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Effects of tetrandrine on free intracellular Ca^{2+} in isolated rat brain cells[1]

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ABSTRACT Using Ca^{2+}-sensitive fluorescent indicator, Fura-2/AM, intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured. Resting $[Ca^{2+}]_i$ was 221±18 nmol·L^{-1} in the presence of Ca^{2+} 1.3 mmol·L^{-1} in Hank's solution. Tetrandrine (Tet) 30 μmol·L^{-1} had no effect on the resting $[Ca^{2+}]_i$ when the extracellular Ca^{2+} were 0—2 mmol·L^{-1}. In the presence of extracellular Ca^{2+} 1.3 mmol·L^{-1}, Tet (1—100 μmol·L^{-1}) concentration-dependently inhibited the high extracellular K^+-induced $[Ca^{2+}]_i$ elevation, with an IC_{50} value of 8.2 μmol·L^{-1} (95% confidence limits: 1.9 — 32.9 μmol·L^{-1}). Low concentrations of Tet (1—10 μmol·L^{-1}) did not alter the norepinephrine-induced $[Ca^{2+}]_i$ elevation. Tet 30 μmol·L^{-1} depressed norepinephrine 10 μmol·L^{-1} induced $[Ca^{2+}]_i$ elevation by 42%. The results suggested that Tet inhibited the Ca^{2+} influx through voltage-dependent ionic channels and, at high concentrations, through receptor-operated ionic channels in the brain cells.

KEY WORDS tetrandrine; norepinephrine; calcium; brain; fluorescent dyes

Tetrandrine (Tet), an alkaloid extracted from *Stephania tetrandra* S Moore, has traditionally been used for the treatment of hypertension. Tet protected cerebral ischemia and decreased the afterhyperpolarization potentials in neurons[1]. Tet blocked the voltage-dependent Ca^{2+} channel in cultured neuroblastoma cells[2]. It has been suggested that the Ca^{2+} channels blockade by Tet played an important role in its effects on hypertension and cerebral ischemia[3]. Although the Ca^{2+} channel blockade effect of Tet has been confirmed, its direct actions on intracellular free Ca^{2+} have not been reported. In this paper, the effects of Tet on free intracellular Ca^{2+} in isolated brain cells were studied using Ca^{2+} sensitive fluorescent probe, Fura-2/AM.

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MATERIALS AND METHODS

Brain cells were isolated according to the mothod of reference[4] with some modifications. Newborn (1—7 d) Sprague-Dawley rats (Jiangsu Laboratory Animal Center) were decapitated in a Petri dish on ice. The isolated brain was rinsed with ice-cold free Ca^{2+} and Mg^{2+} Hank's solution, pH 7.2—7.4, with the following compositions: NaCl 137, KCl 5, glucose 5.6, and HEPES 10 (mmol·L^{-1}). Vessels and meninges were carefully stripped off. After the brain was washed with Ca^{2+}- and Mg^{2+}-free Hank's solution, it was cut into 3 mm^3-pieces, and placed in a 10 ml flask containing 0.125% trypsin and EGTA 0.5 mmol·L^{-1} in Ca^{2+}- and Mg^{2+}-free Hank's solution. The flask was shaken at 37℃ for 20 min. Trypsinization was discontinued by adding 10 ml ice-cold Hank's solution containing 10% bovine serum. Tissue pieces were mechanically dissociated by gently triturating 10—15 times with a polished pipette. The isolated brain cells were filtered through nylon mesh (200 mesh, hole width 95 μm) and collected in a flask. Cells were centrifuged twice at 100×g for 3—4 min each. The supernatant was decanted and the cells were resuspended in warm Hank's solution (pH 7.4) containing: NaCl 137, $CaCl_2$ 1.3, $MgCl_2$ 0.5, KCl 5.0, glucose 5.6, and HEPES 10 mmol·L^{-1}. Trypan blue staining showed a 90—95% cellular viability rate. The cell suapension was further diluted to a total of 8 ml with Hank's solution and divided into 2 aliquots and placed in a water bath for 5 min at 37℃. A final concentration of Fura-2/AM 5 μmol·L^{-1} dissolved in Me_2SO was added to one aliquot and a same volume of Me_2SO was added to the other as a control. The cells were loaded with Fura-2 in water bath for 40 min. The Fura-2-loaded cells and the control cells were cen-

trifuged at $100\times g$ for 3—4 min. The cells were resuspended in 4 ml Hank's solution containing 0.2% bovine serum albumin resulting in approximately 1×10^6 cells·ml^{-1}. Suspension of cells were incubated for 4—5 min at 37℃ prior to measurements.

A RF-540 spectrofluorophotometer (Shimadzu) was used for Ca^{2+} measurement (excitation 340 nm, emission 490 nm). $[Ca^{2+}]_i$ was calculated according to the formular[5] with K_d of 224 nmol·L^{-1}: $[Ca^{2+}]_i = K_d \times (F-F_{min})/(F_{max}-F)$. The maximal fluorescence (F_{max}) was determined by the final concentrations of 0.2% Triton X-100 and Ca^{2+} 2 mmol·L^{-1} added to the sample of cells. The minimal fluorescence (F_{min}) was determined by the final concentration of EGTA 8 mmol·L^{-1} (pH > 8.5). Correction was made for autofluorescence in each experiment.

Fura-2/AM was purchased from Sigma. Tet > 98% pure, was manufactured by Jinhua Pharmaceutical Co. Trypsin and all other chemicals were AR.

RESULTS

Isolated brain cells were resuspended in Hank's solution containing Ca^{2+} 1.3 mmol·L^{-1}. After addition of Triton X-100 (final concentration 0.2%) to lyse the cells, the spectrum was shifted to a peak at 340—350 nm. Through the addition of EGTA (final concentration 8 mmol·L^{-1}) to deplete the calcium, the spectrum of Fura-2 revealed a peak at 370—380 nm (Fig 1). The spectra were similar to those reported by Grynkiewicz[6]. The resting $[Ca^{2+}]_i$ was 221 ±18 nmol·L^{-1} ($n=8$, $\bar{x}\pm s$). This value of resting $[Ca^{2+}]_i$ was well within the expected range for the $[Ca^{2+}]_i$ level[7].

Tet on resting $[Ca^{2+}]_i$ The resting $[Ca^{2+}]_i$ was 78±10 nmol·L^{-1} ($n=6$) in Ca^{2+} free Hank's solution containing EGTA 0.1 mmol·L^{-1}. Resting $[Ca^{2+}]_i$ levels were 104 ±16, 138±17, 216±15, and 251±21 nmol·L^{-1} in the presence of extracellular Ca^{2+} 0.01, 0.1, 1, and 2 mmol·L^{-1}, respectively. $[Ca^{2+}]_i$ was dependent on the extracellular

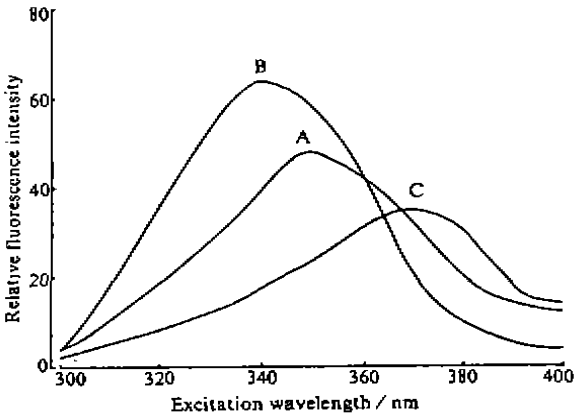

Fig 1. Fura-2 excitation spectrum in isolated rat brain cells. A) Resuspended in Hank's solution containing Ca^{2+} 1.3 mmol·L^{-1}. B) Lysed with Triton X-100. C) Calcium was depleted with EGTA.

Ca^{2+} concentrations. Preincubated with Tet 30 μmol·L^{-1} in Hank's solution containing Ca^{2+} 0, 0.01, 0.1, 1, and 2 mmol·L^{-1} for 15 min. Tet did not induce any significant change in $[Ca^{2+}]_i$. Tet apparently had no effect on the passive diffusible flux of Ca^{2+} through the cytoplasmic membraine of the brain neurons.

Tet on KCl-induced $[Ca^{2+}]_i$ elevation When the brain cells were exposed to high K^+ in Hank's solution containing Ca^{2+} 1.3 mmol·L^{-1}, the $[Ca^{2+}]_i$ increased rapidly and concentration-dependently. KCl 25 and 50 mmol·L^{-1} increased the $[Ca^{2+}]_i$ by 60% and 165%, respectively. Tet 10 μmol·L^{-1} inhibited the KCl (25 and 50 mmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation by 50% and 70%, respectively, but did not change the resting $[Ca^{2+}]_i$ level (Fig 2). Tet 1—100 μmol·L^{-1} inhibited the extracellular high KCl-induced $[Ca^{2+}]_i$ elevation dose-dependently, with IC_{50} of 8.2 (95% confidence limits; 1.9—32.9) μmol·L^{-1}.

Tet on norepinephrine-induced $[Ca^{2+}]_i$ elevation Norepinephrine 0.01, 0.1, 1, and 10 μmol·L^{-1} increased the $[Ca^{2+}]_i$ by 43%,

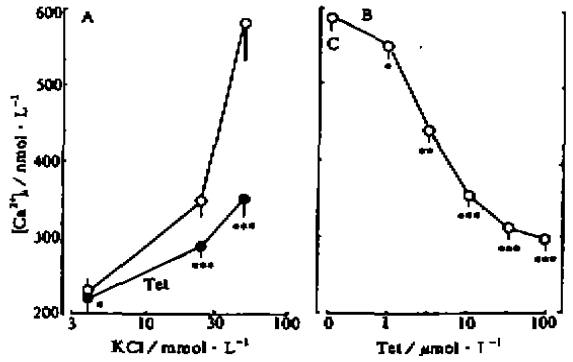

Fig 2. Effects of Tet on KCl-induced $[Ca^{2+}]_i$ increases in rat isolated brain cells. A) Tet 10 μmol·L⁻¹ on KCl-induced $[Ca^{2+}]_i$ increases. B) Tet on KCl (50 mmol·L⁻¹)-induced $[Ca^{2+}]_i$ increases. C = Control. $n=5$, $\bar{x}\pm s$. $^{**}P<0.05$, $^{***}P<0.01$ vs control.

80%, 100%, and 120%, respectively. Tet 30 μmol · L^{-1} inhibited the norepinephrine (0.01 — 10 μmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation by 30%, 37%, 39%, and 42%, respectively (Fig 3). Lower concentrations of Tet (1 — 10 μmol·L^{-1}) did not show noticeable effects on norepinephrine-induced $[Ca^{2+}]_i$ elevation.

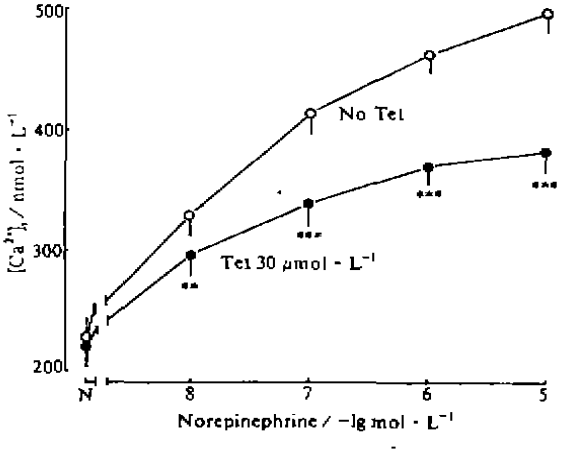

Fig 3. Effects of Tet on norepinephrine-induced $[Ca^{2+}]_i$ elevation in rat brain neurons. (N) without norepinephrine. $n=5$, $\bar{x}\pm s$. $^{**}P<0.05$, $^{***}P<0.01$ vs N.

DISCUSSION

The results showed that Tet did not alter the resting $[Ca^{2+}]_i$ in brain cells. Kanaide *et al*[8] reported that the Ca^{2+} channel blockers, verapamil, and diltiazem had no effects on the plasma membrane permeability to Ca^{2+}. The effects of Tet on resting $[Ca^{2+}]_i$ in brain cells were similar to those of verapamil and diltiazem.

The mechanism for KCl-induced $[Ca^{2+}]_i$ elevation may be that the high extracellular K^+ causes the cell membrane to depolarize to a certain extent which opened the voltage-dependent Ca^{2+} channels. Tet has been demonstrated to have inhibitory effects on voltage-dependent Ca^{2+} channels both of L-type and of T-type[3]. Tet inhibiting the KCl-induced $[Ca^{2+}]_i$ elevation accounted for its Ca^{2+} channel blockade effect. With regard to norepinephrine induced $[Ca^{2+}]_i$ elevation, there has not been a general agreement reached. It has been suggested that there were norepinephrine sensitive receptor-operated Ca^{2+} channels and norepinephrine sensitive Ca^{2+} store in endoplasmic reticulum[9,10]. Many of the Ca^{2+} antagonists have been reported to depress the norepinephrine-induced $[Ca^{2+}]_i$ elevation in various tissues. The molecular mechanism whereby the Ca^{2+} antagonists inhibit the norepinephrine-induced $[Ca^{2+}]_i$ elevation remains unclear. The inhibitory effect of Tet on norepinephrine induced $[Ca^{2+}]_i$ elevation was about 10-fold less potent than on KCl-induced $[Ca^{2+}]_i$ elevation. As reported[8], the inhibitory effect of Ca^{2+} *antagonists (verapamil, diltiazem) on* norepinephrine-induced $[Ca^{2+}]_i$ elevation were about 20 — 30 fold less potent than those on KCl-induced $[Ca^{2+}]_i$ elevation. It seemed that Tet had a more potent effect on norepinephrine-induced $[Ca^{2+}]_i$ elevation than Ca^{2+}

antagonists (verapamil, diltiazem) did in brain cells. The mechanism for Tet on norepinephrine-induced $[Ca^{2+}]_i$ elevation requires further exploration.

Loss of Ca^{2+} homeoatasis was responsible for the ischemic brain cell damage[11,12]. Ischemia causes increased Ca^{2+} influx into cells because the depolarization and transmitter release could open the voltage-dependent Ca^{2+} channels and the receptor-operated Ca^{2+} channels[13,14]. Ca^{2+} influx enhanced the breakdown of proteins and liqids, and resulted in cell damage. Thus, the inhibitory effects of Tet on KCl and norepinephrine induced $[Ca^{2+}]_i$ elevation were closely related to its protective effects on cerebral ischemia.

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粉防己碱对大鼠脑细胞内游离钙的影响

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摘要 利用荧光钙指示剂 Fura-2/AM，测定脑细胞内游离钙的浓度。细胞内静息钙浓度为221±18 nmol·L^{-1}。Tet 30 μmol·L^{-1}对细胞内静息钙无影响。Tet (1—100 μmol·L^{-1})能抑制胞外高钾引起的胞内钙升高，其 IC_{50} 为 8.2 (95% 可信限为 1.89—32.90 μmol·L^{-1})。Tet 30 μmol·L^{-1}可抑制去甲肾上腺素10 μmol·L^{-1}引起脑细胞内钙升高，其幅度为42%。

关键词 粉防己碱；去甲肾上腺素；钙；脑；荧光染料