

## Inhibitory effects of 8-(*N,N*-diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB-8) on intracellular $Ca^{2+}$ elevated by neurotransmitters in brain cells

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**KEY WORDS** brain; cultured cells; nimodipine; Fura-2; sodium glutamate; histamine; serotonin; calcium

### ABSTRACT

**AIM:** To study the effects of TMB-8 on  $[Ca^{2+}]_i$  elevation induced by neurotransmitters in dissociated brain cells. **METHODS:** The brain cell suspension was made using a gentle trituration for 1 min with a polished pipette. The changes of  $[Ca^{2+}]_i$  were detected by the fluorescent indicator, Fura 2-AM. **RESULTS:** In the presence of extracellular  $Ca^{2+}$   $1.3 \text{ mmol} \cdot \text{L}^{-1}$ , sodium glutamate (Glu), histamine (His), and serotonin (5-HT) markedly increased the  $[Ca^{2+}]_i$  which were reduced by TMB-8  $30 \mu\text{mol} \cdot \text{L}^{-1}$ . TMB-8  $3 \mu\text{mol} \cdot \text{L}^{-1}$  produced inhibitory effects on the increase of  $[Ca^{2+}]_i$  by His and 5-HT in a  $Ca^{2+}$ -free Hanks' solution. The increase of  $[Ca^{2+}]_i$  by His and 5-HT was reduced to control level by TMB-8  $10 \mu\text{mol} \cdot \text{L}^{-1}$ . **CONCLUSION:** TMB-8 inhibited the  $[Ca^{2+}]_i$  elevation induced by Glu, 5-HT, and His in brain cells.

### INTRODUCTION

A major factor in triggering brain cell death caused by the cerebral ischemia is intracellular free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) overloading. Ischemia induced release of some neurotransmitters such as glutamate (Glu), histamine (His), and serotonin (5-HT) increased  $[Ca^{2+}]_i$ <sup>[1,2]</sup>. Some calcium antagonists such as

nimodipine (Nim) have been used to treat cerebral ischemia and get some protective role on cerebral ischemic injury. However, these drugs' actions were confined with their marked decrease in mean arterial blood pressure and reduce the ability of autoregulation of cerebral blood flow<sup>[3]</sup>.

8-(*N,N*-Diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB-8) is an inhibitor of  $Ca^{2+}$  release of intracellular store from the sarcoplasmic reticulum (SR) and  $Ca$ -influx from extracellular site in skeletal and smooth muscles<sup>[4]</sup>. TMB-8 can protect/treat cerebral ischemia in rats<sup>[5]</sup>. It is highly possible that some action mechanisms of TMB-8 may reduce the cerebral  $[Ca^{2+}]_i$ . To test this hypothesis, the effects of TMB-8 on the increases of  $[Ca^{2+}]_i$  induced by His, Glu, and 5-HT in dissociated single rat brain cell were investigated.

### MATERIALS AND METHODS

**Materials** TMB-8, Fura 2-AM, Glu, serotonin, and nimodipine were purchased from Sigma. TMB-8 was dissolved in distilled water and Glu in HCl  $0.1 \text{ mol} \cdot \text{L}^{-1}$ . Histamine and HEPES were from BDH. Bovine serum albumin (BSA) was from Boehringer. Dulbecco's modified Eagle medium (DMEM) was from Gibco. The system of measurement of AR-CM-MIC was the product of Spex Co, USA.

**Cerebral cell suspension** Cerebral cells were prepared from newborn Sprague-Dawley rats (from Jiangsu Province Laboratory Animal Center, Certificate No 97001), according to the methods of [6] with some modifications. The rat brains were put into ice-cold Hanks' solution; NaCl 137, KCl 5.0,  $CaCl_2$  1.3,  $MgCl_2$  0.5, glucose 5.6, and HEPES  $10 \text{ mmol} \cdot \text{L}^{-1}$ .

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Received 1999-01-21

Accepted 1999-03-11

pH 7.4. The brain was washed and cut into small pieces, which were mechanically dissociated by gentle trituration for 1 min with a polished pipette. The isolated brain cells were filtered through nylon mesh (200 mesh) and spun at  $300 \times g$  for 5 min. The cells were resuspended in warm DMEM with 10 % bovine serum. Trypan blue staining showed a cellular viability rate of 85 % - 90 %.

**Fura 2-AM loading and fluorescence measurement** The cell suspension was placed at  $37^\circ\text{C}$  for 5 min, then incubated with Fura 2-AM  $5 \mu\text{mol}\cdot\text{L}^{-1}$  in  $\text{Me}_2\text{SO}$  at  $37^\circ\text{C}$  for 40 min with shaking. The loaded cells were spun at  $300 \times g$  for 5 min and washed twice with Hanks' solution containing 0.2 % BSA. Cells were resuspended with Hanks' solution containing 0.2 % BSA.  $\text{Ca}^{2+}$ -free Hanks' solution was replaced with  $\text{Ca}^{2+}$ -free and added egtazic acid  $0.1 \text{ mmol}\cdot\text{L}^{-1}$ . TMB-8 was added 30 min prior to the measurement.

The fluorescence was determined using a system of measurement of AR-CM-MIC at  $\lambda_{\text{ex}}$  340 and 380 nm and  $\lambda_{\text{em}}$  505 nm.  $[\text{Ca}^{2+}]_i$  of single cell was calculated according to the formula:  $[\text{Ca}^{2+}]_i = K_d \times (sf_2/sb_2) \times [(R - R_{\text{min}})/(R_{\text{max}} - R)]$ .  $R (F_{340}/F_{380})$  was fluorescence intensity.  $R_{\text{max}}$  was determined by the addition of ionomycin and  $R_{\text{min}}$  by the addition of egtazic acid.  $K_d$  was  $224 \text{ nmol}\cdot\text{L}^{-1}$ . Values of  $sf_2$  and  $sb_2$  were the fluorescence of Fura-2 at 380 nm in  $\text{Ca}^{2+}$ -free and saturation with  $\text{Ca}^{2+}$ , respectively. The autofluorescence was corrected before calculation.

**Statistical analysis of data** Datas were expressed as  $\bar{x} \pm s$  and analyzed with *t*-test.

## RESULTS

**Resting  $[\text{Ca}^{2+}]_i$**  When the brain cells were incubated in a  $\text{Ca}^{2+}$ -free Hanks' solution,  $[\text{Ca}^{2+}]_i$  was decreased from  $(71 \pm 6) \text{ nmol}\cdot\text{L}^{-1}$  ( $n = 6$ ) to  $[(67 \pm 4) (n = 6, P > 0.05)$  and  $(63 \pm 6)] \text{ nmol}\cdot\text{L}^{-1}$  ( $n = 6, P < 0.05$ ) at TMB-8 concentrations of 3 and  $10 \mu\text{mol}\cdot\text{L}^{-1}$ , respectively. On the other hand, the resting  $[\text{Ca}^{2+}]_i$  of brain cells was  $(125 \pm 13) \text{ nmol}\cdot\text{L}^{-1}$  in the presence of extracellular  $\text{Ca}^{2+}$   $1.3 \text{ mmol}\cdot\text{L}^{-1}$ . Preincubation with TMB-8  $30 \mu\text{mol}\cdot\text{L}^{-1}$  did not induce any obvious change in  $[\text{Ca}^{2+}]_i$ .

**Glu-induced  $[\text{Ca}^{2+}]_i$  elevation** Glu  $10 \mu\text{mol}\cdot\text{L}^{-1}$  increased  $[\text{Ca}^{2+}]_i$  by 44 % in Hanks' solution

containing  $\text{Ca}^{2+}$   $1.3 \text{ mmol}\cdot\text{L}^{-1}$ . This increase was reduced by TMB-8  $30 \mu\text{mol}\cdot\text{L}^{-1}$  from  $185 \pm 19$  to  $152 \pm 8$ . When brain cells were incubated with Nim  $1 \mu\text{mol}\cdot\text{L}^{-1}$  in Hanks' solution, which blocked the voltage-sensitive  $\text{Ca}^{2+}$ -channel. Glu increased  $[\text{Ca}^{2+}]_i$  slightly less than that of without Nim (Tab 1). However, Glu  $10 \mu\text{mol}\cdot\text{L}^{-1}$  did not induce  $[\text{Ca}^{2+}]_i$  rise in  $\text{Ca}$ -free Hanks' solution containing egtazic acid  $0.1 \text{ mmol}\cdot\text{L}^{-1}$ .

**Tab 1. Effect of TMB-8 and Nim on  $[\text{Ca}^{2+}]_i$  elevation induced by Glu of rat brain cells in Hanks' solution containing  $\text{Ca}^{2+}$   $1.3 \text{ mmol}\cdot\text{L}^{-1}$ .  $n = 6$  cells from 6 rats.  $\bar{x} \pm s$ .  $^c P < 0.01$  vs control, without Glu.  $^f P < 0.01$  vs Glu.**

Group	Concentration/ $\mu\text{mol}\cdot\text{L}^{-1}$	$[\text{Ca}^{2+}]_i$ / $\text{nmol}\cdot\text{L}^{-1}$
Control		$129 \pm 18$
Glu	10	$185 \pm 19^c$
Nim + Glu	1 + 10	$175 \pm 16$
TMB-8 + Glu	30 + 10	$152 \pm 8^f$

**His-induced  $[\text{Ca}^{2+}]_i$  elevation** The  $[\text{Ca}^{2+}]_i$  was raised by His 50, 100, and  $200 \mu\text{mol}\cdot\text{L}^{-1}$  in Hanks' solution containing  $\text{Ca}^{2+}$   $1.3 \text{ mmol}\cdot\text{L}^{-1}$  in a concentration-dependent manner. These increases were markedly inhibited by TMB-8  $30 \mu\text{mol}\cdot\text{L}^{-1}$  by 34 %, 38 %, and 49 %, respectively. In the presence of a  $\text{Ca}^{2+}$ -free Hanks' solution with egtazic acid  $0.1 \text{ mmol}\cdot\text{L}^{-1}$ , His 50, 100, and  $200 \mu\text{mol}\cdot\text{L}^{-1}$  was responsible for  $[\text{Ca}^{2+}]_i$   $(79 \pm 4)$ ,  $(89 \pm 6)$  and  $(111 \pm 8) \text{ nmol}\cdot\text{L}^{-1}$ , respectively. TMB-8 3 and  $10 \mu\text{mol}\cdot\text{L}^{-1}$  inhibited the  $[\text{Ca}^{2+}]_i$  elevation caused by His completely (Fig 1).

**5-HT-induced  $[\text{Ca}^{2+}]_i$  elevation** 5-HT 0.1, 1.0, 10,  $100 \mu\text{mol}\cdot\text{L}^{-1}$  showed remarkable increase of  $[\text{Ca}^{2+}]_i$  in Hanks' solution containing  $\text{Ca}^{2+}$   $1.3 \text{ mmol}\cdot\text{L}^{-1}$ , which was reduced by TMB-8 (Fig 2). When cells were incubated in the  $\text{Ca}^{2+}$ -free Hanks' solution with egtazic acid  $0.1 \text{ mmol}\cdot\text{L}^{-1}$ , 5-HT  $10 \mu\text{mol}\cdot\text{L}^{-1}$  induced  $[\text{Ca}^{2+}]_i$  elevation from  $78 \pm 11$  to  $102 \pm 10$  ( $n = 5, P < 0.01$ ). The increase of  $[\text{Ca}^{2+}]_i$  was clearly restrained by TMB-8 10 and  $30 \mu\text{mol}\cdot\text{L}^{-1}$  to  $86 \pm 6$  ( $n = 5, P < 0.01$ ) and  $77 \pm 7$  ( $n = 5, P < 0.01$ ), respectively.

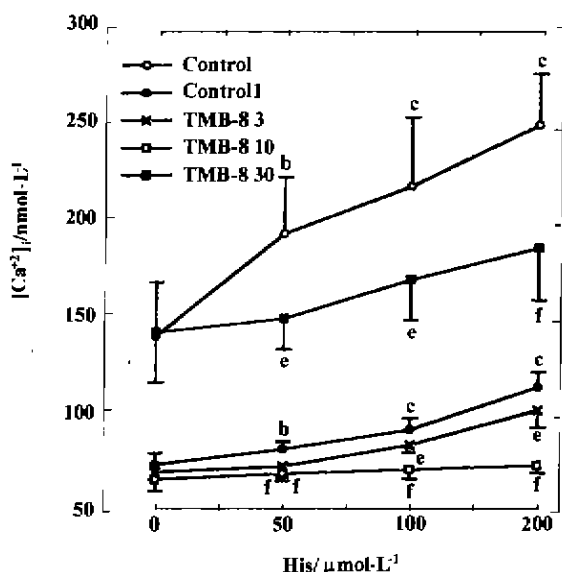


Fig 1. Effect of TMB-8 ( $\mu\text{mol} \cdot \text{L}^{-1}$ ) on  $[\text{Ca}^{2+}]_i$  elevation induced by His in Hanks' solution containing  $\text{Ca}^{2+} 1.3 \text{ mmol} \cdot \text{L}^{-1}$  (control, upper) and  $\text{Ca}^{2+}$ -free Hanks' solution containing egtazic acid  $0.1 \text{ mmol} \cdot \text{L}^{-1}$  (control1, down).  $n = 6$  cells from 6 rats.  $\bar{x} \pm s$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , vs without His. <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  vs His.

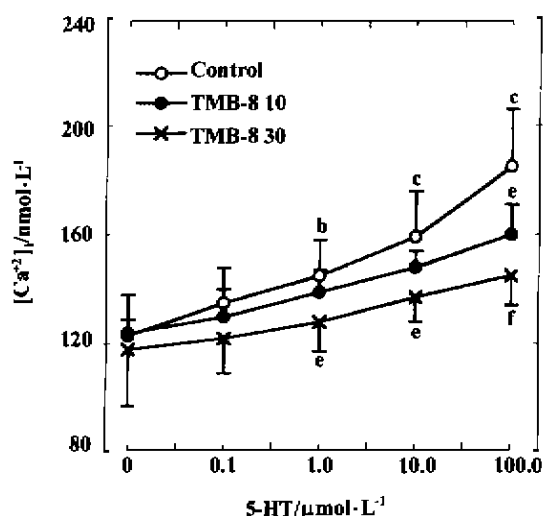


Fig 2. Effect of TMB-8 on  $[\text{Ca}^{2+}]_i$  elevation induced by 5-HT in Hanks' solution containing  $\text{Ca}^{2+} 1.3 \text{ mmol} \cdot \text{L}^{-1}$ .  $n = 6$  cells from 6 rats.  $\bar{x} \pm s$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , vs 5-HT  $0 \mu\text{mol} \cdot \text{L}^{-1}$ . <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  vs 5-HT.

## DISCUSSION

It is clear that the release of excitatory amino acids

and some neurotransmitters play important roles in the neurotoxicity associated with cerebral ischemia, and it has been shown that antagonism of their effects is also potentially neuroprotective<sup>[7]</sup>. Ischemia leads release of transmitters such as Glu, 5-HT and His which cause intracellular  $\text{Ca}^{2+}$  overloading that is the primal responsible for cells death. It has been suggested that Glu could induce the rise of  $[\text{Ca}^{2+}]_i$  by activating the NMDA receptor and opening the receptor-mediate  $\text{Ca}^{2+}$  channels<sup>[8]</sup>. Our results found that Glu induced the elevation of  $[\text{Ca}^{2+}]_i$  via the extracellular Ca-influx, which was inhibited by TMB-8  $30 \mu\text{mol} \cdot \text{L}^{-1}$ . However, Nim did not inhibited the increase of  $[\text{Ca}^{2+}]_i$  induced by Glu in the presence of Hank's solution containing  $\text{Ca}^{2+} 1.3 \text{ mmol} \cdot \text{L}^{-1}$ . It indicated that the inhibited action of TMB-8 on the elevation of  $[\text{Ca}^{2+}]_i$  did not relate to voltage-mediated  $\text{Ca}^{2+}$  channels.

His and 5-HT are important neurotransmitters in the mammalian central neural system. They could increase  $[\text{Ca}^{2+}]_i$  through extracellular  $\text{Ca}^{2+}$ -influx and the release of intracellular  $\text{Ca}^{2+}$  stores<sup>[9-11]</sup>. In our studies, TMB-8 decreased the elevation of  $[\text{Ca}^{2+}]_i$  in the presence of extracellular  $\text{Ca}^{2+}$  and almost completely blocked the increase of  $[\text{Ca}^{2+}]_i$  in the present of  $\text{Ca}^{2+}$ -free extracellular medium induced by His and 5-HT. Our past study have found that TMB-8 reduced  $[\text{Ca}^{2+}]_i$  via increase of the sarcoplasmic reticulum (SR) sequestration, which blocked the release of intracellular store from SR. However, the inhibitory effect of TMB-8 on Ca-influx from extracellular medium seems to be an indirect action from the saturation of SR with calcium<sup>[12]</sup>. It was reported that TMB-8 could block N-receptor<sup>[13]</sup>. But the action of TMB-8 on His or 5-HT receptors still needs more study.

In summary, the  $[\text{Ca}^{2+}]_i$  elevation induced by Glu, His and 5-HT was inhibited by TMB-8. Therefore TMB-8 may become a more effective compound to treat/prevent ischemic stroke.

**ACKNOWLEDGMENTS** To Prof WANG Jin-Xi (The State Key Laboratory of Coordination Chemistry, Nanjing University) for his help in the measurement of  $[\text{Ca}^{2+}]_i$ .

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### TMB-8 抑制神经递质引起的 单个脑细胞内游离钙的升高

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关键词 脑; 培养的细胞; 尼莫地平; Fura-2; 谷氨酸钠; 组织胺; 血清素; 钙

目的: 研究 TMB-8 对神经递质引起的单个脑细胞内游离钙升高的作用. 方法: 应用 AR-CM-MIC 阳离子测定系统测定游离大鼠单个脑细胞内钙离子浓度. 结果: 当细胞外液  $Ca^{2+}$  浓度为  $1.3 \text{ mmol} \cdot \text{L}^{-1}$  时, TMB-8  $30 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  能降低谷氨酸, 组织胺, 5-羟色胺引起的脑  $[Ca^{2+}]_i$  浓度的升高. 而当细胞外液无钙时, TMB-8 能降低细胞内静息  $[Ca^{2+}]_i$ ; TMB-8  $10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  则几乎完全抑制了组织胺和 5-羟色胺引起的脑  $[Ca^{2+}]_i$  升高作用. 结论: TMB-8 能降低谷氨酸, 组织胺, 5-羟色胺引起的脑  $[Ca^{2+}]_i$  升高.

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