

Actions of 8-(*N,N*-diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate in vascular smooth muscle cell cultures

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KEY WORDS TMB-8; vascular smooth muscle; calcium channel blockers; cultured cells; calcium radioisotopes

AIM: To study the effects of 8-(*N,N*-diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB-8) on vascular smooth muscle (VSM) cells A7r5.

METHODS: The effects of TMB-8 were investigated in A7r5 cell cultures with ⁴⁵CaCl₂.

RESULTS: TMB-8 reduced the intracellular free Ca²⁺ concentration, [Ca²⁺]_i in a Ca²⁺-free medium and blocked Ca²⁺ entry from the extracellular site in a regular Ca²⁺ medium. The equilibrated total cellular binding of Ca²⁺ was increased by TMB-8 whereas ⁴⁵Ca²⁺ entry activated by both NE and KCl was inhibited. However, the NE-activated Ca²⁺ entry was not blocked by TMB-8 if TMB-8 was added together with ⁴⁵Ca²⁺ at a later time instead of by pretreatment. Similar to actions of NE and KCl, depletion of Ca²⁺ from sarcoplasmic reticulum (SR) would also activate Ca²⁺ entry, which was blocked by TMB-8. When TMB-8 was rinsed out alone or together with NE after pretreatment with NE plus TMB-8 in VSM cells, the inhibitory effect of TMB-8 was not affected. **CONCLUSION:** TMB-8 not only blocks Ca²⁺ entry from the extracellular site, but also enhances Ca²⁺ uptake into SR which, indirectly inhibits Ca²⁺ entry from the extracellular site.

Some Ca²⁺ antagonists could be used to prevent/treat ischemic strokes^[1-3]. However, none of them show a total prevention or significant efficacy in reversing the neural damage after drug treatments. 8-(*N,N*-diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB-8) is a calcium antagonist capable of reducing intracellular free

Ca²⁺ ([Ca²⁺]_i) by acting dually at cell membrane to reduce Ca²⁺ influx and at intracellular site to sequester free Ca²⁺ into sarcoplasmic reticulum (SR)^[4]. TMB compounds were found to produce inhibitory actions on muscle contractility induced by a variety of stimulating agents in skeletal, smooth and cardiac muscles^[4-6]. These compounds are also reported to have strong effects on prevention and treatment of cerebral necrosis caused by ischemic stroke in rat when they are administered at -1, 0, 1, 6, and even 24 h after ischemic insults^[7].

In spite of the fact that TMB compounds were able to increase Ca²⁺ binding to the isolated skeletal muscle SR and to increase cAMP^[4,7,8], it is not clear how TMB compounds reduce [Ca²⁺]_i in the vascular smooth muscle (VSM) which is critical for the prevention and treatment of ischemic stroke. TMB-8 acts primarily at the intracellular site to reduce [Ca²⁺]_i via enhancement of Ca²⁺ uptake into SR. As for the blockade of Ca-influx by TMB-8, it could be an indirect capacitative effect due to SR saturation with Ca²⁺ by TMB-8 rather than a direct blockade of Ca²⁺-entry at the membrane site. This study was to study the effects of TMB-8 on VSM cells A7r5.

MATERIALS AND METHODS

Materials TMB-8 was purchased from Aldrich Chemical Co, Milwaukee WI. Norepinephrine bitartrate was obtained from Sigma Chemical Co, St Louis MO. A7r5 cells were acquired from American Type Culture Collection, Rockville MD. Dulbecco's modified Eagle's medium (DMEM), benzylpenicillin 10 MU · L⁻¹, streptomycin sulfate 10 g · L⁻¹ and amphotericin B 25 mg · L⁻¹ were obtained from Gibco Lab, Grand Island NY. Fetal bovine serum and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. The ⁴⁵CaCl₂ was obtained from ICN Radiochemicals, Irvine CA.

Cell cultures Embryonic rat thoracic aorta smooth muscle derived A7r5 cells were grown in monolayers in 75 cm²

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flasks in DMEM containing NaHCO_3 $3.7 \text{ g} \cdot \text{L}^{-1}$ + 10 % fetal bovine serum, benzylpenicillin $100 \text{ kU} \cdot \text{L}^{-1}$, and streptomycin $100 \text{ mg} \cdot \text{L}^{-1}$. The cultures were maintained at 37°C in a humidified atmosphere of air supplemented with 5 % CO_2 . The medium was changed every 2–3 d. Cells reached confluence in approximately 6 d and then passaged every 7–10 d.

$^{45}\text{Ca}^{2+}$ uptake In $^{45}\text{Ca}^{2+}$ influx studies, cells were seeded in 6-well dishes and grown to confluence. Before the assay, the culture medium was replaced with serum-free DMEM for 24 h to arrest cell growth and make cells quiescent. All experiments were carried out at 37°C . Prior to the study of $^{45}\text{Ca}^{2+}$ entry, cells were washed with $2 \times 2 \text{ mL}$ aliquots of Tris-buffered physiologic saline solution (PSS) (NaCl 154, KCl 5.4, dextrose 11, tris (hydroxymethyl) aminomethane 6, CaCl_2 2, and MgCl_2 $1.2 \text{ mmol} \cdot \text{L}^{-1}$) or a Ca^{2+} free solution from which Ca^{2+} had been omitted and containing egtazic acid $10 \mu\text{mol} \cdot \text{L}^{-1}$ and gallopamil $1 \mu\text{mol} \cdot \text{L}^{-1}$, and were allowed to equilibrate at 37°C for 30 min. In KCl-induced Ca^{2+} entry, gallopamil was omitted from Ca^{2+} -free solution and phentolamine $10 \mu\text{mol} \cdot \text{L}^{-1}$ was added to block α -receptor activation induced Ca -entry. The cultured monolayer cells in the dishes were incubated with $^{45}\text{Ca}^{2+}$ $185 \text{ MBq} \cdot \text{L}^{-1}$ for varying lengths of time as noted in the presence or absence of the indicated compounds. If the cells were incubated in the Ca^{2+} -free solution, Ca^{2+} $2 \text{ mmol} \cdot \text{L}^{-1}$ was added together with $^{45}\text{Ca}^{2+}$ 185 GBq for the measurement of Ca^{2+} influx. To stop Ca^{2+} fluxes, the $^{45}\text{CaCl}_2$ was quickly removed, the dishes were kept on ice, and the cells were washed with $5 \times 4 \text{ mL}$ aliquots of an ice-cold medium containing LaCl_3 $80.8 \text{ mmol} \cdot \text{L}^{-1}$ and dextrose $11 \text{ mmol} \cdot \text{L}^{-1}$ in Tris buffer $6 \text{ mmol} \cdot \text{L}^{-1}$ at pH 6.2. The cells were lysed by the addition of a solution containing egtazic acid $4 \text{ mmol} \cdot \text{L}^{-1}$ and 1 % sodium dodecylsulfate, and cell lysate 0.5 mL was taken followed by an addition of Universal Cocktail 10 mL into scintillation vials, counted in a Beckman LS 5000 CE beta counter.

Data analysis All values were given in $\bar{x} \pm s$. The number of viable cells was determined and the $^{45}\text{Ca}^{2+}$ uptake was expressed as $\text{Bq}/10^5$ cells. Statistical analysis utilized *t*-test for pooled samples and one-way ANOVA.

RESULTS AND DISCUSSION

In Ca^{2+} free solution, TMB-8 at 3, 10, and $30 \mu\text{mol} \cdot \text{L}^{-1}$ reduced $[\text{Ca}^{2+}]_i$ in VSM cells from $174 \pm 5 \text{ nmol} \cdot \text{L}^{-1}$ to 154 ± 6 , 140 ± 9 , and $135 \pm 15 \text{ nmol} \cdot \text{L}^{-1}$, respectively, measured with Fura-2 method⁽⁹⁾. While in normal Ca^{2+} medium, there was no change in $[\text{Ca}^{2+}]_i$ ⁽⁹⁾. To explore TMB-8 effect on Ca^{2+} influx at the plasma membrane site,

$^{45}\text{Ca}^{2+}$ uptake across the plasma membrane was measured with or without TMB-8. In this experiment $^{45}\text{Ca}^{2+}$ was incubated with VSM cells for 5 min and the amount of $^{45}\text{Ca}^{2+}$ across the plasma membrane was determined. TMB-8 at $30 \mu\text{mol} \cdot \text{L}^{-1}$ significantly decreased Ca^{2+} influx at the plasma membrane of VSM cells incubated either in PSS or in Ca^{2+} free medium when Ca^{2+} and $^{45}\text{Ca}^{2+}$ were added to the medium for measuring Ca^{2+} influx (Tab 1). These results indicate that TMB-8 inhibits Ca^{2+} influx even when $[\text{Ca}^{2+}]_i$ is low.

Tab 1. Effect of TMB-8 ($30 \mu\text{mol} \cdot \text{L}^{-1}$) on $^{45}\text{Ca}^{2+}$ influx in VSM cells in Ca^{2+} free and PSS solutions. $n = 6$ wells, $\bar{x} \pm s$. ^b $P < 0.05$ vs control.

TMB-8 ($\mu\text{mol} \cdot \text{L}^{-1}$)	$^{45}\text{Ca}^{2+}$ uptake in 5 min ($\text{Bq}/10^5$ cells)	
	Ca^{2+} -free medium	Normal Ca^{2+} medium
0	11.8 ± 2.7	20.7 ± 3.1
30	7.0 ± 0.9^b	14.2 ± 1.9

If TMB-8 acts to sequester Ca^{2+} into SR, then the total Ca^{2+} binding capacity of the cells should be increased accordingly. To determine the effects of TMB-8 on equilibrated total cellular binding of Ca^{2+} , 185 GBq of $^{45}\text{Ca}^{2+}$ was incubated with the VSM cells for 120 min in PSS solution with or without TMB-8. The results showed that TMB-8 increased total binding (120 min) of $^{45}\text{Ca}^{2+}$ by about 17 % (Tab 2), supporting the concept that TMB-8 acts by increasing sequestration of Ca^{2+} into SR. As a result, the intracellular store is full which in turn sends a message to the plasma membrane to stop Ca^{2+} influx no matter whether $[\text{Ca}^{2+}]_i$ is low or high, according to the capacitative mechanism.

Tab 2. Effect of TMB-8 ($30 \mu\text{mol} \cdot \text{L}^{-1}$) on $^{45}\text{Ca}^{2+}$ binding in cultured VSM cells in PSS solution. $n = 6$ wells, $\bar{x} \pm s$. ^b $P < 0.05$ vs control.

TMB-8 ($\mu\text{mol} \cdot \text{L}^{-1}$)	$^{45}\text{Ca}^{2+}$ uptake in 120 min ($\text{Bq}/10^5$ cells)
0	92 ± 11
30	108 ± 11^b

NE is known to open the receptor-operated Ca^{2+} channel at the plasma membrane and to release

Ca²⁺ from SR of VSM cells. In this experiment, NE 0.1–10 μmol·L⁻¹ markedly increased ⁴⁵Ca²⁺ entry in a concentration-dependent manner. To find out if TMB-8 has any effect on inhibiting Ca²⁺ influx by NE, the cells were pretreated with TMB-8 for 15 min. The increased Ca²⁺ entry was totally blocked by TMB-8 30 μmol·L⁻¹ (Tab 3), indicating that TMB-8 could inhibit receptor-operated Ca²⁺ influx of NE. This inhibition is very efficient because the inhibitory effects of TMB-8 on NE-induced contraction in basilar artery ring can be seen at 1 μmol·L⁻¹ concentration^[10].

Tab 3. Effects of TMB-8 (30 μmol·L⁻¹) on NE-induced ⁴⁵Ca²⁺ influx in VSM cells in Ca²⁺ free solution. $\bar{x} \pm s$. ^bP<0.05 vs corresponding controls.

NE (μmol·L ⁻¹)	TMB-8 (μmol·L ⁻¹)	Wells	⁴⁵ Ca ²⁺ uptake in 7 min (Bq/10 ⁵ cells)
0	0	12	11.0±0.6
0	30	12	9.6±0.9 ^b
0.1	0	11	12.2±0.7
0.1	30	6	11.2±0.6 ^b
1	0	6	12.7±0.9
1	30	6	10.3±1.3 ^b
10	0	12	13.7±1.2
10	30	12	10.3±1.2 ^b

KCl is known to activate the voltage-operated Ca²⁺ entry. KCl 80 mmol·L⁻¹ significantly raised ⁴⁵Ca²⁺ influx into the cells (Tab 4).

Tab 4. Effect of TMB-8 (30 μmol·L⁻¹) on KCl-induced ⁴⁵Ca²⁺ influx in VSM cells in Ca²⁺ free solution. n = 6 wells, $\bar{x} \pm s$. ^bP<0.05 vs corresponding control.

KCl (mmol·L ⁻¹)	TMB-8 (μmol·L ⁻¹)	⁴⁵ Ca ²⁺ uptake in 7 min (Bq/10 ⁵ cells)
0	0	21.5±3.5
0	30	16.4±2.6 ^b
80	0	27.3±5.5
80	30	15.9±4.6 ^b

When the cells were pretreated with TMB-8 30 μmol·L⁻¹, this increase in uptake was completely inhibited, indicating that TMB-8 could inhibit voltage-operated Ca²⁺ influx. TMB-8 inhibited KCl-induced Ca²⁺ entry to the level lower than the control while in NE-induced Ca²⁺ entry, the

inhibition is at the control level. This may be because NE-induced Ca²⁺ release from SR compensate for part of TMB-8 Ca²⁺ sequestration effect. In the KCl group, there is no release of Ca²⁺ from SR, thus the inhibition is greater. The following experiments were designed to determine if SR Ca²⁺ depletion can activate Ca²⁺ entry in VSM cells. The cells were pretreated with NE to deplete Ca²⁺ from SR in Ca²⁺ free medium. NE was then rinsed out and Ca²⁺ in the SR was kept depleted as the cells were incubated in the Ca²⁺-free medium. The cells were then incubated with ⁴⁵Ca²⁺ for 1.5 min and ⁴⁵Ca²⁺ influx was measured. In one group, NE 10 μmol·L⁻¹ increased ⁴⁵Ca²⁺ influx markedly as expected. In another group, when NE was rinsed out after pretreatment, the depletion of intracellular Ca²⁺ store continued and the Ca²⁺ entry was still higher than control despite the fact that there was no NE present in the medium (Tab 5). These results indicate that Ca²⁺ depletion from SR can activate Ca²⁺ influx across the plasma membrane. This is consistent with Putney's capacitative model in which SR full of Ca²⁺ can inhibit Ca²⁺ entry.

Tab 5. Effect of depletion of intracellular Ca²⁺ store on ⁴⁵Ca²⁺ influx in cultured VSM cells in Ca²⁺ free solution. $\bar{x} \pm s$. ^bP<0.05 vs (A); ^cP<0.05 vs (B).

NE (μmol·L ⁻¹)	Wells	⁴⁵ Ca ²⁺ uptake in 1.5 min (Bq/10 ⁵ cells)
(A) 0	12	8.9±0.4
(B) 30	11	13.6±3.9 ^b
(C) 30 then rinsed	6	9.9±0.7 ^c

The increased Ca²⁺ influx activated *via* Ca²⁺ depletion from SR by NE (control) was completely inhibited by pretreatment of cells with TMB-8 (30 μmol·L⁻¹) to the level that Ca²⁺ uptake was lower than the control. If the cells were pretreated with NE and TMB-8, and both NE and TMB-8 were rinsed out just before the addition of ⁴⁵Ca²⁺, Ca²⁺ influx was still blocked (Tab 6).

These results indicate that TMB-8 inhibits NE-induced Ca²⁺ depletion from SR which leads to the inhibition of Ca²⁺ entry. These results support the hypothesis that TMB-8 acts primarily in the intracellular site.

Tab 6. Effect of TMB-8 ($30 \mu\text{mol}\cdot\text{L}^{-1}$) on $^{45}\text{Ca}^{2+}$ influx induced by depletion of intracellular Ca^{2+} store in cultured VSM cells in Ca^{2+} free solutions. $n = 6$ wells, $\bar{x} \pm s$. $^{\text{b}}P < 0.05$ vs control.

NE ($\mu\text{mol}\cdot\text{L}^{-1}$)	TMB-8 ($\mu\text{mol}\cdot\text{L}^{-1}$)	$^{45}\text{Ca}^{2+}$ uptake in 1.5 min (Bq/ 10^5 cells)
0	0	8.0 ± 0.3
0	30	$6.3 \pm 0.3^{\text{b}}$
10	30	$7.1 \pm 0.3^{\text{b}}$
10	30 then rinsed	$7.1 \pm 0.2^{\text{b}}$

The following experiments were designed to elucidate whether TMB-8 has inhibitory function on Ca^{2+} influx directly or through the capacitative intracellular mechanisms. NE $10 \mu\text{mol}\cdot\text{L}^{-1}$ enhanced $^{45}\text{Ca}^{2+}$ influx, which could be blocked by pretreatment of cells with TMB-8 and NE added together as mentioned before. If TMB-8 used in the pretreatment was rinsed out before $^{45}\text{Ca}^{2+}$ incubation, the Ca^{2+} influx was still low, remaining at the control level, despite the continuous presence of NE. On the other hand, if the cells were pretreated with NE alone instead of NE plus TMB-8 and TMB-8 was added later together with $^{45}\text{Ca}^{2+}$ in the cells (Later TMB-8 in Tab 7), the Ca^{2+} influx was markedly increased and there was no inhibitory effect on Ca^{2+} influx in spite of the presence of TMB-8 during the influx process. These results indicate that the TMB-8's inhibitory action on Ca-influx is through intracellular capacitative mechanism rather than through its direct action on the plasma membrane.

Tab 7. Effect of different TMB-8 ($30 \mu\text{mol}\cdot\text{L}^{-1}$) treatments of SR-depletion-induced and NE-induced $^{45}\text{Ca}^{2+}$ influx in VSM cells in Ca^{2+} free solution. $\bar{x} \pm s$. $^{\text{a}}P > 0.05$, $^{\text{b}}P < 0.05$ vs control.

NE ($\mu\text{mol}\cdot\text{L}^{-1}$)	TMB-8 ($\mu\text{mol}\cdot\text{L}^{-1}$)	Wells	$^{45}\text{Ca}^{2+}$ uptake in 5 min (Bq/ 10^5 cells)
0	0	12	10.1 ± 0.4
10	0	5	$11.7 \pm 1.7^{\text{b}}$
0	30	6	$9.9 \pm 1.1^{\text{a}}$
10 added later	30 then rinsed	6	$9.8 \pm 0.7^{\text{a}}$
10	30 added later	5	$11.1 \pm 0.6^{\text{b}}$

TMB compounds increased cAMP concentration in the cells, which could be the reason for

TMB-8 to decrease $[\text{Ca}^{2+}]_i$ ^[7]. Aminophylline, an inhibitor of phosphodiesterase, was found to enhance the inhibitory effect of TMB-8 on rabbit basilar artery contraction induced by Ca^{2+} released from SR by BHQ^[10]. These results indicate that TMB-8 may act through cAMP at intracellular site.

Contraction of VSM is regulated by $[\text{Ca}^{2+}]_i$ and the rise in $[\text{Ca}^{2+}]_i$ is due to either Ca^{2+} release from intracellular stores or influx of extracellular Ca^{2+} across the plasma membrane or both. The depletion of an inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} pool has been proposed to be the signal for Ca^{2+} entry in agonist-activated cells^[11]. Putney^[12,13] proposed a capacitative model: the state of filling of the intracellular Ca^{2+} pool regulates the Ca^{2+} permeability of the plasma membrane, suggesting that stimulated influx is a direct consequence of intracellular Ca^{2+} release rather than an independent process. The depletion of an intracellular Ca^{2+} pool signals the opening of a pathway for the replenishment from the extracellular space. As long as the pool remains empty, Ca^{2+} would enter across the plasma membrane. The plasma membrane is responsive to the Ca^{2+} content of the intracellular store, regardless of $[\text{Ca}^{2+}]_i$ ^[11,12].

In this study, all of the work was performed on cultured A7r5 VSM cells. It should be noted that these cells are different from intact smooth muscle cells in artery wall, eg, the G-kinase activity in passaged cells is lower than that in the intact cell in the artery wall^[14].

In summary, TMB-8 was found to act on intracellular site to increase Ca^{2+} uptake into SR which in turn indirectly inhibits Ca^{2+} entry from the extracellular site. This is different from previous belief that TMB-8 acts directly on the plasma membrane to block Ca^{2+} entry. This is the possible mechanism for TMB-8 to be more effective in protection and treatment of ischemic stroke than other Ca-antagonists.

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8-(*N,N*-二乙胺)-*n*-辛基-3,4,5-三甲氧基苯甲酸酯在血管平滑肌细胞培养液中的作用

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关键词 TMB-8; 血管平滑肌; 钙通道阻滞剂; 培养的细胞; 钙放射性同位素

Effects of isolation housing and timing of drug administration on amikacin kinetics in mice

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KEY WORDS social isolation; animal housing; drug administration schedule; amikacin; chronobiology; pharmacokinetics

AIM: To study the influences of social condition and drug administration time on amikacin metabolism in mice. **METHODS:** Forty ♂ ICR mice were randomly assigned into 4 groups according to 1) housing condition: individual

housing (I, one mouse in a cage) or aggregated housing (A, 10 mice in a cage) and 2) drug administration time: at midday (D) or at midnight (N), ie I-D, I-N, A-D, and A-N groups. Amikacin was injected sc $15 \text{ mg} \cdot \text{kg}^{-1}$ after 4 wk of raising at D or N. Blood samples were taken at 5, 10, 15, 20, 30, and 60 min after medication in each mouse. Plasma amikacin was measured by enzyme immunoassay. The concentration-time data were fitted with one-compartment open model in each mouse and data were analyzed with group *t* test. **RESULTS:** The clearance (*Cl*) of amikacin

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