was considered statistically significant.

Results

Sanguinarine-induced contraction in rat heart

The effect of sanguinarine (SANG) on cardiac muscle was studied by using preparations of cardiac muscle strip from rat heart (Figure 1). SANG, at 60 μM, enhanced the twitch and followed by contracture in electrical-stimulated right ventricle and left atria (trace a and b) and the spontaneously beating right atria (trace c). The SANG-induced contracture (Figure 2) and twitch enhancement (Figure 3) were both dose-dependent. In isolated right atrium, SANG produced a decrease in spontaneously beating rate in time and dose-dependent fashion (Figure 4).

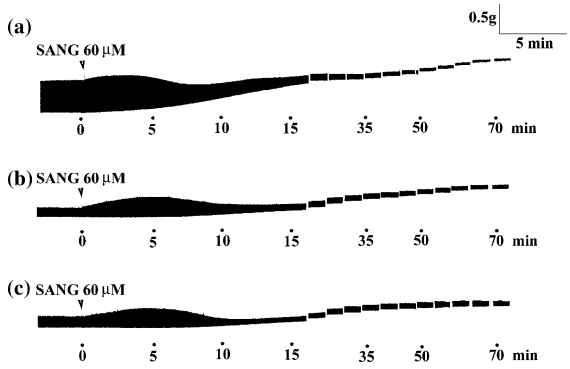

Figure 1. Sanguinarine-induced muscular contracture. The effects of SANG on electrical-stimulated right ventricle (trace a), left atrium (trace b) and spontaneously beating right atrium (trace c) treated with 60 μM sanguinarine. The twitch tension of the muscular strips isolated from rat heart was measured according to the procedure outlined in 'Materials and methods'.

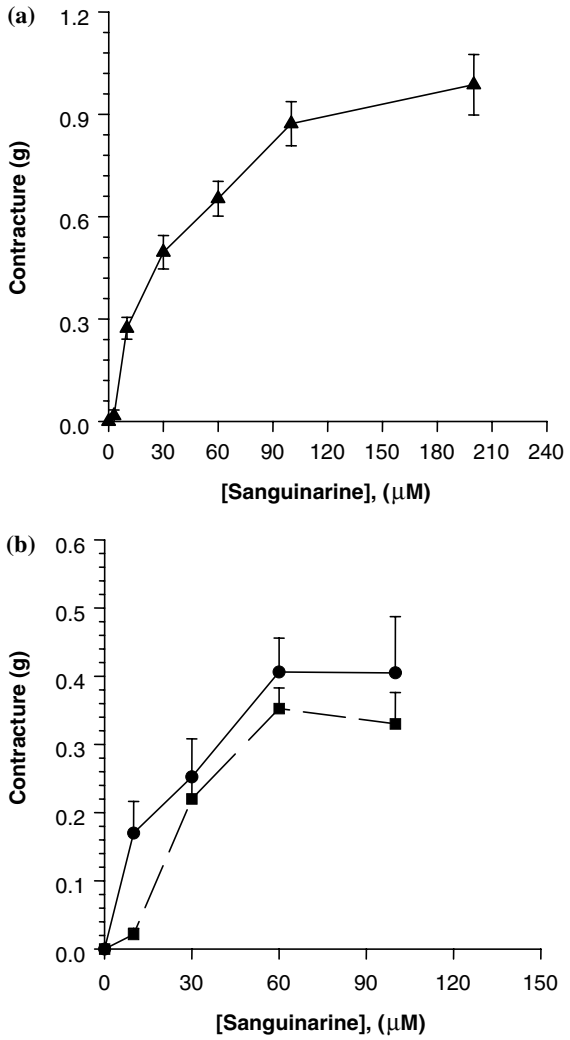

Figure 2. (a) Concentration–response curve of SANG-induced contracture of isolated right ventricle (RV); (b) Concentration–response curves of SANG-induced contracture of isolated left atria (LA, ●) and right atria (RA, ■). Each datapoint represents the mean ± S.E.M. from four preparations.

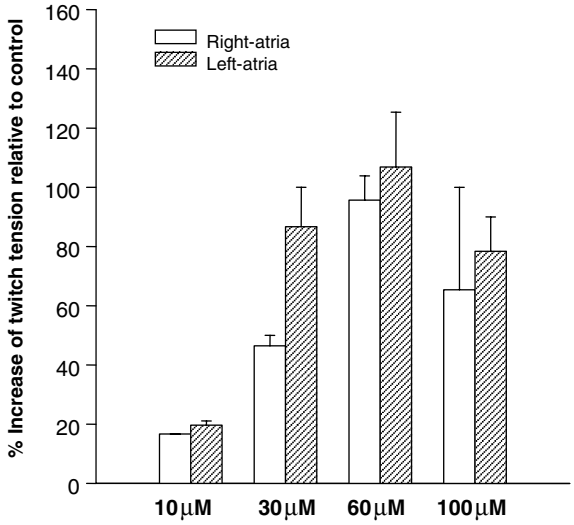

Figure 3. The positive inotropic effect of SANG on right atria and left atria. The isolated atria strips were treated with various concentrations of SANG (10, 30 and 100 μM) and the twitch tension was measured. Data represents the mean ± S.E.M. from four preparations.

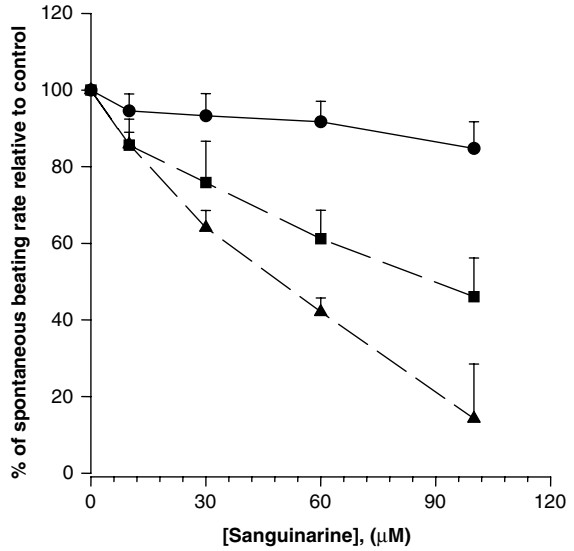

Figure 4. Concentration–response curves on beating rate in SANG-treated right atrium. The spontaneous beating rate of the SANG-treated right atrium was measured at 10 min (●), 20 min (■) and 30 min (▲), respectively. Each data point represents the mean ± S.E.M. from four preparations.

Factors affecting sanguinarine-induced contracture

The involvement of Ca^{2+} in SANG-induced contracture was examined by removal of extracellular Ca^{2+} or pretreatment with different Ca^{2+} channel blockers, and the data are summarized in Table 1. The contracture induced by SANG was reduced by 87% in Ca^{2+} free Tyrode solution. Pretreatment with the L-type Ca^{2+} channel blockers, nifedipine, verapamil and diltiazem, or non-selective Ca^{2+} channel blocker, nickel and manganese, had no effect on the SANG-induced contracture. Pretreatment with ryanodine to deplete the internal Ca^{2+} from SR or ruthenium red to block the internal Ca^{2+} release channel also had no effect on SANG-induced contracture. These data suggested that the contracture induced

Table 1. Factors affecting sanguinarine-induced contracture in rat heart.

	Contracture (g)
Normal Tyrode solution	0.53 ± 0.04
Ca^{2+}-free Tyrode solution	0.07 ± 0.04[a]
Nifedipine	0.40 ± 0.06
Verapamil	0.43 ± 0.15
Diltiazem	0.54 ± 0.12
Nickel	0.65 ± 0.01
Ryanodine	0.56 ± 0.08
Ruthenium red	0.67 ± 0.20
Tetrodotoxin	0.56 ± 0.02
Manganese chloride	0.52 ± 0.10
Amiloride	0.46 ± 0.15
Lanthanum chloride, 2 mM	0.34 ± 0.07[a]
Lanthanum chloride, 4 mM	-0.02 ± 0.01[a]

The contracture of isolated ventricular strips was induced by 30 μM sanguinarine in various conditions, and tension measured according to the procedure outlined in 'Materials and methods'. Nifedipine (10 μM), verapamil (10 μM), diltiazem (10 μM), nickel (2 mM), tetrodotoxin (2 μM), manganese chloride (5 mM), amiloride (200 μM), ryanodine (4 μM), ruthenium red (4 μM) and lanthanum (2 and 4 mM) were added and incubated with the ventricular strips for 10 min prior to the addition of sanguinarine. Data are expressed as mean ± S.E.M. ($n > 3$). [a]$p < 0.01$, as compared with normal Tyrode solution analyzed by Student's *t*-test.

by SANG was primarily due to the influx of extracellular Ca^{2+}. The SANG-induced contractures were not affected by pretreatment with tetrodotoxin, a sodium channel blocker, nor by aminoride, a Na^{+}–Ca^{2+} exchange blocker. Interestingly, when treated with La^{3+}, the SANG-induced contracture was significantly reduced.

Effect of sanguinarine on Ca^{2+} influx of cardiomyocytes

The above data suggested that SANG might be able to induce Ca^{2+} influx in cardiac muscle. The effect of SANG on Ca^{2+} influx was further investigated on isolated primary cultured cardiomyocytes. The $^{45}Ca^{2+}$ influx of cardiomyocyte increased dramatically when incubated in high-K^{+} solution due to membrane depolarization (Table 2). Pretreatment with verapamil significantly inhibited the influx. Treatment with SANG (1–30 μM) resulted in an increase of extracellular $^{45}Ca^{2+}$ influx (Table 2). SANG-induced $^{45}Ca^{2+}$ influx was not blocked by pretreatment with verapamil, nifedipine, Ni^{2+} and Mn^{2+}; however, it was completely blocked when pretreated with La^{3+} (Table 2).

Table 2. Effect of sanguinarine on cardiamyocytes $^{45}Ca^{2+}$ influx.

Treatment	% increase of $^{45}Ca^{2+}$ influx
Resting	–
High-K^{+}	70.92 ± 8.26
+ Verapamil	11.14 ± 8.70[b]
SANG 1 μM	38.45 ± 6.41
3 μM	49.80 ± 11.63
10 μM	58.71 ± 12.19
30 μM	56.34 ± 5.67
+ Nifedipine	65.12 ± 10.96
+ Verapamil	57.96 ± 11.57
+ Ni^{2+}	59.10 ± 19.27
+ Mn^{2+}	69.74 ± 17.68
+ La^{3+}	3.81 ± 3.22[a]

Sanguinarine-induced Ca^{2+} influx was measured in the presence of various concentrations of sanguinarine according to the procedure described in 'Materials and methods' using $^{45}Ca^{2+}$ as tracer. The concentrations of all drugs used were: high-K^{+} (KCl 60 mM), verapamil (10 μM), nifedipine (10 μM), nickel ($NiCl_2$ 2 mM), manganese ($MnCl_2$ 5 mM), lanthanum ($LaCl_3$ 4 mM). The percent increase was calculated by subtracting the radioactivity measured of untreated resting cardiomyocytes. The data are expressed as mean ± S.E.M. ($n > 4$). [a]$p < 0.05$, as compared with 30 μM SANG, and [b]$p < 0.05$, as compared with the high K^{+}, analyzed by Student's *t*-test.

Sanguinarine caused lipid peroxidation of cardiac muscle

The degree of lipid peroxidation was increased in SANG (10–100 μM) treated ventricular strips. Pretreatment with α-tocopherol (Vitamine E) and ascorbic acid (Vitamine C) prevented the SANG-induced lipid peroxidation (Figure 5). However, the SANG-induced contracture was not inhibited by pretreatment of Vitamine E, Vitamine C or superoxide dismutase (Figure 6). Pretreatment of NAC and DTT inhibited the contracture induced by SANG.

Discussion

In this study, we have found that sanguinarine (SANG) could cause a positive inotropic effect followed by contracture on isolated rat heart. Spontaneous beating rate of right atria was also decreased by SANG treatment. The twitch, either

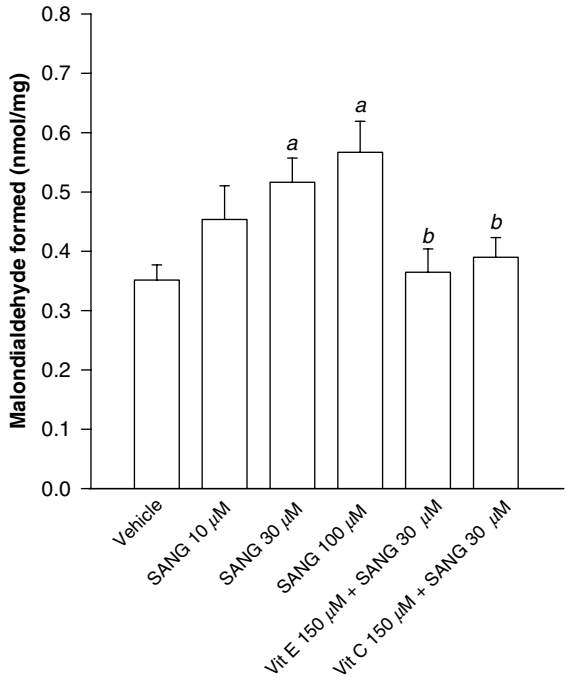

Figure 5. Induction of lipid peroxidation in right ventricle by SANG. The right ventricular strips were treated with vehicle (H_2O) or various concentrations of SANG (10, 30, 100 μM) for 60 min and the degree of lipid peroxidation was measured according to the procedure described in 'Materials and methods'. The antioxidant was preincubated for 10 min before exposure to SANG. All data represent the mean ± S.E.M. from four preparations. $a - p < 0.05$, as compared with vehicle, and $b - p < 0.05$, as compared with 30 μM SANG analyzed by Student's *t*-test.

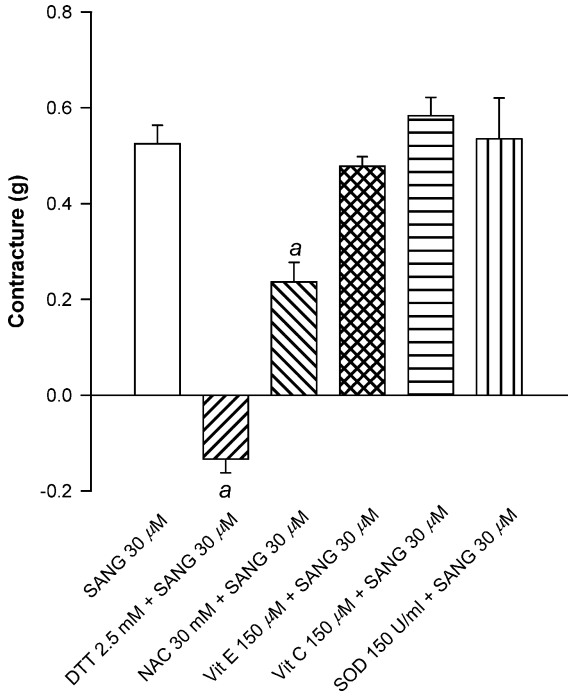

Figure 6. Effect of thiol-reducing agents and antioxidants on SANG-induced contracture. Isolated right ventricular strips were treated with thiol-reducing agents and antioxidants before addition of SANG and tension was measured accordingly. The data were expressed as mean ± S.E.M. from different preparations ($n = 4$) and analyzed with Student's *t*-test. $a - p < 0.05$, as compared with 30 μM SANG.

directly stimulated or spontaneously induced, was inhibited during contracture. Upreti et al. [11] reported that cardiac muscle fibers showed degenerative changes by treating *Argemone mexicana* seed oil orally in rat. And in some extreme cases, sanguinarine poisoning can cause glaucoma and death due to cardiac arrest [15]. The positive inotropic action of SANG observed in this study is compatible with the previous report from Seifen et al. [4] and is possibly due to the inhibition of Na,K–ATPase. In this study, we have presented evidence showing that the influx of extracelluar Ca^{2+} through La^{3+}-sensitive channel might be the cause of contracture induced by SANG.

It is known that Ca^{2+} regulation plays an important role in muscle contraction [25]. The Ca^{2+} regulation of cardiac muscle contraction could be separated into two parts: one part is from extracellular Ca^{2+} influx and another from intracellular Ca^{2+} efflux from the sarcoplasmic reticulum or endoplasmic reticulum [26]. SANG-induced contracture was reduced by approximately 87% when the incubating medium was replaced by Ca^{2+}-free Tyrode solution. These observations suggested that the influx of extracellular Ca^{2+} played a critical role in the contractures induced by SANG. This is further supported by the observation that the $^{45}Ca^{2+}$ influx of cardiomyocyte was increased when treated with SANG. Pretreatment with the L-type Ca^{2+} channel blocker, nifedipine, verapamil [27] and diltiazem [28] or non-specific Ca^{2+} channel blocker, Ni^{2+}, Mn^{2+} [29], Na^+ channel blocker, tetrodotoxin [30], and Na^+-Ca^{2+} exchange blocker, amiloride [31], has no effect on SANG-induced contractures or $^{45}Ca^{2+}$ influx. This indicates that these membrane related channels might not be involved in the SANG-induced cardiac muscle contracture and the influx of Ca^{2+}. However, both SANG-induced contracture and $^{45}Ca^{2+}$ influx were greatly reduced when pretreated with La^{3+}, a blocker of membrane Ca^{2+} permeability [32]. It is known

that La^{3+}, Ni^{2+} and Mn^{2+} are blockers of Ca^{2+} channels [28,33,34]. However, it was also noted by several researchers that these ions might have different sensitivities toward different types of channels in different cells. For example, La^{3+} but not Ni^{2+} inhibit light-induced Ca^{2+} influx into fly photoreceptors [35], and La^{3+} is more potent than Ni^{2+} in P2Y2-receptor-mediated activation of Ca^{2+} entry pathway in the human airway epithelium [36]. Whether the SANG-induced Ca^{2+} influx is specifically inhibited by La^{3+} or expressing differential sensitivity toward these ions is unclear at this moment. La^{3+} has been shown to inhibit the Portuguese Man-of-war (*Physalia physalis*) venom-induced calcium influx into chicken heart cells [37], a polycystin-2-like large conductance cation channel in rat left ventricular myocytes [38] and slow inward Ca^{2+} current in Bullfrog atrial cell [39]. Whether SANG induces the contracture of cardiac muscle through these Ca^{2+} related channels is unclear and is awaited for further investigation.

Pretreated with ryanodine, used in concentrations which will open the ryanodine receptor (RyR) Ca^{2+} release channel and consequently depletion of the SR Ca^{2+} [40], and the Ca^{2+} release channel blocker, ruthenium red [41] did not block the SANG-induced contracture. The present results suggested that SANG might not interact primarily with RyR to induce contracture and to increase intracellular Ca^{2+} in rat cardiomyocytes. This is in contrast with our previous finding in skeletal muscle [16], in which we found that SANG could directly interact with the skeletal muscle RyR resulting in Ca^{2+} release and hence muscle contraction. Different isoforms of RyR, namely RyR1 and RyR2 are expressed predominantly in skeletal and cardiac muscles, respectively [42]. Although sharing some common properties, isoforms of RyRs expressing differences not only in the physiological functions but also in responses toward the endogenous ligands and pharmacological agents are noted. In the excitation-coupling process, the induction of Ca^{2+} release from cardiac RyR is caused by the extracellular Ca^{2+} through the mechanism known as Ca^{2+}-induced Ca^{2+} release, while the mechanical coupling is suggested in skeletal muscle [43,44]. The RyRs of skeletal and cardiac muscles show differential sensitivity toward NADH [45], Ca^{2+} and redox regulation [46], Ca^{2+}, ATP and caffeine [33], and scorpion toxins [47]. The possible mechanism leading to different sensitivity of RyR1 and RyR2 toward SANG is awaited for further investigation.

SANG had been previously reported to cause lipid peroxidation in liver [10]. The toxicity induced by argemone oil can be prevented by antioxidants [48]. We also observed that SANG could cause lipid peroxidation in cardiac muscle, and could be prevented by α-tocopherol and ascorbic acid. However, α-tocopherol, ascorbic acid and superoxide dismutase had no inhibitory effect against SANG-induced muscle contracture. These data suggested that although SANG could induce lipid peroxidation in cardiac muscle, this is unrelated to the SANG-induced contracture. On the other hand, we observed that DTT and NAC, the thiol-reducing agent, inhibited SANG-induced cardiac muscle contractures. This evidences that interaction with proteins which have an essential SH group is crucial in SANG-induced contracture in heart as previously seen in other tissues [1,16,17].

In summary, the present study demonstrates the ability of SANG to induce contracture in isolated cardiac muscle in a concentration-dependent manner. This effect appears to be associated with Ca^{2+} influx through La^{3+}-sensitive channels. The direct effect of SANG on heart might be responsible for the cardiac arrest and death from SANG intoxication.

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