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The Mechanism of Antihypertensive Effect of Stevioside isolated from the Herb Stevia

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Running title ¡ GAntihypertensive effect of stevioside on dogs

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Abstract

Stevioside is a sweet-tasting glycoside isolated from the leaves of *Stevia rebaudiana*. It has been used as a non-caloric sugar substitute in Japan and Brazil for decades. Previous studies have shown that it lowered blood pressure in spontaneously hypertensive rats by intravenous injection. This study was designed to evaluate the hypotensive effect of stevioside on dogs and to define the underlying mechanism. After nasogastric feeding with stevioside powder (200 mg/kg), blood pressure of healthy Mongrel dogs began to decrease significantly at 60 min and returned to baseline level at 180 min. The reduction of blood pressure was more rapid (at 5-10 min) and was effective under intravenous injection. However, no significant change of blood pressure was noted after injection though left vertebral artery, implicating that the hypotensive effect was not related to central nervous system. Stevioside also showed significant hypotensive effect on renal hypertensive dogs in a dose-dependent manner. In cultured rat aortic smooth muscle cells (A7r5 cell line), stevioside can dose-dependently inhibit the stimulatory effect of vasopressin and phenylephrine on intracellular Ca^{2+} under calcium-containing medium. However, no intracellular Ca^{2+} inhibitory effect was observed in calcium-free medium, implicating that stevioside may inhibit Ca^{2+} influx from extracellular fluid. Our present data show that stevioside did not influence calcium ionophore (A23187) induced Ca^{2+} influx, showing the antagonistic effect was through Ca^{2+} channels. This study confirmed that stevioside is an effective antihypertensive natural product, and its hypotensive mechanism may be probably due to inhibition of Ca^{2+} influx.

Key Words¡GStevioside, Hypotension, Anesthetized dogs

Introduction

Hypertension is one of the most common cardiovascular disease causing major mortality and morbidity in many epidemiological studies [1]. However, inadequate blood pressure control still persists as a major public health problem [2]. Compliance of patients to antihypertensive treatment may be an important barrier to improve blood pressure control, since antihypertensive drugs commonly have a negative effect on quality of life [3]. Diuretics and beta-blockers are most commonly prescribed as initial therapy and have been proven to reduce cardiovascular mortality [2]. Unfortunately, these two classes of the drugs usually have side effects especially causing sexual dysfunction [4]. It would be of considerable benefits for patients with hypertension if some natural products could lower blood pressure effectively with fewer side effects.

Stevioside is a sweet-tasting glycoside isolated from the herb *Stevia rebaudiana Bertoni* (Compositae), which is widely used as a sugar substitute in Japan and Brazil for about 20 years [5]. Its safety in human usage has been well established. Our previous report has shown that a large dose of stevioside administered intravenously has effective hypotensive action without changing serum catecholamines in spontaneously hypertensive rats (SHRs) [6].

Calcium ions play a fundamental role in the activation of cells. An influx of Ca^{2+} into the cell through specific ion channels is required for myocardial contraction and determining peripherial vascular resistance. Clinically, Ca^{2+} antagonists remain the most commonly used agents for treatment of angina and hypertension, due to their ability to induce smooth muscle relaxation. Our previous study reported that stevioside could endothelium-independently relax isolated rat thoracic aortic ring pretreated with vasoconstrictors [7]. Melis et al. showed that

infusing $CaCl_2$ could attenuate the vasodilating response of stevioside in rats.[8]. These data indicated that stevioside can act directly on vascular smooth muscle cells and this effect is calcium-related.

The hypotensive effect of stevioside on other mammalian animals such as dog or pig is still unclear. In present study, we evaluate the hypotensive effect of stevioside on healthy dogs, surgery-induced renal hypertensive dogs and investigate whether the mechanism of antihypertension of stevioside is through Ca^{2+} influx inhibition of smooth muscle cells.

Materials and Methods

Experimental Animals

The investigation conforms to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health [NIH Publication No. 85-23, revised 1985]. Healthy Mongrel dogs (weight 18 ± 3kg) of both sexes were anesthetized with pentobarbital 30 mg/kg intravenously. Left fore limb femoral vein was implanted with polyethylene catheter (PE 50) for administering drugs. Right femoral artery was implanted catheter for measurement of systolic and diastolic blood pressure. The implanted catheter was connected to a Statham P23 pressure transducer (Gould Inc., Calif., USA) with the display on a Gould RS-3200 physiological recorder (Gould Inc., Ohio, USA). After stabilization for 30 min, the experiments were proceeded.

Experiments

There are four different experiments groups performed: (1) Nasogastric feeding with stevioside (fig. 1) powder (200 mg/kg) and recorded blood pressure at 30, 60, 90, 120,180 min. (2) Intravenous administration of stevioside 50 mg/kg and recorded blood pressure. (3) Referring previous method [9], renal hypertensive dogs were made. Briefly, laparotomy of anesthetized dog was performed and left renal artery was isolated and ligated. After 4 hours, the left renal artery ligation was released. After the blood pressure risen to a steady level, different dosages of stevioside were given and recorded its blood pressure. (4) Referring previous model [10], left vertebral artery was isolated and cannulated; 5 mg/kg stevioside was administered and then recorded blood pressure changes. Eight dogs were used for experiment in each group.

Culture of Rat Aortic Smooth Muscle Cells (A7r5)

The A7r5 aortic smooth muscle cell line [11], obtained from the Food Industry Institute (Hsin-Chu, Taiwan), were cultured as described previously [11].

Measurement of Cytosolic Ca^{2+} in A7r5 with Fura-2/AM

Measurements of Ca^{2+} in aortic smooth muscle cells (A7r5) were performed at room temperature using the calcium-sensitive dye fura 2-acetoxymethyl ester (Fura-2/AM, Molecular Probes), as described previously [12-13]. Cells were kept in ice for 15 min before incubation with 4 μmol Fura-2/AM in phosphate buffer saline for 60 min in the dark at room temperature. Then, the solution was centrifuged for 2-3 min to remove Fura-2/AM. The pellet of cells was put on ice for 10-15 min and 300 μl of physiological salt solution (PSS) were then added slowly back to the cells over 2-3 min. Harvested cells were suspended in Ca^{2+}-containing PSS for 30 min up to 4 hours before Fura-2/AM determinations. The cells were maintained on ice until immediately before an experiment.

For measurements of Ca^{2+}, drug at the desired concentration was then added into the 10 μl of cell solution during the stable state of fluorescence recorded in Hitachi F-2000 spectrophotometer; an excitation and emission wavelength of 340 and 380 nm was used, respectively. The value of Ca^{2+} was calculated based on the ratio at 340/380 nm, as described previously [11].

The role of Ca^{2+} influx in the responses to stimulating agents (vasopressin, phenylephrine) at different concentrations (10^{-8} to 10^{-6} mol/l) was evaluated using normal PSS containing Ca^{2+} and Ca^{2+}-free PSS. Then, stevioside was administered to observe its Ca^{2+} antagonistic effect.

Drugs and Solutions

Drugs used in this study were: HEPES, L-phenylephrine hydrochloride (Sigma

Chemical Co., Mo, USA); fetal bovine serum, FBS (Hyclone, Utah, USA); Fura-2/AM (Molecular Probes Inc., Eugene, USA). Stevioside was purchased from Nankang Chemical Company, Shanghai, China. The standard PSS contained (in mmol/l): 140 NaCl, 5.9 KCl, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 1.4 $MgCl_2$, 1.8 $CaCl_2$, 11.5 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH). In the preparation of Ca^{2+}-free solution, $CaCl_2$ was replaced by 1.8 mmol/l $MgCl_2$ (total 3.2 mmol/l) with an addition of 0.5 mmol/l EDTA.

Statistics

All values were presented as mean ± standard error of mean. ANOVA and Dunnetts post-hoc test were used to evaluate data between different experimental groups. A p value less than 0.05 was regarded as significant.

Results

Effect of Stevioside on Blood Pressure by Nasogastric Feeding

After nasogastric feeding for 30 min, blood pressure was observed to lower effectively but did not reach statistical significance. Blood pressure began to decrease significantly at 60 min and to a maximun at 90 min, and returned to baseline level at 180 min (table1).

Effect of Stevioside on Blood Pressure by Intravenous Injection

The effect of stevioside on blood pressure was decreased more effective by intravenous administration than nasogastric feeding. Blood pressure decreased reaching a maximum at 5-10 min. The reduction of diastolic pressure was greater than that of systolic pressure (table 2).

Effect of Stevioside on Renal Hypertension

Initially, intravenous administration of 10 mg/kg stevioside did not lower blood pressure significantly. From the dosage of 20 mg/kg, significant hypotension effect on renal hypertensive dogs was noticed in a dose-dependent manner. (table 3).

Effect of Stevioside on Blood Pressure via Vertebral Artery Injection

For evaluation of the direct effect on central nervous system (CNS), stevioside was injected through left vertebral artery to diminish the metabolic effect of liver on this compound. Blood pressure did not change significantly after stevioside was administered via vertebral artery (table 4). The hypotensive effect of stevioside was not related to the central nervous system.

Effect of Stevioside on Ca^{2+} Influx in A7r5

Figure 2**A** shows that in Ca^{2+}-containing medium, vasopressin increased the intracellular Ca^{2+} concentration dose-dependently in A7r5 cell suspension from 192.8 ± 20.2 nmol/l (vasopressin:0 mol/l) to 442.8 ± 32.8 nmol/l (vasopressin: 10^{-6} mol/l). But, vasopressin had no effect in Ca^{2+}-free cell suspension,. This finding suggests that intracellular Ca^{2+} increase induced by vasopressin in A7r5 cells was mediated through Ca^{2+} influx from extracellular space.

In Ca^{2+}-containing cell suspension, stevioside could dose-dependently inhibit the stimulatory effect of 10^{-6} mol/l vasopressin on intracellular Ca^{2+} from 346.8 ± 24.6 nmol/l (stevioside: 0 mol/l) to 112.4 ± 12.4 nmol/l (stevioside: 10^{-7} mol/l) (Fig.2**B**). Figure 2**C** shows that phenylephrine increased the intracellular Ca^{2+} in A7r5 whether the medium contained Ca^{2+} or not. Phenylephrine caused the intracellular Ca^{2+} concentration to increase from 186.8 ± 18.8 nmol/l (phenylephrine: 0 mol/l) to 826.6 ± 28.8 nmol/l (phenylephrine: 10^{-7} mol/l) in Ca^{2+}-containing medium, and to 410.4 ± 20.8 nmol/l (phenylephrine: 10^{-7} mol/l) in Ca^{2+}-free medium. Stevioside caused intracellular Ca^{2+} concentration decrease by a dose-dependent manner in A7r5 pretreated with phenylephrine (10^{-8} mol/l) under Ca^{2+}-containing medium (Fig. 2D). The intracellular Ca^{2+} was decreased from 464.8 ± 40.8 nmol/l (stevioside 0 mol/l) to 220.6 ± 19.8 nmol/l (stevioside 10^{-6} mol/l) in Ca^{2+}-containing medium, whereas no significant change was observed in Ca^{2+}-free medium, the intracellular Ca^{2+} concentration remained unchanged at 438.6 ± 38.4 nmol/l (stevioside 10^{-6} mol/l).

A23187 can increase intracellular Ca^{2+} concentration without depolarization.[ref] Data show that stevioside had no inhibitory effect on A23187 induced Ca^{2+} influx, the intracellular Ca^{2+} was still at the concentration of 1046.4 ± 110.8 nmol/l.

Discussion

The present data confirm that intravenous stevioside is an effective antihypertensive agent in anesthetized dogs. Previous studies have also shown that the Stevia extract is an effective hypotensive agent [14], and Melis et al. have shown that intravenous adminstration of pure stevioside (8,12, and 16mg/kg/h) resulted in a significant dose-dependent decrease in mean arterial pressure from 121 to 72 mm Hg in anesthetized Wistar rats [15]. Our previous report also showed dose-dependnet hypotensive effect of stevioside on SHR [6]. In this study, our data showed that intravenous administration of stevioside to renal hypertensive dogs resulted in significantly and dose-dependently decrease of blood pressure. SHR is a common animal model for studying genetic hypertension. The mechanism of blood pressure elevation may be related to the abnormality of neurohormonal system (ACTH-corticoid and TSH-thyroxine) [16]. The plasma catecholamine value of immature SHR is higher than normotensive rats and increase of serum sodium concentration does not induce renin secretion. Animal model in this study is acquired hypertension by using renal hypertensive dogs through surgical ligation of one side renal artery. The development of hypertension is related to renin-angiotensin system. So, stevioside not only reduces blood pressure in genetic hypertensive rats but also in acquired hypertensive dogs in which the mechanism of hypertension is different.

In our previous studies, we found that the effective dose of hypotension of stevioside when administering intravenously was around 50 mg/kg in hypertensive rats [6,17], so we chose to use this dose in the experiment using dogs as animal model. We also reported a double-blind placebo-controlled study of oral stevioside in human hypertension [18]. We found the dose of a 250 mg/capsule by oral administration trice a day is effective and safe in human, so we chose the dose of 200 mg/kg by nasogastric feeding to dogs in the present study.

Nasogastric administration was ineffective in lowering blood pressure, implicating that the

efficacy of stevioside was not good in blood pressure control by oral administration. The absorption of stevioside from gastrointerstinal tract may be low. Injection via vertebral artery did not result in significant blood pressure lowering implicates that the hypotensive action of stevioside was probably not through central effect.

The mechanism of the hypotensive action of stevioside has also been investigated previously, but proved to be inconclusive. Earlier studies have shown that hypotensive response to stevioside appears to occur through a Ca^{2+} antagonist mechanism similar to that with verapamil [8]. These investigators also showed that hypotension induced by stevioside in normotensive rat is inhibited by indomethacin, which is a potent inhibitor of prostaglandin (PG) synthesis [19]. Purdy et al have shown that PGI_2 attenuated angiotensin II-mediated increases in cytosolic calcium in preglomerular vascular smooth muscle cells. This effect may be related to cAMP activated protein kinase C (PKC) pathway [20]. Thus, the inhibitory effect of calcium influx of stevioside might be also related to prostaglandin activities.

Our data also showed that stevioside could inhibit vasopressin or phenylephrine-induced intracellular calcium increase on cultured smooth muscle cells under calcium-contained medium. Szmigielski et al. have reported that phenylephrine-induced translocation of PKC from the cytosol to the membrane and this translocation was blocked by prazosin and verapamil [21]. It seems that the influx of calcium ions through calcium channel is probably necessary for alpha1-adrenoceptor mediated activation and translocation of PKC. We need further study to evaluate whether stevioside has blocking actions against vasopressin or alpha-1 adrenergic receptors.

Fura-2/AM Ca^{2+} concentration measurement is a standard method in performing experiment concerning Ca^{2+} influx. Our data also revealed that stevioside was effective in inhibiting Ca^{2+} influx in aortic smooth muscle cells. Calcium ionophore (A23187) can induce Ca^{2+} influx without involving Ca^{2+} channels [22], stevioside was ineffective in inhibiting Ca^{2+} influx induced by A23187, showing that the Ca^{2+} antagonistic effect of stevioside was through

Ca^{2+} channels.

In conclusion, the present study confirmed that stevioside is an effective antihypertensive agent, and its mechanism of antihypertension is probably through Ca^{2+} antagonism.

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Table 1. Time course of blood pressure changes in anesthetized dogs after nasogastric feeding of stevioside

	Stevioside (200 mg/kg)					
	Baseline	30 min	60 min	90 min	120 min	180 min
SBP	148.0 ± 33.3	145.0 ± 32.6	135.4 ± 32.6**	132.5 ± 31.8**	139.1 ± 30.3*	146.5 ± 33.3
DBP	104.3 ± 19.2	101.4 ± 17.8	94.7 ± 17.0*	91.0 ± 18.5**	94.0 ± 21.5*	102.9 ± 15.5
MAP	119.1 ± 23.7	116.2 ± 22.2	108.0 ± 22.9**	105.1 ± 22.9**	116.9 ± 25.2*	117.7 ± 24.4

Values are mean ± SD. SBP=systolic blood pressure, DBP=diastolic blood pressure, MBP=mean arterial blood pressure.

* p <0.05; ** p <0.01. (*vs* baseline); the number of experimental animals = 8.

Table 2. Hypotensive effect of stevioside on anesthetized dogs by administering intravenously

	SBP (mmHg)	DBP (mmHg)	MBP (mmHg)
Baseline	165.8 ± 16.3	108.0 ± 21.5	127.3 ± 18.5
Vehicle (saline)	165.0 ± 17.0	107.3 ± 19.2	126.5 ± 17.8
Stevioside 50 mg/kg	130.2 ± 39.2**	65.1 ± 20.0**	86.6 ± 30.3**

Abbreviations as in Table 1. ** p <0.01. (*vs* vehicle); the number of experimental animals = 8.

Table 3. Dose-dependentl hypotensive effect of stevioside administered intravenously on renal hypertensive dogs

Dose (mg/kg)	SBP	DBP	MBP
Baseline	201.3 ± 28.9	156.9 ± 24.4	171.7 ± 28.1
Vehicle (saline)	201.3 ± 29.6	157.6 ± 26.6	172.4 ± 27.4
10	191.7 ± 30.3	148.7 ± 31.1	162.8 ± 30.3
20	185.0 ± 31.1*	136.9 ± 32.6*	153.2 ± 31.1*
40	175.4 ± 33.3**	126.5 ± 31.8**	142.8 ± 32.6**
80	162.8 ± 42.9**	109.5 ± 39.2**	127.3 ± 40.7**
160	118.4 ± 52.5**	71.0 ± 40.0**	86.6 ± 50.3**

Abbreviations as in Table 1. * p <0.05; ** p <0.01 (*vs* vehicle); the number of experimental animals = 8.

Table 4. Changes of blood pressure in anesthetized dogs after stevioside administering via left vertebral artery

Dose (mg/kg)	SBP	DBP	MBP
Baseline	144.3 ± 29.6	102.9 ± 18.5	116.9 ± 23.4
Vehicle (saline)	145.0 ± 30.3	102.9 ± 18.5	116.9 ± 23.7
Stevioside (5 mg/kg)	143.6 ± 31.1	102.1 ± 17.8	116.2 ± 24.4

Abbreviations as in Table 1; the number of experimental animals = 8.

Legends of Figures

Fig. 1. The chemical structure of stevioside.

Fig. 2. Using Fura-2/AM to evaluate intracellular Ca^{2+} in A7r5 cells. (**A**) Vasopressin-induced dose-dependent increase of intracellular Ca^{2+} in A7r5 cultured with calcium-contained solution; (**B**) stevioside dose-dependently inhibited 10^{-6} mol/l vasopressin-induced intracellular Ca^{2+} increase; (**C**) phenylephrine-induced intracellular increase of Ca^{2+} on A7r5 cultured with calcium-contained or calcium-free solution dose-dependently; (D) stevioside dose-dependently inhibited 10^{-8} mol/l phenylephrine-induced intracellular Ca^{2+} increase in A7r5 cultured with calcium-contained solution. -Δ- = Ca^{2+}-containing solution; -▲- = Ca^{2+}- free solution.

* $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

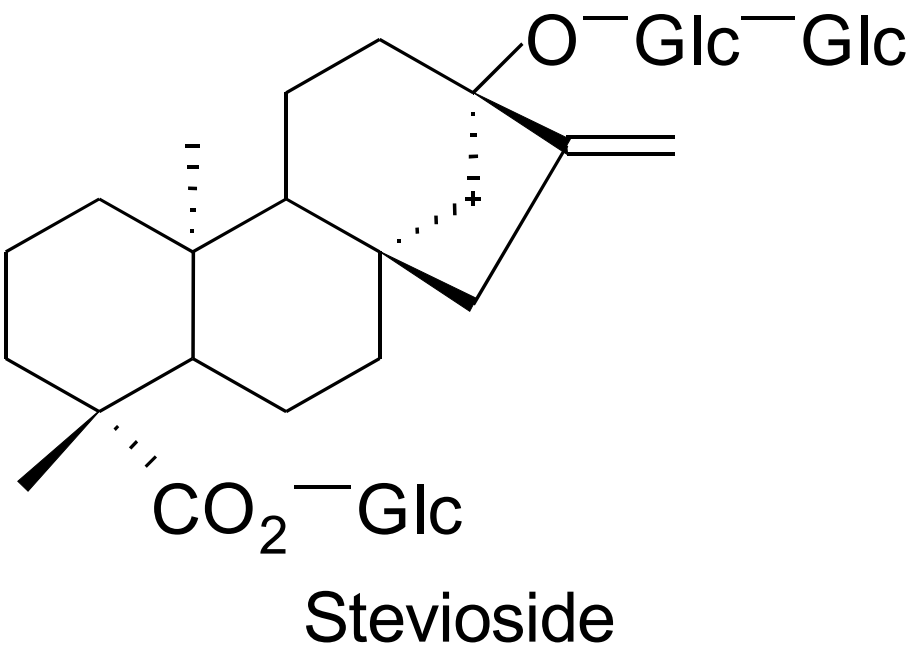

Fig. 1.

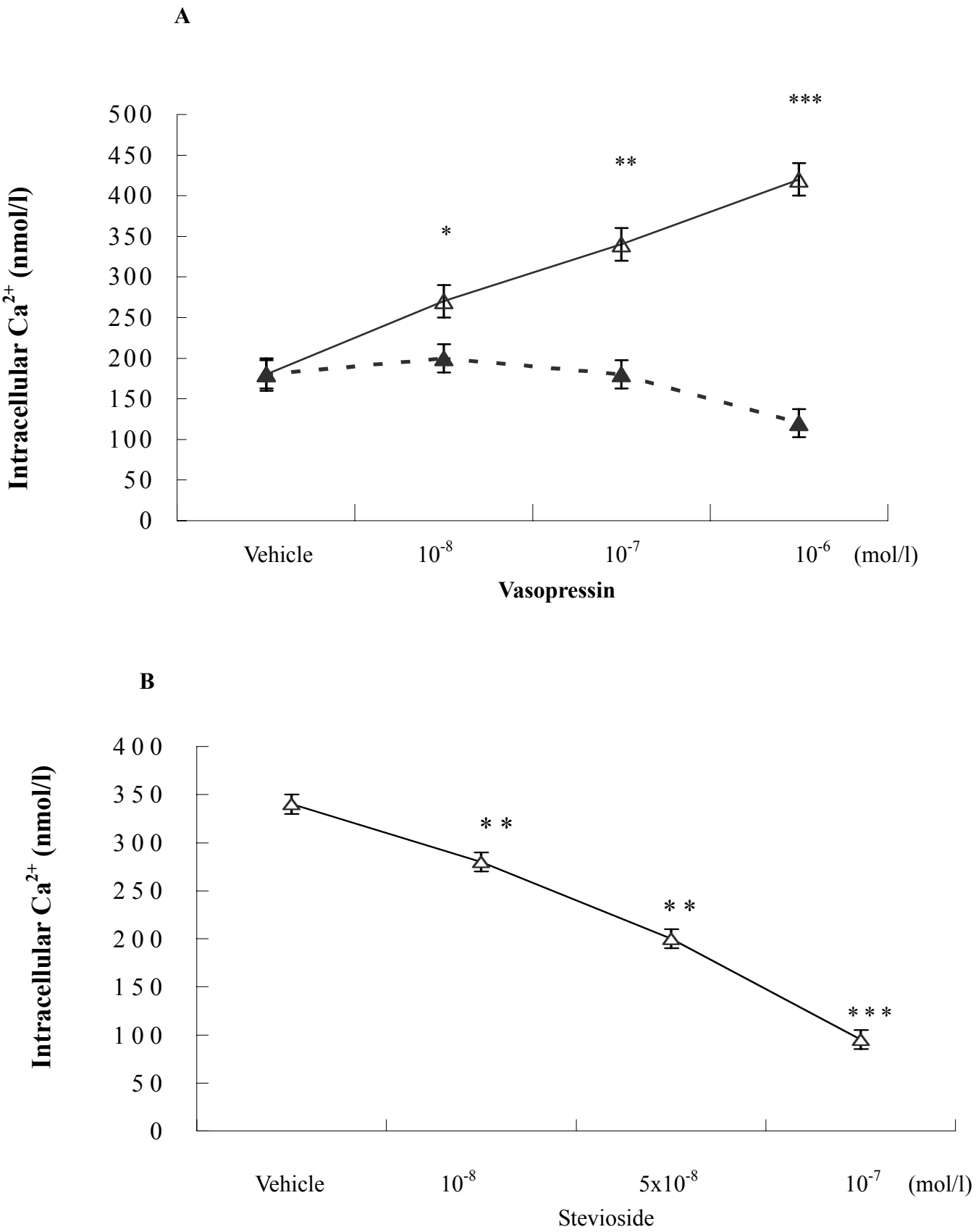
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Fig. 2

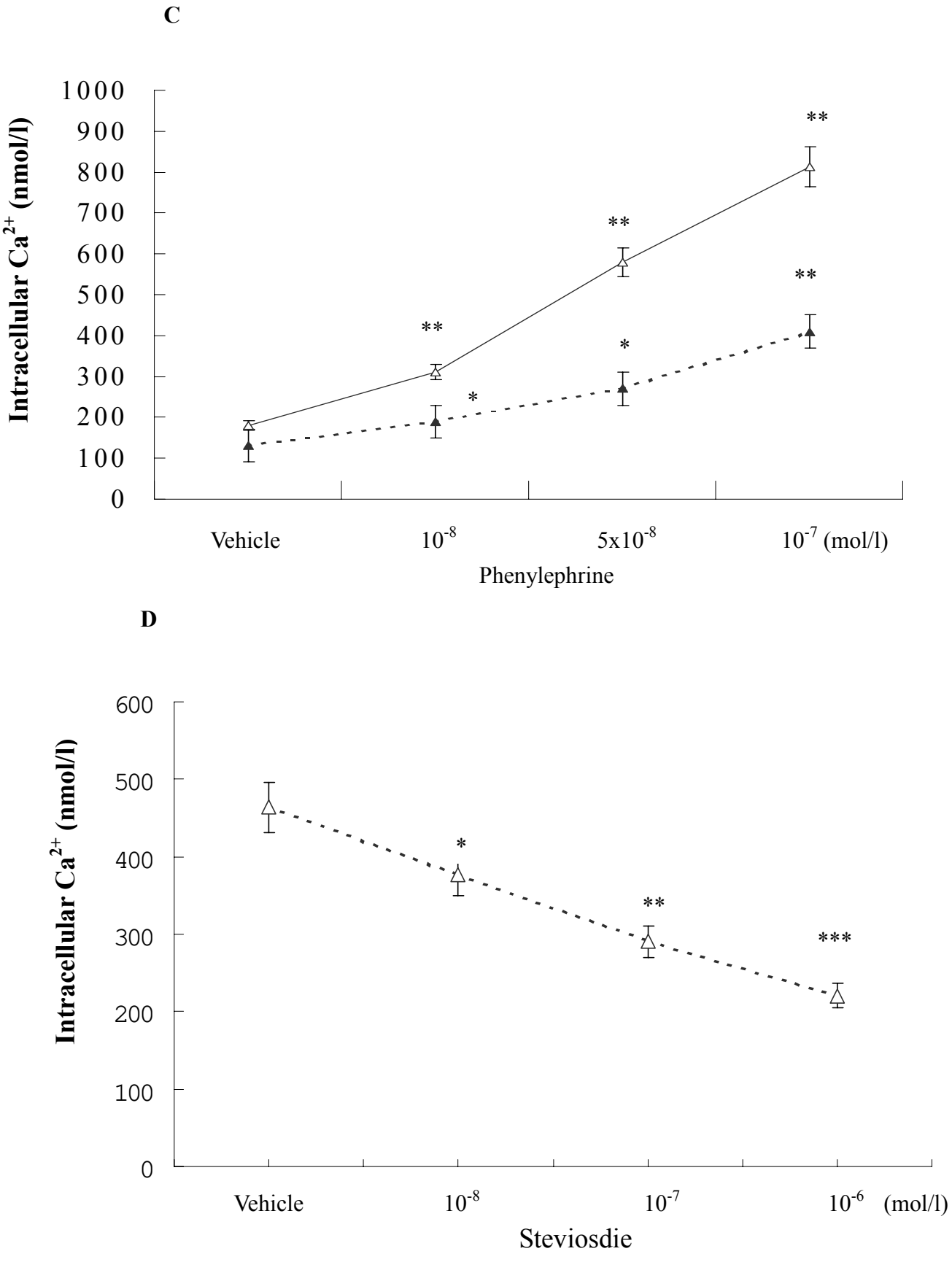
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Fig 2.