Toosendanin increases free-Ca^{2+} concentration in NG108-15 cells *via* L-type Ca^{2+} channels[1]

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ABSTRACT

AIM: To examine if toosendanin (TSN) affects intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$) in neuroblastoma×glioma hybrid cells (NG108-15 cells). **METHODS:** The $[Ca^{2+}]_i$ was determined by laser-scanning confocal microscopic imaging technique in which Fluo-3 was used as Ca^{2+} indicator. **RESULTS:** TSN induced an increase in resting $[Ca^{2+}]_i$ and in high K^+-evoked Ca^{2+} transient in differentiated NG108-15 cells. The TSN-induced increase in $[Ca^{2+}]_i$ was dose-dependent and disappeared in $CdCl_2$-, nifedipine-containing or Ca^{2+}-free solution, and appeared after washing out the Ca^{2+} channel blockers or adding Ca^{2+}. **CONCLUSION:** TSN increased $[Ca^{2+}]_i$ in differentiated NG108-15 cells. The $[Ca^{2+}]_i$ enhancement was due to the influx of extracellular Ca^{2+} and related to L-type Ca^{2+} channels.

INTRODUCTION

Previous studies suggest that toosendanin ($C_{30}H_{38}O_{11}$, 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 antibotulismic effect. For example, TSN could prevent mice from death that had been administrated 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 $[Ca^{2+}]_i$ by using confocal microscopy and using Fluo-3 as the Ca^{2+} indicator in NG108-

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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, $CaCl_2$ 2, $MgCl_2$ 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 Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mmol/L ethylene glycol-*bis* (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was added. Nifedipine was prepared in Me_2SO 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 ºC in a humidified atmosphere of 5 % CO_2 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 2×10^4-4×10^4 cells per dish, and the culture medium was refreshed every 1-3 d.

Fluoresence measurement of Ca^{2+} 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 ºC. 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 (Me_2SO) 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 Ca^{2+} were not calibrated to Ca^{2+} concentration, but the term of Ca^{2+} 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 Ca^{2+}-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 $[Ca^{2+}]_i$ in NG108-15 cells under different conditions shown in Tab 1, the $[Ca^{2+}]_i$ of the cells in standard physiological solution was defined as control.

RESULTS

Increasing high K^+-evoked $[Ca^{2+}]_i$ transient in differentiated cells In response to 35 mmol/L K^+-induced depolarization, a transient rise of $[Ca^{2+}]_i$ 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 Ca^{2+} signal was observed. The high K^+-induced Ca^{2+} 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 Ca^{2+}-free solution, no $[Ca^{2+}]_i$ change could be induced by the high K^+-induced depolarization.

Increasing resting $[Ca^{2+}]_i$ in differentiated cells *via* extracellular Ca^{2+} influx Besides the enhancement of high-K^+ evoked transient, a TSN-induced in-

Tab 1. TSN (50 μmol/L)-induced $[Ca^{2+}]_i$ 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 μmol/L Cd^{2+} or 10 μmol/L nifedipine, or Ca^{2+}-free solution) or 2 min after further elevating $[Ca^{2+}]_i$ in the solution to 2 mmol/L or after washing out channel blockers with TSN-containing standard physiological solution. $^aP>0.05$, $^cP<0.01$ *vs* control. See text for detail.

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

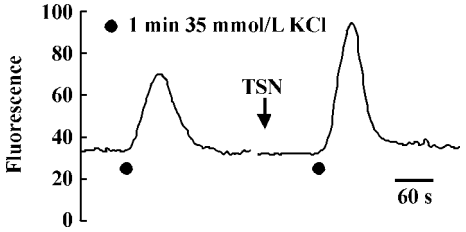

Fig 1. Effect of TSN on high K^+-induced Ca^{2+} transient. After treatment with 35 μmol/L TSN for 2 min (the arrow), the amplitude of high K^+-induced Ca^{2+} transient was evidently increased. The increase of $[Ca^{2+}]_i$ in fluo-3 loaded cells is represented as an increase of dye fluorescence shown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

crease in resting level of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increased to 117.7 %±10.5 % after 2 min and 133.1 %± 17.5 % after 4 min 35 μmol/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 $[Ca^{2+}]_i$ was observed at micromolar range of TSN. TSN at the concentrations of 0.5, 5, 35, and 50 μmol/L caused $[Ca^{2+}]_i$ to increase by 4.6 %± 4.2 % (n=10, P<0.01), 28.6 %±7.3 % (n=21, P<0.01), 33.1 %±17.5 % (n=12, P<0.01), and 37.1 %±30.0 % (n=12, P<0.01, Fig 2A), respectively.

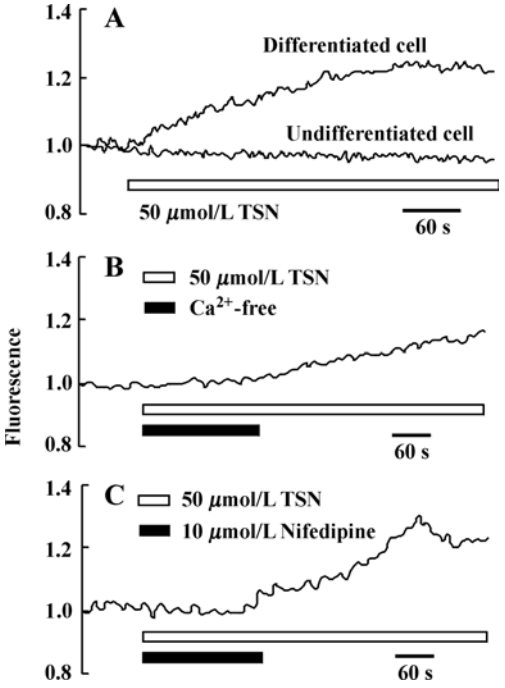

Fig 2. Effect of TSN on resting $[Ca^{2+}]_i$. A: 50 μmol/L TSN-induced changes of $[Ca^{2+}]_i$ in differentiated and undifferentiated cells. B: TSN-induced increase of $[Ca^{2+}]_i$ in differentiated cell appeared after elevating $[Ca^{2+}]_i$ in the solution to 2 mmol/L. C: The TSN-induced increase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

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

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

When performing the similar experiments in undifferentiated NG108-15 cells, it was found that the $[Ca^{2+}]_i$ 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 %±3.1 % (n=29,

P>0.05, Fig 2A) of control after 50 μmol/L TSN application.

Inhibition of TSN-induced increase in $[Ca^{2+}]_i$ 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 μmol/L), a L-type Ca^{2+} channel antagonist, the $[Ca^{2+}]_i$ maintained 100.9 %±4.6 % of the control (n=17, P> 0.05) after 50 μmol/L TSN application for 4 min. However, when the nifedipine was washed out, the TSN-induced increase of $[Ca^{2+}]_i$ appeared, and 2 min later the $[Ca^{2+}]_i$ increased to 146.0 %±31.1 % of the control (n=17, P<0.001, Fig 2C). For the convenience of comparison, Tab 1 summarized the effects of 50 μmol/L TSN on $[Ca^{2+}]_i$ under different experimental conditions.

DISCUSSION

The present study showed that TSN increased high K^+-evoked Ca^{2+} transient and resting $[Ca^{2+}]_i$ in differentiated NG108-15 cells, and that the TSN-induced $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$.

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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 ^{3}H-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×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.