ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Effect of tetrandrine on free intracellular calcium in cultured calf basilar artery smooth muscle cells[1]

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KEY WORDS tetrandrine; calcium; basilar artery; vascular smooth muscle; cultured cells; caffeine; phenylephrine

ABSTRACT

AIM: To study the effects of tetrandrine (Tet) on extracellular Ca^{2+} influx and intracellular Ca^{2+} release in cultured calf basilar artery smooth muscle cells. **METHODS:** Free intracellular calcium was examined by a system of measurement of AR-CM-MIC, using Fura 2-AM as a fluorescent indicator. **RESULTS:** In the presence of extracellular Ca^{2+} 1.3 mmol/L, no significant effect of Tet on resting $[Ca^{2+}]_i$ was found. KCl 20, 40, and 60 mmol/L triggered a sustained rise in $[Ca^{2+}]_i$, pretreatment with Tet inhibited the elevation of $[Ca^{2+}]_i$ induced by KCl in concentration-dependent manner, Tet at high concentration (100 μmol/L) almost abolished the rise of $[Ca^{2+}]_i$ evoked by KCl. Caffeine 10 mmol/L only produced a transient increase of $[Ca^{2+}]_i$, which spontaneously declined back to resting levels. Tet 10-30 μmol/L had no effect on caffeine-induced $[Ca^{2+}]_i$ transient peak. Tet at high concentration (100 μmol/L), however, reduced the $[Ca^{2+}]_i$ transient peak induced by caffeine. Phenylephrine (PE) 10 mmol/L produced a rapid transient peak and a distinct sustained elevation in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+} containing egtazic acid (EGTA), PE only produced a rapid transient peak in $[Ca^{2+}]_i$. Pretreatment of Tet (10-100 μmol/L) inhibited the sustained elevation in $[Ca^{2+}]_i$ induced by PE in a concentration-dependent manner. However, only 100 μmol/L of Tet inhibited the transient peak in $[Ca^{2+}]_i$ induced by PE both in the presence of extracellular Ca^{2+} 1.3 mmol/L and in the absence of extracellular Ca^{2+} containing EGTA. **CONCLUSION:** Tet inhibited the Ca^{2+} influx from the extracellular site via voltage-activated Ca^{2+} channel and PE-receptor-operated Ca^{2+} channel. At a high concentration, Tet may inhibit the Ca^{2+} release from sarcoplasmic reticulum (SR) or refilling of intracellular calcium store in cerebral artery smooth muscle cells.

INTRODUCTION

Tetrandrine (Tet) is the major active component in the root of *Stephania tetrandra* S. Tet has been used for the treatment of various diseases, including hypertension, cardiac arrhythmia, angina and inflammation[1-3]. Recently, it was reported that Tet had a protective effect on the brain ischemia/reperfusion model in rats and gerbils[4,5]. A series of studies have revealed that Tet blocked Ca^{2+} channel in cardiac cells, neuroblastoma cells, neurohypophysial nerve terminals, and mesenteric artery, which largely accounted for its antihypertensive, antiarrhythmic and antimyocardial ischmeia actions[1,3]. In addition to its direct effect on neurons, Tet may relax cerebral artery to protect from

[1] Project supported by the National Natural Science Foundation of China (No 30000207), the Natural Science Foundation of Education Department, Jiangsu Province (No KJB310001), and Commercialization of Science and Technology Foundation of Education Department, Jiangsu Province (No JH01-050).
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Received 2002-07-18 Accepted 2002-10-18

brain ischmia. However, the effect and mechanism of Tet on cerebral artery remain unclear. In the pesent study, we examined the effect of Tet on Ca^{2+} influx from extracellular space and Ca^{2+} release from sarcoplasmic reticulum (SR) or refilling of intracellular calcium store in cultured calf basilar artery smooth muscle cells.

MATERIALS AND METHODS

Materials Fura 2-AM, dimethyl sulfoxide, caffeine and phenylephrine were purchased from Sigma Chemical Co. Fura 2-AM was dissolved in dimethyl sulfoxide. Tet was obtained from Jin Hua Pharmaceutical Factory, it was dissolved in HCl 0.1 mol/L, then diluted with D-Hanks' medium to desired concentration. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco. The system of measurement of AR-CM-MIC was the product of Spex Co, USA.

Cell culture Calf basilar artery smooth muscle cells were cultured according to our previous method[6]. Briefly, the basilar artery of newborn calf (Nanjing Weigang Calf Serum Factory) was isolated rapidly. The connective tissue was removed under a dissecting microscope. The endothelium was removed first by erasing when the blood vessels were cut longitudinally, the pieces (2 mm^2) of the blood vessels were placed endosurface downright in culture dishes and were incubated at 37 °C in a humidified incubator with 95 % air and 5 % CO_2 in DMEM with 10 % (v/v) fetal calf serum(FCS). Cells migrated from the blood vessels in about 7 d. When cells covered the culture dish, small pieces were removed and cells were washed with D-Hanks' solution twice, then incubated at 37 °C for 10 min in D-Hanks' solution with 0.125 % trypsin. The trypsinization was discontinued by adding ice-cold DMEM 10 mL containing 10 % (v/v) FCS. The cell pellets were resuspended in fresh medium with FCS to 1×10^8 cells/L and cultured. The medium was changed every 2-3 d. Cells reached confluence in about 5 d and then passaged every 5-6 d. The single cell was cultured in culture dish with a glass cover slip 2 d before experiment. $[Ca^{2+}]_i$ was measured in passages 4 and 5.

$[Ca^{2+}]_i$ measurement Cells grown on glass coverslip were incubated with DMEM containing Fura 2-AM 3 μmol/L at 37 °C for 40 min. The loaded cells were washed twice with Hanks' solution or D-Hanks' solution. The fluorescence was recorded in a system of measurement of AR-CM-MIC at λ_{ex}=340 nm/380 nm, λ_{em}=505 nm, directly after the addition of an agonist. $[Ca^{2+}]_i$ of single cell was calculated according to the formula: $[Ca^{2+}]_i=K_d\ (sb_1/sb_2)\ [(R-R_{min})/(R_{max}-R)]$ nmol/L. Where, the dissociation constant K_d=224 nmol/L at 37 °C. R was fluorescence intensity, R_{max} and R_{min} were obtained by the addition of ionomycin and egtazic acid respectively.

Statistical analysis Date were analyzed with *t*-test. Differences were considered significant when $P<0.05$.

RESULTS

Effect of Tet on resting $[Ca^{2+}]_i$ The resting $[Ca^{2+}]_i$ was (100±9) nmol/L in the presence of extracellular Ca^{2+} 1.3 mmol/L. When 10, 30, and 100 μmol/L of Tet were added, the $[Ca^{2+}]_i$ was (101±10), (98±8), and (97±10) nmol/L, respectively ($P>0.05$ *vs* 0 μmol/L of Tet). No significant effect of Tet on resting $[Ca^{2+}]_i$ was found in cultured calf basilar artery smooth muscle cells.

Effect of Tet on elevation of $[Ca^{2+}]_i$ induced by KCl KCl 20, 40, and 60 mmol/L triggered a sustained rise in $[Ca^{2+}]_i$ without transient component in the presence of extracellular Ca^{2+} 1.3 mmol/L. In the absence of extracellular Ca^{2+} containing egtazic acid, addition of KCl had no effect on $[Ca^{2+}]_i$. This result suggested that KCl increased $[Ca^{2+}]_i$ via Ca^{2+} channel in the plasma membrane rather than in SR in this condition. Pretreatment with Tet inhibited the elevation of $[Ca^{2+}]_i$ induced by KCl in a concentration-dependent manner. High concentration of Tet almost abolished the rise of $[Ca^{2+}]_i$ evoked by KCl (Fig 1).

Effect of Tet on transient elevation of $[Ca^{2+}]_i$ induced by caffeine Caffeine 10 mmol/L only produced a transient increase of $[Ca^{2+}]_i$, which spontaneously declined back to resting levels. To clarify whether the SR stores of Ca^{2+} were refilled completely, the repetitive addition and washout of caffeine was performed. When the interval of repetitive addition and washout of caffeine was 5 min, the transient peak in $[Ca^{2+}]_i$ evoked by subsequent repetitive addition of caffeine declined, suggesting that the Ca^{2+} store in the SR was depleted. With the interval of washout of caffeine was increased to 25 min, the transient peak in $[Ca^{2+}]_i$ evoked by repetitive addition of caffeine was kept stable, indicating that the Ca^{2+} store in the SR was refilled completely within 25 min. We used 25 min as the interval of washout. Tet 10-30 μmol/L had no effect on caffeine-induced $[Ca^{2+}]_i$ transient peak. Tet at a high concentra-

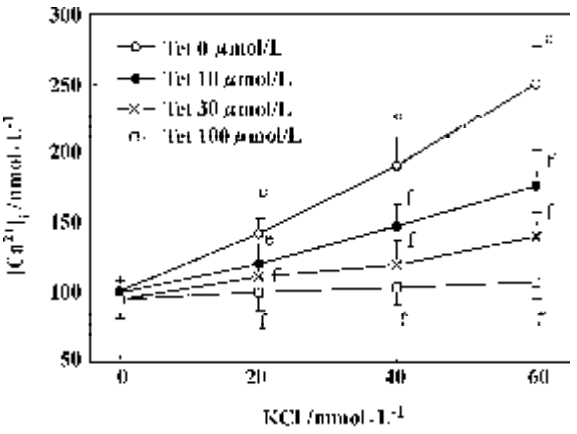

Fig 1. Efffect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by KCl in the presence of extracellular Ca^{2+} 1.3 mmol/L. *n*=6. Mean±SD. $^cP<0.01$ *vs* KCl 0 mmol/L. $^cP<0.05$, $^fP<0.01$ *vs* 0 mmol/L of Tet.

tion (100 μmol/L) , however, reduced the $[Ca^{2+}]_i$ transient peak induced by caffeine (Fig 2).

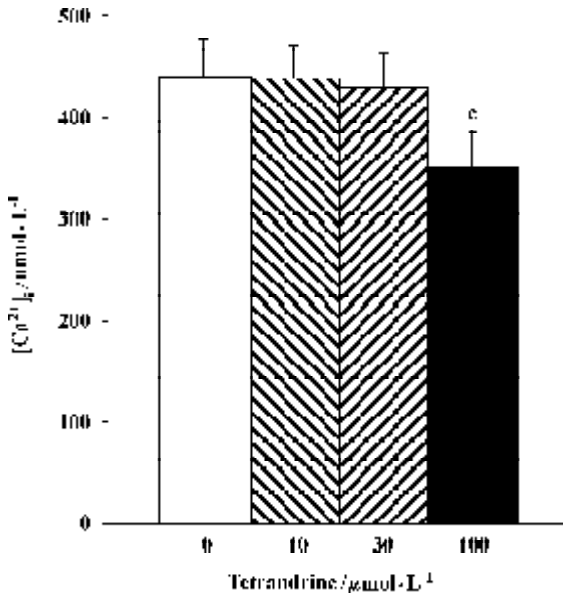

Fig 2. Effect of tetrandrine on transient elevation of $[Ca^{2+}]_i$ induced by caffeine (10 mmol/L) in the presence of extracellular Ca^{2+} 1.3 mmol/L. *n*=6. Mean±SD. $^cP<0.01$ *vs* 0 mmol/L of Tet.

Effect of Tet on PE-induced elevation of $[Ca^{2+}]_i$

PE 10 μmol/L produced an increase in $[Ca^{2+}]_i$, consisting of a rapid transient peak and a distinct sustained component in the presence of extracellular Ca^{2+} 1.3 mmol/L. In the absence of extracellular Ca^{2+} containing egtazic acid, PE only produced a rapid transient peak in $[Ca^{2+}]_i$ without the sustained component, which indicated that the rapid transient peak induced by PE represented Ca^{2+} release from Ca^{2+} store. To clarify whether the sustained elevation in $[Ca^{2+}]_i$ induced by PE represented Ca^{2+} influx from extracellular site, caffeine was pretreated to deplete Ca^{2+} store. When PE was added 3 min after pretreatment of caffeine (20 mmol/L), only a sustained elevation in $[Ca^{2+}]_i$ induced by PE was observed. These data verified that the rapid transient peak and sustained elevation in $[Ca^{2+}]_i$ induced by PE represented Ca^{2+} release and Ca^{2+} influx respectively in cultured calf basilar artery smooth muscle cells.

In the presence of extracellular Ca^{2+} 1.3 mmol/L, pretreatment of Tet (10-100 μmol/L) inhibited the sustained elevation in $[Ca^{2+}]_i$ induced by PE in a concentration-dependent manner and high concentration of Tet (100 μmol/L) inhibited the transient peak in $[Ca^{2+}]_i$ induced by PE (Fig 3). In the absence of extracellular Ca^{2+} containing egtazic acid, 10 and 30 μmol/L of Tet showed no significant effect on the transient peak in $[Ca^{2+}]_i$ induced by PE and 100 μmol/L of Tet inhibited the transient peak in $[Ca^{2+}]_i$ induced by PE (Fig 4).

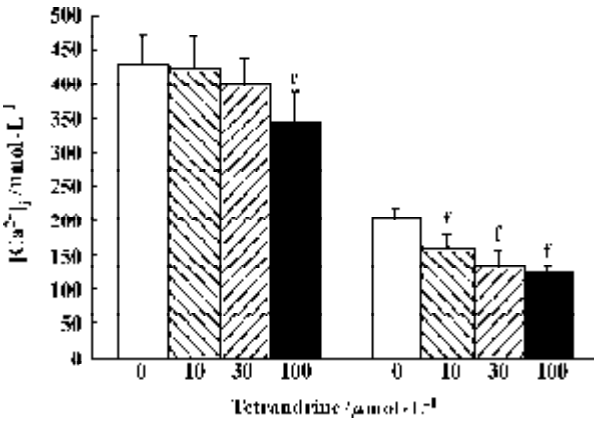

Fig 3. Efffect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by phenylephrine (PE, 10 mmol/L) in the presence of extracellular Ca^{2+} 1.3 mmol/L. Left: transient peak in $[Ca^{2+}]_i$, Right: sustained component in $[Ca^{2+}]_i$. *n*=6. Mean±SD. $^cP<0.01$ *vs* Tet 0 mmol/L in $[Ca^{2+}]_i$ transient peak. $^fP<0.01$ *vs* Tet 0 mmol/L in $[Ca^{2+}]_i$ sustained component.

DISCUSSION

The actions of Tet on a series of systems have been investigated, including cardiac cells, cardiac papillary muscle, arotic vessel, mesenteric artery, pulmonary artery, B cells, human leukaemic HL-60 cells, cornea and retina cells[3, 7-10]. However little has been reported on the effect of Tet on cerebral artery. Tet was

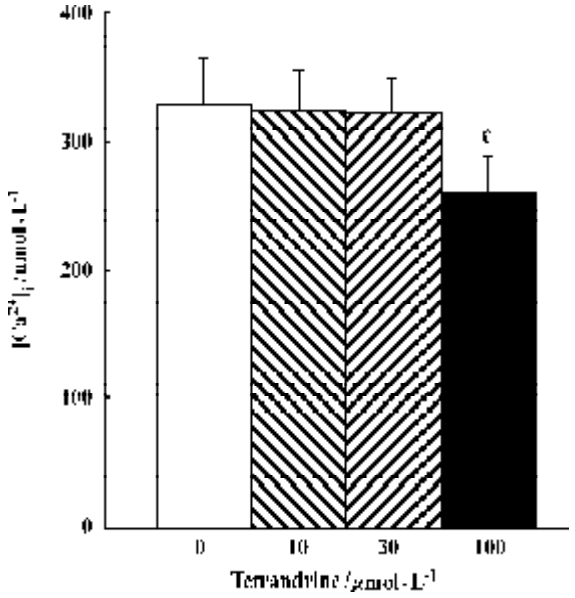

Fig 4. Efffect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by phenylephrine (PE, 10 mmol/L) in the absence of extracellular Ca^{2+} containing egtazic acid. *n*=6. Mean±SD. $^cP<0.01$ *vs* 0 mmol/L of Tet.

reported to decrease brain injury on brain ischemia/reperfusion model in rats and gerbils[4,5]. Neural tissues are highly dependent on glucose and oxygen supplies, an adequate continuous blood supply to the brain is essential to ensure the normal function of the brain. In addition to its direct effect on neurons, Tet was able to inhibit pial artery constriction induced by KCl and PE in rats (unpublished data), which may partially account for its protective effect on cerebral ischemia. However, the effect and mechanism of Tet on cerebral artery remain unclear. In the present study, we demonstrated that Tet blocked voltage-activated Ca^{2+} channel and PE receptor-gated Ca^{2+} channel on the plasma membrane in a concentration-dependent manner and Tet at a high concentration may inhibited Ca^{2+} release or Ca^{2+} uptake via caffeine- and PE-sensitive Ca^{2+} store in cerebral basilar artery smooth muscle cells in rats.

Tet relaxed the high K^+-induced constrictions in isolated arteries, including pulmonary, coronary, renal arteries[3,11]. Electrophysiological study revealed that Tet declined a high-voltage-threshold, long-lasting (L-type) Ca^{2+} channel current in GH3 anterior hypophysial cells, cardiac ventricular muscles , which demonstrated that Tet was an L-type Ca^{2+} channel blocker[3,12]. On the cerebral basilar artery smooth muscle cells (this study), KCl only produced a sustained increase in $[Ca^{2+}]_i$ whithout the transient component, indicating that KCl increased Ca^{2+} entry but did not influence the Ca^{2+} store. Tet inhibited markedly KCl-induced rise in $[Ca^{2+}]_i$ and high concentration of Tet almost abolished the rise of $[Ca^{2+}]_i$, suggesting that Tet acted as a voltage-activated Ca^{2+} channel antagonist on cerebral artery smooth muscle cells.

Kwang *et al*[13] reported that Tet inhibited the binding of ^{3}H-prazosin (an α_1-adrenoceptor antagonist) to dog aortic muscle plasmalemma-rich membranes and Tet was shown as an α_1-adrenoceptor antagonist. In addition to inhibiting voltage-activated Ca^{2+} channel, Tet could inhibit receptor-dependent Ca^{2+} channel, including PE-, norepinephrine-, histamin-, glutamate-sensitive Ca^{2+} channel on plasma membrane[9,10]. In the present study, Tet concentration-dependently inhibited PE-induced sustained rise in $[Ca^{2+}]_i$ in Ca^{2+} -containing medium, indicating that Tet inhibited Ca^{2+} influx via PE-sensitive Ca^{2+} channel in cerebral artery smooth muscle cells. Considering the system difference, whether Tet directly interacted with α_1-adrenoceptor on cerebral artery will require further studies.

The results of studies of Tet on Ca^{2+} release or Ca^{2+} uptake sensitive to caffeine are still confusing: Tet facilitated the contraction induced by caffeine in rabbit aortic strip[14], however, elicited no effect on caffeine-induced transient contraction in rat aortic rings[13], it has been also shown that Tet reduced the magnitude of the caffeine-induced $[Ca^{2+}]_i$ elevation in rat cardiomyocyte[15] and the refilling of intracellular calcium stores sensitive to caffeine was inhibited by Tet in rat aortic strips[16]. The differences of experimental procedures or preparations used might in part account for the results.

The intracellular Ca^{2+} store depletion by caffeine elicited Ca^{2+} entry from extracellular space via specific channels or by passive diffusion or exchange with other ions and intracellular Ca^{2+} can be uptaken by Ca^{2+} store. Tet inhibited the Ca^{2+} uptake to SR in rat cardiomyocyte and the refilling of intracellular calcium stores in rat aortic strips[15-17], Tet might influence the refilling of intracellular calcium stories via affecting plasma membrane channels indirectly or via affecting intracellular Ca^{2+} store directly, the exact mechanisms remain unclear.

Caffeine inhibited cyclic nucleotide phosphodiesterases and stimulated Ca^{2+} release[18]. The present study showed that the Ca^{2+} storage sites sensitive to caffeine can not be completely refilled within 5 min with 1.3 mmol/L extracellular Ca^{2+}. When caffeine was added

again after incubation period of 25 min in extracellular Ca^{2+} 1.3 mmol/L, the magnitude of $[Ca^{2+}]_i$ transient was similar to that of the first one, which indicated that Ca^{2+} store was refilled completely. We used 25 min as the interval of washout, and found that 100 μmol/L of Tet had the inhibitory effect on the transient peak in $[Ca^{2+}]_i$ evoked by caffeine. Although we can not explain the exact mechanisms, these results also implied that Tet might inhibit refilling of intracellular calcium store in cerebral artery smooth muscle cells.

PE elicited a rapid transient peak companied with a more sustained increase in $[Ca^{2+}]_i$ in Ca^{2+} -containing medium. But in absence of Ca^{2+}, only rapid transient peak was induced by PE. After caffeine depleted the SR Ca^{2+} store, subsequent addition of PE only elicited a sustained rise in $[Ca^{2+}]_i$ without the transient component. These data implied that the caffeine-inducible Ca^{2+} pool and PE-inducible Ca^{2+} pool might overlap in cerebral artery smooth muscle cells. Tet inhibited the transient peak in $[Ca^{2+}]_i$ induced by PE both in presence and absence of Ca^{2+}. These result indicated that Tet at a high concentration might inhibit Ca^{2+} release or Ca^{2+} uptake via caffeine- or PE-inducible Ca^{2+} pool. The exact mechanism of Tet on Ca^{2+} release or Ca^{2+} uptake in cerebral artery smooth muscle cells will warrant further investigation.

In summary, Tet inhibited the Ca^{2+} influx from the extracellular site via voltage-activated Ca^{2+} channel and PE-receptor-operated Ca^{2+} channel. At high concentration, Tet may inhibit the Ca^{2+} release from SR or refilling of intracellular calcium store in cerebral artery smooth muscle cells.

REFERENCES

1 Liu QY, Li B, Gang JM, Karpinski E, Pang PK. Tetrandrine, a Ca^{++} antagonist: effects and mechanisms of action in vascular smooth muscle cells. J Pharmacol Exp Ther 1995; 273: 32-9.

2 Xiao JG, Chiou GC. Tetrandrine inhibits breakdown of blood-aqueous barrier induced by endotoxin and interleukin-1 alpha in rats. J Ocul Pharmacol Ther 1996; 12: 323-9.

3 Wang G, Lemos JR. Tetrandrine: a new ligand to block voltage-dependent Ca^{2+} and Ca^{2+}-activated K^+ channels. Life Sci 1995; 56: 295-306.

4 Liu SJ, Zhou SW, Xue CS. Effect of tetrandrine on neutrophilic recruitment response to brain ischemia/reperfusion. Acta Pharmacol Sin 2001; 22: 971-5.

5 Sun F, Liu TP. Tetrandrine *vs* nicardipine in cerebral ischemia-reperfusion damages in gerbils. Acta Pharmacol Sin 1995; 16: 145-8.

6 Wang B, Zhang XQ, Liu TP, Xiao JG. 8-(*N*, *N*-diethylamino)-*n*-octyl-3, 4, 5-trimethoxybenzoate reduced $[Ca^{2+}]_i$ elevation induced by histamine, serotonin, and glutamate in cultured calf basilar artery smooth muscle cells. Acta Pharmacol Sin 1998; 19: 251-3.

7 Kwan CY, Leung YM, Kwan TK, Daniel EE. Tetrandrine inhibits Ca^{2+} release-activated Ca^{2+} channels in vascular endothelial cells. Life Sci 2001; 68: 841-7.

8 Leung YM, Kwan CY, Loh TT. Dual effects of tetrandrine on cytosolic calcium in human leukaemic HL-60 cells: intracellular calcium release and calcium entry blockade. Br J Pharmacol 1994; 113: 767-74.

9 Xuan B, Liu F, Zhang MY, Xiao JG. Inhibitory effects of tetrandrine on intracellular free Ca^{2+} increase induced by glutamate, serotonin and histamine in dissociated retina cells. J Ocul Pharmacol Ther 1996; 12: 331-6.

10 Xuan B, Zhang MY, Liu F, Xiao JG. Inhibition of high K^+ and norepinephrine-induced free intracellular calcium by tetrandrine in dissociated rabbit retina cells. J Ocul Pharmacol Ther 1996; 12: 151-8.

11 Kwan CY, Ma FM, Hui SC. Inhibition of endothelium-dependent vascular relaxation by tetrandrine. Life Sci 1999; 64: 2391-400.

12 Wu SN, Hwang TL, Jan CR, Tseng CJ. Ionic mechanisms of tetrandrine in cultured rat aortic smooth muscle cells. Eur J Pharmacol 1997; 30; 327: 233-8.

13 Kwan CY, Chen YY, Ma MF, Daniel EE, Hui SC. Tetrandrine, a calcium antagonist of Chinese herbal origin, interacts with vascular muscle alpha 1-adrenoceptor. Life Sci 1996; 59: PL 359-64.

14 Kai L, Xue CS, Zhou QX. Effects of tetrandrine, rhychophylliine and verapamil on transference of intraceullar and extracellular calcium in rabbit aorta smooth muscle. Chin J Pharmacol Toxicol 1995; 9: 180-3.

15 Wang HX, Kwan CY, Wong TM. Tetrandrine inhibits electrically induced $[Ca^{2+}]_i$ transient in the isolated single rat cardiomyocyte. Eur J Pharmacol 1997; 319: 115-22.

16 Anselmi E, Gomez-Lobo MD, Blazquez MA, Zafra-Polo MC, D' Ocon MP. Influence of the absolute configuration on the vascular effects of tetrandrine and isotetrandrine in rat aorta. Pharmazie 1994; 49: 440-3.

17 Noguera MA, D'Ocon MP. Different and common intracellular calcium-stores mobilized by noradrenaline and caffeine in vascular smooth muscle. Naunyn Schmiedebergs Arch Pharmacol 1992; 345: 333-41.

18 Baro I, O'Neill SC, Eisner DA. Changes of intracellular $[Ca^{2+}]_i$ during refilling of sarcoplasmic reticulum in rat ventricular and vascular smooth muscle. J Physiol 1993; 465: 21-41.