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Toosendanin increases free-Ca²⁺ concentration in NG108-15 cells *via* L-type Ca²⁺ channels¹

Tong-hui XU, Jun DING, Yu-liang SHI²

*Key Laboratory of Neurobiology, Shanghai Institutes for Biological Sciences,
Chinese Academy of Sciences, Shanghai 200031, China*

KEY WORDS toosendanin; intracellular calcium concentration; confocal microscopy; calcium indicator; neuroblastoma×glioma hybrid cells; L-type Ca²⁺ channels

ABSTRACT

AIM: To examine if toosendanin (TSN) affects intracellular free-Ca²⁺ concentration ([Ca²⁺]_i) in neuroblastoma×glioma hybrid cells (NG108-15 cells). **METHODS:** The [Ca²⁺]_i was determined by laser-scanning confocal microscopic imaging technique in which Fluo-3 was used as Ca²⁺ indicator. **RESULTS:** TSN induced an increase in resting [Ca²⁺]_i and in high K⁺-evoked Ca²⁺ transient in differentiated NG108-15 cells. The TSN-induced increase in [Ca²⁺]_i was dose-dependent and disappeared in CdCl₂-, nifedipine-containing or Ca²⁺-free solution, and appeared after washing out the Ca²⁺ channel blockers or adding Ca²⁺. **CONCLUSION:** TSN increased [Ca²⁺]_i in differentiated NG108-15 cells. The [Ca²⁺]_i enhancement was due to the influx of extracellular Ca²⁺ and related to L-type Ca²⁺ channels.

INTRODUCTION

Previous studies suggest that toosendanin (C₃₀H₃₈O₁₁, FW 574, TSN), a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb et Zucc, is a selective presynaptic blocker^[1,2]. Having no effects on the nerve impulse conduction, resting potential and transmitter sensitivity of postsynaptic membrane, TSN blocks synaptic transmission at the neuromuscular and central synapses by interfering with evoked and spontaneous transmitter release. Interestingly, before the final blockage there is always an initial facilitation of

transmitter release^[3-6]. TSN-induced decrease in the number of synaptic vesicles in mice and TSN-specific binding in rat cerebral cortex synaptosomes was also found^[7,8]. In spite of sharing some similar actions with botulinum neurotoxins (BoNT), TSN was shown to have a dramatic antbotulismic effect. For example, TSN could prevent mice from death that had been administered with several lethal doses of BoNT/A or B, and the effect was still observed by applying the drug 18 h after BoNTs injection^[9]. The tolerance to BoNT of the neuromuscular junction preparations, which were isolated from rats after a single injection of TSN, was enhanced significantly, and the high tolerance was associated with the TSN-induced facilitation of neurotransmitter release in the time course^[10].

Calcium ion is closely related to transmitter release. In the present study, we design to examine if TSN induces the change of [Ca²⁺]_i by using confocal microscopy and using Fluo-3 as the Ca²⁺ indicator in NG108-

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² Correspondence to Prof Yu-liang SHI. Phn 86-21-5492-0270. Fax 86-21-5492-0276. E-mail ylshi@server.shnc.ac.cn

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15 cells, which have been used widely as a neuron model in electrophysiological and pharmacological research on ion channels^[11,12].

MATERIALS AND METHODS

Solutions and reagents Standard physiological solution used in this work contained (in mmol/L) NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 11, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 5^[13]. To obtain high K⁺ solution, Na⁺ in the solution was replaced by an equivalent amount of K⁺. For Ca²⁺-free solution, CaCl₂ was omitted and 2 mmol/L ethylene glycol-*bis* (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was added. Nifedipine was prepared in Me₂SO at 50 mmol/L and diluted to its final concentration in test solutions. The pH of all solutions was adjusted to 7.4 with NaOH. TSN used in this work was a sample recrystallized in ethanol with a purity >98 %^[14,15]. HEPES, EGTA, nifedipine, and dBcAMP were all Sigma products (St Louis, MO, USA). All other reagents were of analytical grade. TSN was dissolved directly in test solutions.

Cell culture The NG108-15 cells were cultured at a glass cover-slip laid in a 35-mm plastic dish (Nunc) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % ambient air as described previously^[14,15]. For maintaining the cells in an undifferentiated state, Dulbecco's modified Eagle's medium (DMEM) containing 10 % new natal bovine serum, 100 μmol/L hypoxanthine, 1 μmol/L aminopterin and 16 μmol/L thymidine (HAT) was used. To induce neural differentiation, 1 mmol/L dBcAMP was added to the culture medium, and the concentration of the new natal bovine serum was reduced to 5 %. The inoculation was 2×10⁴-4×10⁴ cells per dish, and the culture medium was refreshed every 1-3 d.

Fluorescence measurement of Ca²⁺ level The glass coverslip with adherent cells was attached to a flow-through chamber (0.2 mL)^[16]. Then the cells were loaded with 10 μmol/L Fluo-3-AM (acetoxymethylester, Molecular Probe Inc, USA) in darkness for 30 min at 37 °C. The cells were then continuously perfused with dye-free standard physiological solution at a rate of 2 mL/min and incubated for further 30 min for complete hydrolysis of the dye. The stock solution of Fluo-3-AM was prepared by mixing 50 μg of the fluorescent dye into 50 μL dimethyl sulfoxide (Me₂SO) and kept frozen in aliquots until use. The perfusion chamber

was mounted on the stage of an invert microscope (Zeiss Axiovert100M, Carl Zeiss Co, Germany) and the tested agents were applied by perfusion.

Fluo-3 loaded NG108-15 cells were imaged with a Zeiss LSM-510 laser scanning confocal microscope (excitation 488 nm, emission 525 nm). Two-dimensional confocal images were acquired by scanning an image of 512×512 pixels. The data of fluorescence intensity were collected at a rate of 0.2-0.5 Hz. The fluorescence excitation and image acquisition were monitored by software (LSM 510, Version 2.01 Carl Zeiss Co, Germany). Time course of fluorescence changes of the cells was obtained automatically with analysis software (TimeSeries, Carl Zeiss Co, Germany). All data were stored in a computer and later processed by Microsoft Excel 2000. The fluorescence signals of Ca²⁺ were not calibrated to Ca²⁺ concentration, but the term of Ca²⁺ concentration was still used in this study for convenience^[16]. The fluorescence signals were normalized as $\Delta F/F_0$, where F_0 was the resting fluorescence and symbol ΔF was the Ca²⁺-dependent rise over the resting fluorescence.

Statistical analysis All data were expressed as mean±SD (n =number of cells). Student's *t*-test was used for statistical analysis, considering $P<0.05$ as significant. In the experiments to compare the effects of TSN on [Ca²⁺]_i in NG108-15 cells under different conditions shown in Tab 1, the [Ca²⁺]_i of the cells in standard physiological solution was defined as control.

RESULTS

Increasing high K⁺-evoked [Ca²⁺]_i transient in differentiated cells In response to 35 mmol/L K⁺-induced depolarization, a transient rise of [Ca²⁺]_i in differentiated NG108-15 cells was evoked. When the repetitive high K⁺ exposures with an interval of 3-4 min were applied, no evident change in successive high K⁺-induced Ca²⁺ signal was observed. The high K⁺-induced Ca²⁺ transients were enhanced by TSN (Fig 1). After 35 μmol/L TSN pretreatment for 2 min, the peak response ($\Delta F/F_0$) evoked by 35 mmol/L K⁺ exposure for 1 min was significantly increased to 132.4 %±21.4 % ($n=18$, $P<0.05$) of the high K⁺-induced depolarization control. But in the Ca²⁺-free solution, no [Ca²⁺]_i change could be induced by the high K⁺-induced depolarization.

Increasing resting [Ca²⁺]_i in differentiated cells via extracellular Ca²⁺ influx Besides the enhancement of high-K⁺ evoked transient, a TSN-induced in-

Tab 1. TSN (50 $\mu\text{mol/L}$)-induced $[\text{Ca}^{2+}]_i$ change in NG108-15 cells. The data were obtained 4 min after TSN application under different mediums (standard physiological solution with 2 mmol/L Ca^{2+} , 10 $\mu\text{mol/L}$ Cd^{2+} or 10 $\mu\text{mol/L}$ nifedipine, or Ca^{2+} -free solution) or 2 min after further elevating $[\text{Ca}^{2+}]_i$ in the solution to 2 mmol/L or after washing out channel blockers with TSN-containing standard physiological solution. ^a $P>0.05$, ^c $P<0.01$ vs control. See text for detail.

	Percent of control (mean \pm SD)	n (cells)
Undifferentiated cells	98.6 $\% \pm 3.1$ % ^a	29
Differentiated cells in:		
Standard solution	137.1 $\% \pm 30.0$ % ^c	12
Ca^{2+} -free solution	98.4 $\% \pm 3.4$ % ^a	15
Elevating $[\text{Ca}^{2+}]_i$ to 2 mmol/L	110.2 $\% \pm 6.9$ % ^c	15
Cd^{2+} 10 $\mu\text{mol/L}$	101.7 $\% \pm 2.1$ % ^a	14
Wash out Cd^{2+}	108.4 $\% \pm 4.6$ % ^c	15
Nifedipine 10 $\mu\text{mol/L}$	100.9 $\% \pm 4.6$ % ^a	17
Wash out nifedipine	146.0 $\% \pm 31.1$ % ^c	17

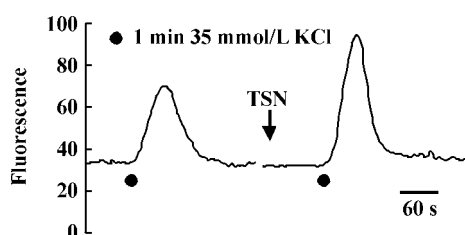


Fig 1. Effect of TSN on high K^+ -induced Ca^{2+} transient. After treatment with 35 $\mu\text{mol/L}$ TSN for 2 min (the arrow), the amplitude of high K^+ -induced Ca^{2+} transient was evidently increased. The increase of $[\text{Ca}^{2+}]_i$ in fluo-3 loaded cells is represented as an increase of dye fluorescence shown in arbitrary unit (au).

crease in resting level of $[\text{Ca}^{2+}]_i$ was observed in differentiated NG108-15 cells (Fig 2). We normalized the response of each experiment to remove the variability among different cells and reveal the average increase rate (%). When compared with the control, the $[\text{Ca}^{2+}]_i$ increased to 117.7 $\% \pm 10.5$ % after 2 min and 133.1 $\% \pm 17.5$ % after 4 min 35 $\mu\text{mol/L}$ TSN application to the cells bathed in standard physiological solution (with 2 mmol/L Ca^{2+}) respectively ($n=12$, $P<0.01$). The increase effect was concentration-dependent and a significant increase in $[\text{Ca}^{2+}]_i$ was observed at micromolar range of TSN. TSN at the concentrations of 0.5, 5, 35, and 50 $\mu\text{mol/L}$ caused $[\text{Ca}^{2+}]_i$ to increase by 4.6 $\% \pm$

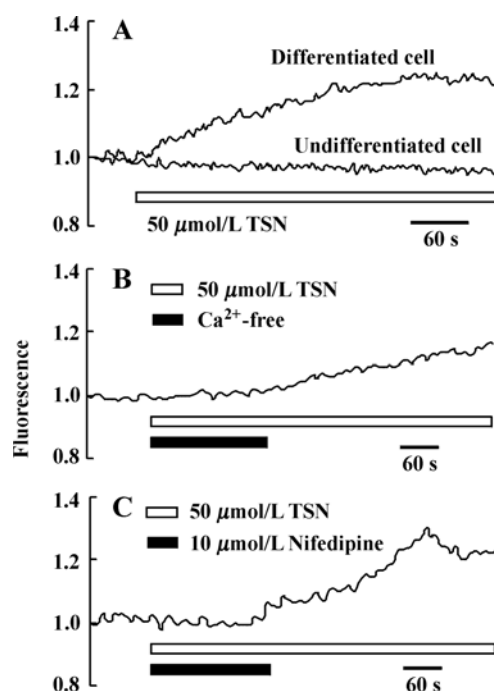


Fig 2. Effect of TSN on resting $[\text{Ca}^{2+}]_i$. **A:** 50 $\mu\text{mol/L}$ TSN-induced changes of $[\text{Ca}^{2+}]_i$ in differentiated and undifferentiated cells. **B:** TSN-induced increase of $[\text{Ca}^{2+}]_i$ in differentiated cell appeared after elevating $[\text{Ca}^{2+}]_i$ in the solution to 2 mmol/L. **C:** The TSN-induced increase of $[\text{Ca}^{2+}]_i$ disappeared in nifedipine-containing solution, but appeared after washing out the channel blocker. The change of $[\text{Ca}^{2+}]_i$ is represented as normalized fluorescence. Horizontal bars below the traces indicate the periods of drug application.

4.2 $\%$ ($n=10$, $P<0.01$), 28.6 $\% \pm 7.3$ $\%$ ($n=21$, $P<0.01$), 33.1 $\% \pm 17.5$ $\%$ ($n=12$, $P<0.01$), and 37.1 $\% \pm 30.0$ $\%$ ($n=12$, $P<0.01$, Fig 2A), respectively.

It was observed that the TSN-induced increase in Ca^{2+} level was completely inhibited by Ca^{2+} channel blocker CdCl_2 (10 $\mu\text{mol/L}$, $n=14$) or disappeared in Ca^{2+} -free solution ($n=15$). Exposure of the cells to 50 $\mu\text{mol/L}$ TSN containing Cd^{2+} or Ca^{2+} -free solution for 4 min caused Ca^{2+} level to maintain 101.7 $\% \pm 2.1$ $\%$ ($n=14$, $P>0.05$) and 98.4 $\% \pm 3.4$ $\%$ ($n=15$, $P>0.05$) of the control. When the solution was changed to standard physiological solution containing 50 $\mu\text{mol/L}$ TSN, the $[\text{Ca}^{2+}]_i$ increased to 108.4 $\% \pm 4.6$ $\%$ ($P<0.01$) and 110.2 $\% \pm 6.9$ $\%$ ($P<0.01$, Fig 2B) of their respective controls after 2 min.

No effect on $[\text{Ca}^{2+}]_i$ in undifferentiated cells

When performing the similar experiments in undifferentiated NG108-15 cells, it was found that the $[\text{Ca}^{2+}]_i$ did not increase after application of TSN. Ca^{2+} level in undifferentiated NG108-15 cells, which only express T-type Ca^{2+} channels^[12], was still 98.6 $\% \pm 3.1$ $\%$ ($n=29$,

$P > 0.05$, Fig 2A) of control after 50 $\mu\text{mol/L}$ TSN application.

Inhibition of TSN-induced increase in $[\text{Ca}^{2+}]_i$ by nifedipine When the differentiated NG108-15 cells, which express T-, L-, N-, and residual deduced to be Q-type Ca^{2+} channels^[12], were exposed to nifedipine (10 $\mu\text{mol/L}$), a L-type Ca^{2+} channel antagonist, the $[\text{Ca}^{2+}]_i$ maintained $100.9 \pm 4.6 \%$ of the control ($n=17$, $P > 0.05$) after 50 $\mu\text{mol/L}$ TSN application for 4 min. However, when the nifedipine was washed out, the TSN-induced increase of $[\text{Ca}^{2+}]_i$ appeared, and 2 min later the $[\text{Ca}^{2+}]_i$ increased to $146.0 \pm 31.1 \%$ of the control ($n=17$, $P < 0.001$, Fig 2C). For the convenience of comparison, Tab 1 summarized the effects of 50 $\mu\text{mol/L}$ TSN on $[\text{Ca}^{2+}]_i$ under different experimental conditions.

DISCUSSION

The present study showed that TSN increased high K^+ -evoked Ca^{2+} transient and resting $[\text{Ca}^{2+}]_i$ in differentiated NG108-15 cells, and that the TSN-induced $[\text{Ca}^{2+}]_i$ increase disappeared in Cd^{2+} -containing or Ca^{2+} -free solution. These data indicated that TSN-induced effects resulted from extracellular Ca^{2+} influx and should be mediated by voltage-gated channels located at the cell membrane. The result that TSN increased $[\text{Ca}^{2+}]_i$ was in accordance with previous observation that TSN increased resting Ca^{2+} -conductance in the differentiated NG108-15 cells^[17] and our recent experiment results obtained by patch clamp technique in the cells^[18]. In the experiments, we observed that TSN increased depolarization-evoked Ca^{2+} current and the effect was demonstrated to be mediated by L-type Ca^{2+} channels^[18]. The TSN-induced $[\text{Ca}^{2+}]_i$ increase should be a facilitation background for transmitter release, and could be related to its anti-botulismic effect.

The result that TSN did not affect $[\text{Ca}^{2+}]_i$ in undifferentiated NG108-15 cells, in which only T-type Ca^{2+} channels were expressed, indicated that T-type Ca^{2+} channels were not involved in the TSN-induced $[\text{Ca}^{2+}]_i$ increase. Differentiation of the cells led to appearance of the high-threshold Ca^{2+} channel, L-, N-, and Q-type^[12]. The present observations that TSN increased $[\text{Ca}^{2+}]_i$ of differentiated NG108-15 cells and the effect was blocked by nifedipine but appeared after washing the blocker out suggested that Ca^{2+} influx through L-type Ca^{2+} channels should be responsible for the TSN-induced increase in $[\text{Ca}^{2+}]_i$.

As we know, L-type Ca^{2+} channel, a kind of high

voltage-activated Ca^{2+} channel, is under inactivated state at resting membrane potential. Previous data showed that TSN did not affect Na^+ channel and resting membrane potential in NG108-15 cells^[17]. How can TSN mediate Ca^{2+} -influx *via* L-type Ca^{2+} channels to increase $[\text{Ca}^{2+}]_i$ at resting membrane level? A possibility was that the activated threshold of the channel could be shifted after applying TSN. This needs to be demonstrated by patch-clamp experiments to analyze the effect of TSN on kinetic property of L-type Ca^{2+} channel.

In conclusion, the present study demonstrated for the first time that TSN induced an intracellular free $[\text{Ca}^{2+}]_i$ increase in differentiated NG108-15 cells, and the influx of extracellular Ca^{2+} *via* L-type Ca^{2+} channel was responsible for the TSN-induced effect.

REFERENCES

- 1 Chang CC, Hsie TH, Chen SF, Liang HT. The structure of Chuanliansu. *Acta Chim Sin* 1975; 33: 35-47.
- 2 Shu GX, Liang XT. A correction of the structure of Chuanliansu. *Acta Chim Sin* 1980; 38: 196-8.
- 3 Chen WY, Yin PB, Ye WL, Shi YL. Toosendanin-induced change of dopamine level detected by microdialysis *in vivo* at rat striatum. *Chin Sci Bull* 1999; 44: 502-6.
- 4 Shi YL, Chen WY. Effect of toosendanin on acetylcholine level of rat brain, a microdialysis study. *Brain Res* 1999; 850: 173-8.
- 5 Shi YL, Wang WP, Xu K. Electrophysiological analysis on the presynaptic blocking effects of toosendanin on neuromuscular transmission. *Acta Physiol Sin* 1981; 33: 259-65.
- 6 Shi YL, Wang WP, Yang SC, Xu K. Effects of calcium ions and nerve impulses on changes in miniature end-plate potential frequency produced by toosendanin. *Acta Physiol Sin* 1982; 34: 304-9.
- 7 Huang SK, Song XE, Shi YL. Effects of toosendanin on the fine structure of the mouse neuromuscular junction. *Acta Physiol Sin* 1980; 32: 385-90.
- 8 Shen GG, Zhuo XL, Shi YL. The binding site of ^3H -Toosendanin in rat cerebral cortex homogenate. *Acta Physiol Sin* 1994; 46: 546-52.
- 9 Li PZ, Zhou J, Miao WY, Ding FH, Meng JY, Jia GR, *et al*. Therapeutic effect of toosendanin on animal botulism. *Chin Tradit Herb Drugs* 1982; 13: 28-30.
- 10 Shi YL, Xu K. Anti-botulismic effect of toosendanin and its facilitatory action on miniature end-plate potential. *Jpn J Physiol* 1983; 33: 677-80.
- 11 Docherty RJ, Robbins J, Brown DA. NG108-15 neuroblastoma \times glioma hybrid cell line as a model neuronal system, In: Chad J, Wheal H, editors. *Cellular neurobiology, a practical approach*. Oxford: Oxford University Press; 1992. p 75-95.
- 12 Lukyanetz EA. Diversity and properties of calcium channel types in NG108-15 hybrid cells. *Neuroscience* 1998; 87:

- 265-74.
- 13 Frange R, Meunier F, Molgo J, Šupt D. Equinatoxin II increases intracellular Ca^{2+} in NG108-15 cells. *Pflügers Arch* 2000; 439 Suppl 3: R100-1.
 - 14 Hu Q, Huang FS, Shi YL. Inhibition of toosendanin on the delayed rectifier potassium current in neuroblastoma×glioma NG108-15 cells. *Brain Res* 1997; 751: 47-53.
 - 15 Hu Q, Shi YL. Characterization of an inward-rectifying potassium current in NG108-15 neuroblastoma×glioma cells. *Pflügers Arch* 1997; 433: 617-25.
 - 16 Wu D, Zhu PH. Caffeine-sensitive Ca^{2+} stores in carp retinal bipolar cells. *Neuroreport* 1999; 10: 3897-901.
 - 17 Shi YL, Furuya K, Wang WP, Terakawa S, Xu K, Yamagashi S. Toosendanin-induced Ca^{2+} conductance increase in neuroblastoma×glioma hybrid cells. *Chin Sci Bull* 1993; 38: 1489-93.
 - 18 Li MF, Wu F, Wang ZF, Shi YL. Toosendanin, a triterpenoid derivative, increases Ca^{2+} current in NG108-15 cells *via* L-type channels. *Neurosci Res* 2004; in press.