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Effects of tetrandrine on cytosolic free calcium in cultured rat myocardial cells

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KEY WORDS tetrandrine; myocardium; Fura-2; calcium; calcium channel blockers; berbines; cultured cells

AIM: To study the effects of tetrandrine (Tet) on myocardium. **METHODS**: Using Fura 2-AM and AR-CM-MIC cation measurement system, cytosolic free calcium ($[Ca^{2+}]_i$) was examined in cultured rat single myocardial cells. **RESULTS**: The resting $[Ca^{2+}]_i$ was 90 ± 12 nmol·L^{-1} in the presence of Ca^{2+} 1.3 mmol·L^{-1} in Hanks' solution. Tet 1—100 μmol·L^{-1} had no effect on the resting $[Ca^{2+}]_i$, but 10—100 μmol·L^{-1} depressed the $[Ca^{2+}]_i$ elevation when extracellular Ca^{2+} was 5 mmol·L^{-1}. Tet 1—100 μmol·L^{-1} inhibited KCl (30 and 60 mmol·L^{-1}) induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner, the IC_{50} value was 8.8 μmol·L^{-1} (95 % confidence limits: 3.3—23.7 μmol·L^{-1}) and 6.9 μmol·L^{-1} (95 % confidence limits: 2.8—17.4 μmol·L^{-1}), respectvely. Norepinephrine (NE) 10 μmol·L^{-1} caused a rapid increase in $[Ca^{2+}]_i$ in the presence or abscence of extracellular Ca^{2+}, Tet 30—100 μmol·L^{-1} only decreased the former. Tet 10—100 μmol·L^{-1} also decreased ouabain (Oua)-induced elevation in $[Ca^{2+}]_i$. **CONCLUSION**: Tet had inhibitory effects on Ca^{2+} transmembrane movement, but it is not a selective calcium channel blocker in rat myocardial cells.

Tetrandrine (Tet), an alkaloid extracted from the roots of *Stephania tetrandra* S Moore, has been used for the treatment of hypertension. Tet exerted a negative inotropic action on the cardiac muscle[1], depressed V_{max} in K^+-depolarized papillary muscle in a frequency-dependent manner[2], blocked the slow inward Ca^{2+} currents in canine cardiac Purkinje fibers[3], inversed positive staircase phenomena, and depressed post-rest potentiation of contraction[4]. Its pharmacological characterizations in cardiovascular tissues have been specially focused on its Ca^{2+} antagonistic action at the voltage-dependent channels (VOC)[5]. This suggested that Ca^{2+} channels blocked by Tet play an important role in its effects on cardiovascular tissues. In this paper, cytosolic free calcium ($[Ca^{2+}]_i$) was examined in cultured rat single myocardial cells loaded with Fura 2-AM to test the effects of Tet on heart cells.

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MATERIALS AND METHODS

Myocardial cells were cultured[6] with some modifications. Sprague-Dawley newborn (2—4 d) rat (Jiangsu Laboratory Animal Center) hearts were rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' solution, (NaCl 137, KCl 5, glucose 5.6, Na_2HPO_4 1.1, KH_2PO_4 6.1 mmol·L^{-1}, pH 7.2—7.4). Vessels and atria were carefully pruned off. The ventricles were minced into 1—3 mm^3-pieces, and disaggregated at 37 ℃ in Ca^{2+}-free modified Hanks' solution containing 0.06 % trypsin. The proportion of myocytes in culture was enriched by a preplating method that made preferential attachment of non-muscle cells[7]. After 1-h incubation, viable myocytes were seeded 1×10^5 per 35-mm dish with a glass cover-slip on its bottom. Cells were incubated at 37 ℃ in a medium containing 90 % RPMI-1640, 10 % fetal bovine serum in humidified 95 % air+5 % CO_2 for 36—48 h. Attaching to glass cover-slip, the cells were loaded with fluorescent probes, which was started by adding fura 2-AM dissolved in Me_2SO to Hanks' solution (NaCl 137, KCl 5, $MgCl_2$ 0.5, CaCl 1.3, glucose 5.6, HEPES-NaOH 10 mmol·L^{-1}, pH 7.2—7.4), with a final concentration of Fura 2-AM of 3 μmol·L^{-1}. Having been incubated at 37 ℃ in the dark 40 min and rinsed with fresh Hanks' solution for 3 times, the cells were available for Ca^{2+} measurement in 2 h at 20—25 ℃.

Loaded cells attaching to cover-slip were studied on AR-CM-MIC cation measurement system with DM3000 software (Spex Industries Inc, USA) λ_{ex} 340 nm and 380 nm, λ_{em} 505 nm; time increment 2 s. Put the cover-slip in a special-made chamber with Hanks' solution (37 ℃, pH 7.2—7.4). Chose one myocardial cell each time under the phase-contrast microscope, before and after Tet-treatment, monitored fluorescence at 340 and 380 nm under protection from light. Autofluorescence was measured

with unloaded cells. $[Ca^{2+}]_i$ was calculated by the following equation [8]: $[Ca^{2+}]_i = K_D \cdot (sb_1/sb_2) \cdot (R - R_{min})/(R_{max} - R)$ nmol·L^{-1}. The R_{max} and R_{min} were determined by ionomycin and egtazic acid, respectively.

Fura 2-AM, ouabain, RPMI Medium 1640, and ionomycin were purchased from Sigma. Tet (>98 % pure) was made by Jinhua Pharmaceutical Co. Trypsin and all other chemicals were AR. High K solution was made by substituting NaCl with equimolar KCl, so did high Ca^{2+} solution. Ca^{2+}-free solution was made by removing $CaCl_2$ from Hanks' solution. All solutions were prepared with distilled and deionized water. Treated groups were added Tet and agonist sequentialy, while control groups were added agonist only. Tet (1 — 100 μmol·L^{-1}) had no effect on the fluorescent intensity at 340 and 380 nm.

Data were expressed as $\bar{x} \pm s$. Statistical difference was evaluated by t-test.

RESULTS

Resting $[Ca^{2+}]_i$ The resting $[Ca^{2+}]_i$ was 90 ± 12 nmol·L^{-1} ($n = 8$) in Hanks' solution containing Ca^{2+} 1.3 mmol·L^{-1}. Preincubation with Tet 1, 10, 30, and 100 μmol·L^{-1} for 5 min, did not induce any significant change in $[Ca^{2+}]_i$. Tet had no effects on the passive diffusible flux of Ca^{2+} through the cytoplasmic membrane of myocardial cells.

Tet on KCl-induced $[Ca^{2+}]_i$ elevation When the cells were exposed to high K^+ in Hanks' solution containing Ca^{2+} 1.3 mmol·L^{-1}, the $[Ca^{2+}]_i$ increased rapidly. KCl 30 and 60 mmol·L^{-1} increased the $[Ca^{2+}]_i$ by 291 % and 450 %, respectively. Preincubation with Tet 1, 10, 30, 100 μmol·L^{-1} for 5 min inhibited the KCl (30 and 60 mmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation by 37 %, 48 %, 61 %, 65 %; and 32 %, 55 %, 66 %, 70 %, respectively. The IC_{50} were 8.8 (95 % confidence limits: 3.3 — 23.7) μmol·L^{-1} and 6.9 (95 % confidence limits: 2.8 — 17.4) μmol·L^{-1}, respectively (Tab. 1).

Tab 1. Effects of Tet on KCl (30, 60 mmol·L^{-1})-induced $[Ca^{2+}]_i$ in cultured rat myocardial cells. $n = 8$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

KCl/ mmol·L^{-1}	$[Ca^{2+}]_i$/ nmol·L^{-1}	Tet/μmol·L^{-1} 1	10	30	100
30	352±9	255±12^c	226±11^c	192±8^c	147±14^c
60	495±16	365±9^c	272±8^c	237±13^c	211±9^c

Norepinephrine-induced $[Ca^{2+}]_i$ elevation Norepinephrine (NE) 10 μmol·L^{-1} increased the $[Ca^{2+}]_i$ by 140 % in the $[Ca^{2+}]_i$-free Hanks' solution containing egtazic acid 0.05 mmol·L^{-1}, and by 191 % in the presence of $[Ca^{2+}]_o$ 1.3 mmol·L^{-1}, respectively. Tet (1—100 μmol·L^{-1}) had no significant effect on the NE-induced $[Ca^{2+}]_i$ elevation with extracellular Ca^{2+} free, while Tet 30 and 100 μmol·L^{-1} inhibited the NE-induced $[Ca^{2+}]_i$ elevation with extracellular Ca^{2+} by 29 % and 43 %, respectively (Tab 2).

Tab 2. Effects of Tet on norepinephrine (10 μmol·L^{-1})-, $CaCl_2$ (5 mmol·L^{-1})-, Ouabain (1 μmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation in cultured rat myocardial cells. $n = 8$, $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Control	$[Ca^{2+}]_i$/ nmol·L^{-1}	Tet/μmol·L^{-1} 1	10	30	100
NE	294±10	290±12	283±12	238±9^c	211±13^c
$CaCl_2$	285±8	271±19	189±10^c	171±14^c	160±10^c
Ouabain	199±8	189±14	167±10^b	177±10^c	166±10^c

High extracellular Ca^{2+}-induced $[Ca^{2+}]_i$ elevation When the extracellular Ca^{2+} was 5 mmol·L^{-1}, the $[Ca^{2+}]_i$ was increased by 217 %. Preincubation with Tet, 10, 30, and 100 μmol·L^{-1} depressed the $[Ca^{2+}]_i$ elevation by 49 %, 58 %, and 64 %, respectively (Tab 2).

Ouabain-induced $[Ca^{2+}]_i$ elevation Oua 1 μmol·L^{-1} caused a rapid increase in $[Ca^{2+}]_i$ by 131 %. With prior addition of Tet 10, 30, and 100 μmol·L^{-1}, the Ouabain (1 μmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation was inhibited by 19 %, 28 %, and 29 %, respectively (Tab 2).

DISCUSSION

In this work, we have demonstrated the effects of Tet in cultured rat single myocardial cells using Fura-2 and AR-CM-MIC cation measurement system. As one of the improved Ca^{2+}-sensitive flurorescent indicator, Fura-2 promises to expand the role of changes in cytosolic free Ca^{2+} underlying cell function. AR-CM-MIC cation measurment system provides the non-imaging capability to measure the changes of cytosolic free Ca^{2+} in single cell quantitatively.

Our study showed that Tet had no effect on the resting $[Ca^{2+}]_i$ in myocardial cells. The Ca^{2+} channel blockers, such as verapamil, did not alter the plasma membane permeability to

Ca^{2+} [9, 10]. Tet was similar to that of verapamil.

In this paper, we have utilized several different approaches to increase the $[Ca^{2+}]_i$. This included (1) KCl-induced $[Ca^{2+}]_i$ elevation via membrane depolarization which opened the voltage-dependent Ca^{2+} channel (VOC). (2) norepinephrine-induced $[Ca^{2+}]_i$ elevation via norepinephrine sensitive receptor-operated Ca^{2+} channel (ROC) and norepinephrine sensitive Ca^{2+} store in endoplasmic reticulum in the presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$), without $[Ca^{2+}]_o$, the $[Ca^{2+}]_i$ increase only depended the latter[11-12]. (3) high $CaCl_2$-induced $[Ca^{2+}]_i$ elevation via the increase of chemical permeability or VOC or other non-specific way[13]. (4) Ouabain-induced $[Ca^{2+}]_i$ elevation via blockade effects on Na^+, K^+-ATPase and following increment in Na^+-Ca^{2+} exchanges[14].

The results showed that (1) the inhibiting effects of Teton KCl-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner accounted for its VOC blockade effects in myocardial cells, and this was quite identical with the reference[5]. (2) The only depressing effects of Tet on norepinephrine-induced $[Ca^{2+}]_i$ elevation in the present $[Ca^{2+}]_o$ suggested its inhibition by interfering with the Ca^{2+} entry but without effect on Ca^{2+} store. The mechanism for Tet on norepinephrine-induced $[Ca^{2+}]_i$ elevation requires further exploration. (3) Tet depressing the high Ca^{2+}-induced $[Ca^{2+}]_i$ elevation further accounted for its inhibition by interfering with the Ca^{2+} entry, whether it was in specific manner or not remains unclear. (4) The inhibiting effects of Tet on ouabain-induced $[Ca^{2+}]_i$ elevation well demonstrated its antagonism on ouabain. This may be one of its pharmacologic mechanism on heart cells.

As the results have shown above, Tet does have noticeable effects on myocardial cells. Indeed, we have confirmed that Tet interfered the Ca^{2+} entry via L-type VOC. However, the inhibitory effects of Tet on Ca^{2+} channels in rat myocardial cells appeared to be non-selective.

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粉防己碱对培养大鼠心肌细胞胞内游离钙的影响

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关键词 粉防己碱；心肌；Fura-2；钙；钙通道阻滞剂；小檗因类；培养的细胞

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大鼠

目的：研究粉防己碱对心肌的作用. **方法**：采用 Fura-2 和 AR-CM-MIC 阳离子测定系统测定培养大鼠单个心肌细胞胞内游离钙. **结果**：外钙 1.3 mmol·L^{-1}时，细胞静息钙为90±12 nmol·L^{-1}. 粉防己碱不影响静息钙，但可明显抑制 $CaCl_2$，KCl，哇巴因引起的胞内钙增高；对于去甲肾上腺素引起的胞内钙增高，粉防己碱只有在外钙存在时，方对其有抑制作用. **结论**：粉防己碱抑制钙离子的跨膜运动，但在心肌细胞，它并非是选择性的钙通道阻滞剂.

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Suberogorgin *vs* *N*-cyclohexyl suberogorgamide effects on urine, respiration, and blood pressure in rat and cat

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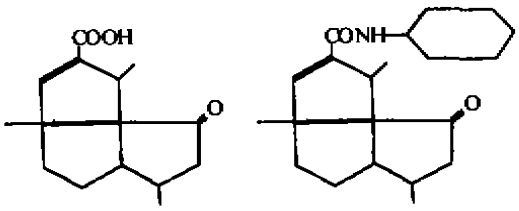
KEY WORDS suberogorgin; *N*-cyclohexyl suberogorgamide; urine; respiration; blood pressure

AIM: To compare the pharmacological actions of suberogorgin (Sub) and *N*-cyclohexyl suberogorgamide (*N*-CS). **METHODS**: Urine was collected from rats and anesthetized cats which had been loaded with water. The concentrations of Na^+ and K^+ in urine were determined in ICAP-9000 atomic emission spectrometry. An equitoxic (1/50 LD_{50}) dose of Sub and *N*-CS was used in cats. **RESULTS**: The cat urine was decreased by 63 % after iv Sub 0.4 mg·kg^{-1}, but increased by 25 % after iv *N*-CS 1.5 mg·kg^{-1}, lasting at least 9 h. Sub and *N*-CS increased the respiratory rate and tidal volume, but did not change the blood pressure. The rat urine was decreased by 48 % after ip Sub 1.3 mg·kg^{-1}, but increased by 14 % after ip *N*-CS 3.2 mg·kg^{-1}. Sub and *N*-CS increased the concentrations of Na^+ and K^+ in rat urine. These effects lasted at least 24 h. **CONCLUSION**: Sub is an antidiuretic, while *N*-CS is a diuretic drug.

Suberogorgin (Sub) was isolated from *Gorgoniae suberogorgia* sp from South China Sea, with iv LD_{50} of 22.8±1.8 mg·kg^{-1} in mice[1]. It inhibited acetylcholinesterase (AChE)[2]. *N*-Cyclohexyl suberogorgamide (*N*-CS) showed a very weak inhibition on AChE, but a diuretic action was accidentally observed. In this study, the effects of Sub and *N*-CS on urine, respiration and blood pressure in rats and anesthetized cats were compared.



Sub *N*-CS

MATERIALS AND METHODS

Sub was isolated by Department of Chemistry, Sun Yat-Sen University; *N*-CS was synthesized in our laboratory. Both were spectrum pure.

Sprague Dawley rats (n=15) weighing 280±s 15 g, bred in our laboratory, were fasted in metabolic cages for 18 h. Then, they were given ig with water 30 mL·kg^{-1}, and the urine recorded in 2 h was 11.4—13.5 mL·kg^{-1}. The rats were normally raised for 1 d and then fasted for 18 h. After that, they were injected ip with Sub or *N*-CS 5 mL·kg^{-1}, and given ig with water 25 mL·kg^{-1}. Their urine volume in 24 h was recorded. The concentrations of Na^+ and K^+ in urine were determined in ICAP-9000 atomic emission spectrometry after the urine was digested with nitric acid[3].

Cats (n = 3), ♀, weighing 3.5±s 0.4 kg, were anesthetized with sodium pentobarbital 30 mg·kg^{-1}. NaCl 0.15 mol·L^{-1} solution was injected gtt into saphenous vein at 0.34 mL·kg^{-1}·min^{-1}. Urine was collected by bladder intubations. The urine in 10 min was recorded for 9 h. Respiration was recorded with respiratory belts. Tidal volume (V_t), calculated with square-method, time

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