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8-(*N*, *N*-Diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate actions on calcium dynamics in cultured vascular smooth muscle cells

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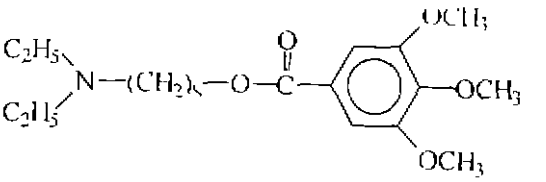
KEY WORDS vascular smooth muscle; TMB; calcium; norepinephrine; hydroquinones; ryanodine; nifedipine

AIM: To study 8-(*N*, *N*-Diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB), a potent Ca^{2+}-antagonist, actions on cellular calcium dynamics in vascular smooth muscle cell (VSMC) cultures. **METHODS**: A7r5 VSMC were cultured with Fura-2 measurements of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. **RESULTS**: TMB reduced $[Ca^{2+}]_i$ from control levels and blocked $[Ca^{2+}]_i$ increase caused by norepinephrine (NE) and 2,5-di(t-butyl)-1,4-benzohydroquinone (BHQ). $[Ca^{2+}]_i$ reduction by TMB was further enhanced by ryanodine. **CONCLUSION**: TMB is an effective agent for blocking the $[Ca^{2+}]_i$ increase caused by NE and BHQ and for enhancing the $[Ca^{2+}]_i$ reduction caused by ryanodine.

ω-(*N*, *N*-Diethylamino)-*n*-alkyl-3,4,5-trimethoxybenzoate compounds were found to protect/reverse ischemic stroke in rats very effectively when administered at −1, 0, 1, 6, and 24 h after stroke[1]. These compounds also produced inhibitory actions in skeletal, smooth, and cardiac muscle contractilities induced by various stimulating agents, such as histamine, acetylcholine, nicotine, KCl, $BaCl_2$, and norepinephrine[2-7]. These compounds increase intracellular cAMP and reduce intracellular free calcium concentrations, $[Ca^{2+}]_i$[1-8].

Although these compounds were found to increase cAMP and to increase Ca^{2+} binding to isolated sarcoplasmic reticulum (SR) in skeletal muscle[1,4], it was not clear how the $[Ca^{2+}]_i$ was reduced in the vascular smooth muscle (VSM) which is critical for the treatment/prevention of ischemic stroke. These compounds can reduce $[Ca^{2+}]_i$ via increasing Ca^{2+} sequestration into SR and decrease Ca-influx from the extracellular site. However, whether the decreasing of Ca-influx at plasma membrane is due to direct blockade of Ca-influx at plasma membrane or due to indirect action of these compounds after saturation of SR by Ca (Putney's capacitative mechanism)[9,10] is uncertain. This study was to study the possible mechanism of $[Ca^{2+}]_i$ reduction by 8-(*N*, *N*-diethylamino)-*n*-octyl-3, 4, 5-trimethoxybenzoate (TMB), the most potent compound among these compounds.



8-(*N*, *N*-Diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB)

MATERIALS AND METHODS

Materials TMB and 2, 5-di(tert-butyl)-1, 4-benzohydroquinone (BHQ) were purchased from Aldrich Chemical Co (Milwaukee WI). Norepinephrine bitartrate, nifedipine, and Fura 2-AM were obtained from Sigma Chemical Co (St Louis MO). Ryanodine was purchased from AgriSystems (Wind Gap PA).

A7r5 VSMC were acquired from American Type Culture Collection (Rockville MD). Dulbecco's modified Eagle's medium (DMEM) and antibiotic-antimycotic (benzylpenicillin 10 $MU \cdot L^{-1}$, streptomycin sulfate 10 $g \cdot L^{-1}$, amphotericin B 25 $mg \cdot L^{-1}$) were obtained from Gibco Lab (Grand Island NY). Fetal bovine serum and its albumin (BSA) were purchased from Sigma Chemical Co.

Cell Cultures Embryonic rat thoracic aorta smooth muscle-derived A7r5 cells were grown in monolayers in 75 cm^2 flasks in DMEM containing $NaHCO_3$ 3.7 $g \cdot L^{-1}$ + 10 % fetal bovine serum, benzylpenicillin 100 $KU \cdot L^{-1}$, streptomycin 100 $mg \cdot L^{-1}$, and amphotericin B 0.25 $mg \cdot L^{-1}$. The

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cultures were maintained at 37 ℃ in a 100 % humidified air supplemented with 5 % CO_2. The medium was changed every 2 – 3 d. VSMC reached confluence in about 6 d and then passaged every 7 – 10 d. Experiments were performed in passages 6 and 7.

Monolayers of confluent cells were treated with trypsin and, subsequently, suspended in Hanks' buffered saline containing: NaCl 137, $CaCl_2$ 1.3, $MgCl_2$ 0.5, KCl 5.0, dextrose 5.6, and HEPES 10 mmol·L^{-1} (pH 7.4) with 0.2 % fatty acid-free BSA. When Ca-free solution was needed, Hanks' buffer was replaced with Ca-free and egtazic acid 0.1 mmol·L^{-1} but, otherwise, was identical to Hanks' solution. After washing, cells were incubated in the Fura 2-AM 5 μmol·L^{-1} at 37 ℃ for 40 min with shaking. The loaded cells were centrifuged at low speed for 5 min and washed twice with Hanks' solution. Cells resuspended in buffer were diluted with Hanks' solution to approximately 5 × 10^7 cells·L^{-1} which were stored in ice. Immediately prior to use, the cuvettes containing cell suspension 2 mL were preincubated at 37 ℃ for 5 min.

The fluorescence was determined using a Perkin-Elmer 650-40 Fluorescence Spectrophotometer at λ_{ex} 340 nm (5 nm slits) and λ_{em} 485 nm (10 nm slits). Maximal fluorescence (F_{max}) was recorded by the addition of $CaCl_2$ 2 mmol·L^{-1} and 0.2 % Triton X-100. Minimal fluorescence (F_{min}) was determined by the addition of $MnCl_2$ 1 mmol·L^{-1}. $[Ca^{2+}]_i$ was calculated with the formula:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where K_d is 224 nmol·L^{-1}[11]. TMB was added 30 min before the fluorescence measurement, while NE, BHQ, ryanodine, and nifedipine were added immediately prior to the fluorescence determinations.

RESULTS AND DISCUSSION

TMB compounds reduce $[Ca^{2+}]_i$ via 2 mechanisms: (a) by blocking Ca-influx from the extracellular space and (b) by enhancing $[Ca^{2+}]_i$ uptake into the SR or inhibiting Ca^{2+} release from the SR[4].

In a Ca-free medium, A7r5 VSMC contained 174 ± 11 nmol·L^{-1} of $[Ca^{2+}]_i$. This was reduced significantly by TMB 3, 10, and 30 μmol·L^{-1}, indicating that TMB may increase the $[Ca^{2+}]_i$ uptake into the SR to reduce $[Ca^{2+}]_i$ (Tab 1).

The same concentrations of TMB 3, 10 μmol·L^{-1} also showed inhibitory effects on rabbit basal artery contraction induced by NE[12].

In a medium containing a normal concentration of calcium A7r5, VSMC contained $[Ca^{2+}]_i$ about 201 ± 9 nmol·L^{-1}; however, they were not significantly affected by TMB (30 μmol·L^{-1}) (Tab 1). It is possible that the uptake of intracellular Ca^{2+} by the SR is compensated by the influx of Ca^{2+} from extracellular site to make up the reduced $[Ca^{2+}]_i$. These results suggest that TMB acts primarily in the intracellular site to enhance Ca^{2+} uptake by the SR but not the blockade of Ca-influx from the extracellular site.

Tab 1. $[Ca^{2+}]_i$ (nmol·L^{-1}) of VSM in TMB. $\bar{x} \pm s$. $^bP < 0.05$ *vs* 0 μmol·L^{-1}.

TMB/μmol·L^{-1}	Ca-free medium ($n = 5$)	Normal Ca medium ($n = 6$)
0	174 ± 11	201 ± 9
3	154 ± 14^b	–
10	140 ± 21^b	–
30	135 ± 34^b	214 ± 14

It has been proposed that saturation of the SR with Ca^{2+} can indirectly inhibit the Ca-influx from the extracellular site[9,10]. Depletion of $[Ca^{2+}]_i$, on the other hand, somehow triggers the opening of a passageway for its replenishment from the extracellular space. TMB compounds were first noted to block Ca-influx in 1975[4]. Not knowing Putney's theory[9], it was thought that TMB blocked Ca-influx directly at the plasma membrane site[4]. The Ca-influx can be triggered by a wide range of NE (0.1 – 10 nmol·L^{-1}) which can be markedly inhibited by TMB at 30 and 100 μmol·L^{-1} (Tab 2), indicating that saturation of the SR with calcium by TMB does block the Ca-influx induced by NE.

Tab 2. $[Ca^{2+}]_i$ after TMB and NE in VSMC in normal calcium medium. $n = 6$, $\bar{x} \pm s$. $^bP < 0.05$ *vs* control.

NE/ nmol·L^{-1}	Control	TMB (30 μmol·L^{-1})	Control	TMB (100 μmol·L^{-1})
0	185 ± 38	180 ± 32	211 ± 27	235 ± 70
0.1	238 ± 22	210 ± 14^b	270 ± 25	187 ± 35^b
1	234 ± 14	213 ± 12^b	348 ± 115	192 ± 54^b
10	273 ± 24	208 ± 7^b	302 ± 77	118 ± 46^b

In the force development studies on rabbit basal artery, TMB is more effective in relaxing NE-

induced contractions versus high-K-induced contractions[12], while high-K^+-induced contractions do not involve calcium release from SR, which supports that the main TMB action site is at intracellular SR. The $^{45}Ca^{2+}$ experiments conducted in our Lab also support this theory, in which the depletion of Ca^{2+} from SR triggers $^{45}Ca^{2+}$ influx, and NE + TMB do not inhibit $^{45}Ca^{2+}$ influx induced by NE[13].

Similar results were obtained in Ca-free medium and nifedipine containing normal calcium medium. In Ca-free medium, NE increased $[Ca^{2+}]_i$ significantly which was antagonized by TMB 3, 10, and 30 $\mu mol \cdot L^{-1}$ (Tab 3).

Tab 3. Effects of TMB on $[Ca^{2+}]_i$ of VSMC in Ca-free *vs* nifedipine-treated normal Ca-medium. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$ *vs* control; $^cP < 0.05$ *vs* NE alone.

NE/ nmol·L^{-1}	TMB/ μmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1} Ca-free medium ($n = 5$)	Normal Ca-medium ($n = 6$)	Nifedipine-treated normal Ca-medium
0	0	186 ± 11	220 ± 26	–
10	0	187 ± 21^a	314 ± 30^b	255 ± 26^b
10	3	154 ± 54	–	–
10	10	147 ± 34^b	–	–
10	30	114 ± 44^b	–	221 ± 23^c

These results clearly indicate that the release of calcium from the SR by NE can be blocked by TMB. Nifedipine containing medium at a normal concentration of calcium is considered to be similar to Ca-free medium, because the voltage-operated Ca entry was blocked. In this condition, intracellular release of calcium from the SR by NE was again blocked by TMB treatment (Tab 3). These results clearly indicate that TMB can enhance calcium uptake into the SR to reverse the release of calcium from the SR by NE.

BHQ is known to release calcium from the SR *via* inhibition of the SR Ca-ATPase. By this action, BHQ appears to deplete intracellular calcium stores without affecting Ca^{2+} pumps located in the plasma membrane[14] or formation of inositol phosphates[15]. BHQ does not affect mitochondrial Ca^{2+} flux nor Ca^{2+}-independent Mg^{2+}-ATPase[14].

In a medium containing normal calcium concentration, BHQ increased $[Ca^{2+}]_i$ markedly as expected (Tab 4). There was a tendency of $[Ca^{2+}]_i$ reduction by TMB from the stimulated value by BHQ. However, the inhibition was not statistically significant. It could be due to the fact that the influx of extracellular calcium from the plasma membrane nullified the intracellular action of TMB. In order to observe the intracellular action of TMB on the SR calcium stores, Ca-free medium was used for the experiments. $[Ca^{2+}]_i$ was significantly increased by BHQ which was reduced significantly to the control level by TMB (Tab 4). These results indicate that TMB can reverse BHQ and NE-induced release of calcium from the SR possibly *via* increase of Ca-uptake into the SR[1].

Tab 4. Effects of TMB on BHQ actions on $[Ca^{2+}]_i$ of VSMC in normal Ca *vs* Ca-free medium $\bar{x} \pm s$.
$^bP < 0.05$ *vs* A; $^cP < 0.05$ *vs* B; $^bP < 0.05$ *vs* F.

Group	BHQ/ nmol·L^{-1}	TMB/ nmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1} Normal Ca-medium ($n = 6$)	Ca-free medium ($n = 8$)
A	0		182 ± 26	156 ± 8
B	10	0	212 ± 19^b	186 ± 15^b
C	10	3	202 ± 17	–
D	10	10	190 ± 25	–
E	10	30	173 ± 43	159 ± 25^c
F	100		–	216 ± 11^c
G	100	30	–	181 ± 12^b

TMB seems to decrease $[Ca^{2+}]_i$ through both intracellular and extracellular mechanisms[4]. TMB was also shown to inhibit phosphodiesterase and to raise the level of cAMP[8]. cAMP activates protein kinase A and protein kinase G, which results in a decrease in both $[Ca^{2+}]_i$ and the sensitivity of vascular smooth muscle contractility[15].

Ryanodine is known to irreversibly open up the calcium channel for depleting calcium at low concentrations and to block calcium release from the SR at high concentrations. Tab 5 shows no effects of ryanodine alone on $[Ca^{2+}]_i$ when A7r5 VSMC were incubated in a Ca-free medium. When BHQ was added to the medium, $[Ca^{2+}]_i$ increased markedly as expected. The increase of $[Ca^{2+}]_i$ by BHQ was reversed by the addition of TMB and/or

ryanodine. The combined use of TMB and ryanodine showed further reduction of $[Ca^{2+}]_i$ by BHQ, indicating that TMB and ryanodine act through different mechanisms to produce additional inhibitory actions caused by individual agents (Tab 5).

Tab 5. Effects of TMB on BHQ plus Ryanodine action in $[Ca^{2+}]_i$ of VSM cells in Ca-free medium. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* A; $^cP < 0.05$ *vs* B.

Group	BHQ/ nmol·L^{-1}	TMB/ nmol·L^{-1}	Ryanodine/ nmol·L^{-1}	$[Ca^{2+}]_i$/ nmol·L^{-1}
A	0	0	0	142 ± 8
B	100	0	0	216 ± 6^b
C	100	30	0	170 ± 34^c
D	100	0	0.5	177 ± 5^c
E	100	30	0.5	133 ± 25^c

In conclusion, TMB was shown to reduce $[Ca^{2+}]_i$ *via* increase of the SR sequestration, and blockade of the release of intracellular Ca^{2+} from the SR by NE and/or BHQ. The Ca-influx from extracellular medium across the plasma membrane also can be blocked by TMB; however, its action mechanism seems to be an indirect action from the saturation of the SR with calcium.

REFERENCES

1 Chiou GCY, Hong SJ. Prevention and reduction of neural damage in ischemic strokes by ω-(*N*,*N*-diethylamino)-*n*-alkyl-3,4,5-trimethoxybenzoate compounds. J Pharmacol Exp Ther 1995; **275**: 474 - 8.

2 Malagodi MH, Chiou CY. Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8); studies in smooth muscles. Eur J Pharmacol 1974; **27**: 25 - 33.

3 Malagodi MH, Chiou CY. Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8); studies in skeletal muscles. Pharmacology 1974; **12**: 20 - 31.

4 Chiou CY, Malagodi MH. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Br J Pharmacol 1975; **53**: 279 - 85.

5 Posner P, Chiou CY. Electrophysiological studies of ω-(*N*,*N*-diethylamino)-hexyl-3,4,5-trimethoxybenzoate on ventricular muscle and conduction system. Pharmacology 1976; **14**: 97 - 113.

6 Chiou CY, Malagodi MH, Sastry BVR, Posner P. Effects of calcium antagonist, 6-(*N*,*N*-diethylamino)-hexyl-3,4,5-trimethoxybenzoate, on digitalis-induced arrhythmias and cardiac contractions. J Pharmacol Exp Ther 1976; **198**: 444 - 9.

7 Chiou GCY, McLaughlin MA. Effects of 5-(*N*,*N*-diethylamino)-*n*-pentyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-5) on cardiovascular systems. Eur J Pharmacol 1982; **81**: 587 - 94.

8 Vassalle M, Barnabei O. Norepinephrine and potassium fluxes in cardiac Purkinje fibers. Pflugers Arch 1971; **322**: 287 - 303.

9 Putney JW. A model for receptor-regulated calcium entry. Cell Calcium 1986; **7**: 1 - 12.

10 Putney JW. Capacitative calcium entry revisited. Cell Calcium 1990; **11**: 611 - 24.

11 Grynkiewicz G, Poenie M, Tsien RY. A new general of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; **260**: 3440 - 50.

12 Xiao J, Liu XL, Chen Z, Chiou GCY. Vasodilation effects and action mechanisms of TMB-8 on basal artery in rabbits. J Cardiovasc Pharmacol Ther 1996; in press.

13 Chen Z, Liu XL, Chiou GCY. Studies on action mechanisms of 8-(*N*,*N*-diethylamino)-*n*-octyl-3,4,5-trimethoxybenzoate (TMB-8) in vascular smooth muscle cell cultures. J Cardiovasc Pharmacol Ther 1996; in press.

14 Moore GA, McConkey DJ, Kass GEN, O'Brien PJ, Orrenius S. 2,5-di-(*Tert*-butyl)-1,4-benzohydroquinone — a novel inhibitor of liver microsomal Ca^{2+} sequestration. FEBS Lett 1987; **224**: 331 - 6.

15 Schilling WP, Cabello OA, Rajan L. Depletion of the inositol 1,4,5-triphosphate-sensitive intracellular Ca^{2+} store in vascular endothelial cells activates the agonist-sensitive Ca^{2+}-influx pathway. Biochem J 1992; **284**: 521 - 30.

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8-(*N*,*N*-二乙铵)-*n*-辛基-3,4,5-三甲氧基苯甲酸酯对培养的血管平滑肌细胞内钙动力学的作用

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关键词 血管平滑肌; TMB; 钙; 去甲肾上腺素; 氢醌; 杀罗定; 硝苯地平