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INHIBITION OF CALCIUM UPTAKE AND CATECHOLAMINE RELEASE BY 8-(*N*,*N*-DIETHYLAMINO)-OCTYL-3,4,5-TRIMETHOXYBENZOATE HYDROCHLORIDE (TMB-8) IN CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

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Abstract—Effects of intracellular calcium antagonists, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and 1-(5-(*p*-nitrophenyl)-furfurylidene-amino) hydantoin sodium hydrate (dantrolene sodium), on catecholamine release and $^{45}Ca^{2+}$ uptake were studied using cultured bovine adrenal chromaffin cells. TMB-8 inhibited carbamylcholine-evoked catecholamine release and $^{45}Ca^{2+}$ uptake in a concentration-dependent manner with a similar potency. On the contrary, dantrolene sodium did not show obvious inhibitory effects of catecholamine release and $^{45}Ca^{2+}$ uptake. Although TMB-8 inhibited the high K^+-evoked catecholamine release and $^{45}Ca^{2+}$ uptake, the potency of the drug was approximately 100-fold less than when used to inhibit the carbamylcholine-evoked catecholamine release and $^{45}Ca^{2+}$ uptake. The inhibitory effect of TMB-8 on the carbamylcholine-evoked catecholamine release was not overcome by an increase in an extracellular calcium concentration, and was not due to competitive antagonism at the nicotinic receptor site. Moreover, TMB-8 inhibited the carbamylcholine-stimulated $^{45}Ca^{2+}$ efflux, but dantrolene sodium failed to affect it. These results suggest that TMB-8, a well-known intracellular calcium antagonist, prevents the cellular calcium uptake in cultured adrenal chromaffin cells, and thus prevents catecholamine release.

The key role of Ca^{2+} in stimulus-secretion coupling in adrenal medulla was first recognized by Douglas and Rubin [1, 2]. Catecholamines are released from adrenal chromaffin cells by a Ca^{2+}-dependent exocytotic process and the Ca^{2+}channels play an important role for the entry of extracellular Ca^{2+} into the cells [3, 4]. Recently several investigators have reported that Ca^{2+} channel antagonists (D-600, verapamil, diltiazem) or calmodulin antagonists (trifluoperazine, pimozide, W-7*) inhibit the catecholamine release by blocking the cellular Ca^{2+} uptake in adrenal chromaffin cells [5–8]. However, little is known concerning the effects of putative intracellular Ca^{2+} antagonists, such as TMB-8 and dantrolene sodium, in these cells. Therefore, in the present study we examined the effects of TMB-8 and dantrolene sodium on catecholamine release and $^{45}Ca^{2+}$ uptake evoked by carbamylcholine or high K^+ using cultured bovine adrenal chromaffin cells.

MATERIALS AND MATERIALS

Materials. The following materials were obtained from the companies indicated: $^{45}CaCl_2$ (32 Ci/g), New England Nuclear, Boston, MA; TMB-8, Aldrich Chemical Company, Milwaukee, WI; dantrolene sodium, Yamanouchi Pharm. Co, Ltd., Tokyo, Japan; carbamylcholine chloride, *d*-tubocurarine chloride, collagenase type I, 5-fluorodeoxyuridine, cytosine arabinoside and uridine, Sigma Chemical Company, St. Louis, MO; Dulbecco's Modified Eagle's Medium, mycostatin and fetal calf serum, Gibco, Grand Island, NY; penicillin G, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; streptomycin sulfate, Meijiseika Kaisha, Ltd., Tokyo, Japan; gentamicin sulfate, Schering Co., U.S.A., Kenilworth, NJ.

Primary culture of adrenal chromaffin cells. Fresh bovine adrenal glands were obtained from a local slaughterhouse, and chromaffin cells were isolated by retrograde perfusion with 0.025% collagenase as described previously [8, 9]. The purified chromaffin cells were cultured by the method described by Kilpatrick *et al.* [3] with a slight modification. The cells were plated on 16 mm dia wells of a 4-well plastic multidish at a concentration of 3–6 × 10^5 cells/ml in Dulbecco's modified Eagle's Medium, supplemented with 10% fetal calf serum, and were cultured at 37° in an atmosphere of 95% air/5% CO_2. The culture medium contained the following antibiotics: penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), gentamicin sulfate (40 μg/ml) and mycostatin (25 units/ml). The medium also contained fluorodeoxyuridine (10 μM), cytosine arabinoside (10 μM) and uridine (5 μM) to prevent the proliferation of non-neuronal cells, and was replaced every 3 to 4

* Abbreviations: TMB-8, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride; dantrolene sodium, 1-(5-(*p*-nitrophenyl)-furfurylidene-amino) hydantoin sodium hydrate; D-600, methoxyverapamil; W-7, *N*-(6 - aminohexyl) - 5 - chloro - 1 - naphthalenesulfonamide; Hepes, 4 - (2 - hydroxyethyl) - 1 - piperazineethanesulfonic acid.

days. Cell viability estimated by a trypan blue exclusion test was above 95% after 10 days of culture. The cells were used for experiments between 4 and 10 days of culture.

Catecholamine release. Cultured cells were first washed with Locke's solution of the following composition: 154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5.0 mM Hepes (pH 7.5) and were incubated with 0.5 ml of the same solution. To determine the effects of drugs the cells were preincubated at 37° for 5 min with different concentrations of each drug. After preincubation, carbamylcholine (final concentration: 0.3 mM) or KCl (final concentration: 56 mM) was added to the medium and the incubation was continued for another 2 min to measure catecholamine release. Cellular catecholamines (noradrenaline plus adrenaline) and released catecholamines (noradrenaline plus adrenaline) in the medium were extracted with 0.4 N perchloric acid and analyzed by a high performance liquid chromatography unit (Waters Assoc., Milford, MA) equipped with an electrochemical detector (Bioanalytical Systems Inc., W. Lafayette, IN) [10].

$^{45}Ca^{2+}$ *uptake.* After preincubation of the cells in 0.45 ml Locke's solution in the presence and absence of drugs at 37° for 5 min, a 0.05 ml buffer containing $^{45}CaCl_2$ (1 μCi/well) and each secretagogue was added to each well. After 1 min, the radioactive medium was removed to stop the uptake, and the cells were immediately washed 4 times with the ice-cold buffer. The cells were solubilized in 1% Triton X-100 solution in water for 30 min and the radioactivity was determined.

$^{45}Ca^{2+}$ *efflux.* Cells were preincubated for 20 hr in a 0.5 ml of culture medium containing $^{45}CaCl_2$ (2 μCi/well). The cells were rapidly washed 4 times with Locke's solution and were used as $^{45}Ca^{2+}$-loaded cells. Incubation was carried out at 37° with 0.5 ml of Locke's solution containing either carbamylcholine or carbamylcholine plus each Ca^{2+} antagonist for the indicated time periods. After incubation, the medium was transferred to a plastic test tube in ice and centrifuged at 1000 *g* for 15 min. The radioactivity in the supernatant was determined.

RESULTS

Figure 1 shows the effects of various concentrations of TMB-8 and dantrolene sodium on catecholamine release from cultured adrenal chromaffin cells. When the cells were preincubated with TMB-8 for 5 min, the carbamylcholine-evoked catecholamine release was inhibited in a concentration-dependent manner. The apparent IC_{50} value for the inhibition was 1 μM. Although TMB-8 also inhibited the high K^+-evoked catecholamine release, approximately 100-fold higher concentrations of TMB-8 were required to inhibit the release. On the other hand, dantrolene sodium did not show an obvious inhibitory effect on catecholamine release evoked either by carbamylcholine or high K^+ (Fig. 1). Both TMB-8 and dantrolene sodium, at the concentrations which we used, failed to affect the spontaneous release of catecholamines (data not shown).

Figure 2 shows the effects of TMB-8 and dan-

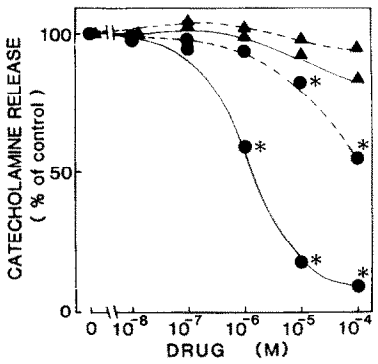

Fig. 1. Effects of TMB-8 and dantrolene sodium on the carbamylcholine- or the high K^+-evoked catecholamine release from cultured adrenal chromaffin cells. The cells were preincubated with various concentrations of TMB-8 or dantrolene sodium for 5 min. After preincubation, the cells were stimulated by carbamylcholine (0.3 mM) or high K^+ (56 mM) for 2 min, and the amount of released catecholamine was 15.2 ± 0.6% and 9.2 ± 0.7% of the total catecholamine content, respectively. The spontaneous release was 3.6 ± 0.4% of the total content. Each stimulant-evoked fraction of catecholamine release in the presence of an inhibitor is expressed as a percentage of the release determined in the absence of an inhibitor. The data for carbamylcholine-evoked (———) and high K^+-evoked (– – –) catecholamine release represent the means of triplicate determinations. Typical data obtained from two or three different cell preparations are presented. Symbols: (●), + TMB-8; (▲), + dantrolene sodium. *, $P < 0.01$ vs control value (no inhibitor).

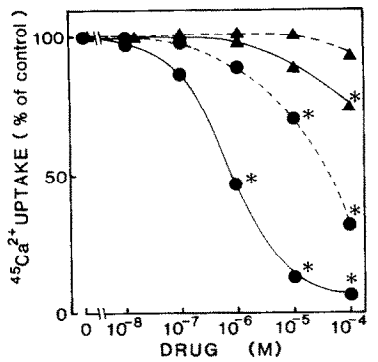

Fig. 2. Effects of TMB-8 and dantrolene sodium on the carbamylcholine- or the high K^+-evoked $^{45}Ca^{2+}$ uptake in cultured adrenal chromaffin cells. The cells were preincubated with various concentrations of TMB-8 or dantrolene sodium for 5 min. After preincubation, the cells were stimulated by carbamylcholine (0.3 mM) or high K^+ (56 mM) for 1 min. $^{45}Ca^{2+}$ in the cells was extracted with 1% Triton X-100 solution and was counted by a liquid scintillation counter. Each stimulant-evoked fraction of $^{45}Ca^{2+}$ uptake in the presence of an inhibitor is expressed as a percentage of the uptake determined in the absence of an inhibitor. The data for carbamylcholine-evoked $^{45}Ca^{2+}$ uptake (———) and high K^+-evoked $^{45}Ca^{2+}$ uptake (— — —) represent the means of triplicate determinations. Typical data obtained from two or three different cell preparations are presented. Symbols: (●), + TMB-8; (▲), + dantrolene sodium. *, $P < 0.01$ vs control value (no inhibitor).

trolene sodium on the $^{45}Ca^{2+}$ uptake of cultured adrenal chromaffin cells. TMB-8 inhibited the uptake of $^{45}Ca^{2+}$ stimulated either by carbamylcholine or high K^+ at the concentrations similar to those inhibiting the secretion of catecholamines. Dantrolene sodium slightly but significantly reduced the carbamylcholine-evoked $^{45}Ca^{2+}$ uptake at the highest concentration tested, i.e. 100 μM. The high K^+-evoked $^{45}Ca^{2+}$ uptake was not affected by dantrolene sodium. TMB-8 and dantrolene sodium showed no effect on the basal $^{45}Ca^{2+}$ uptake at the concentrations which we tested (data not shown). These results suggest that the inhibitory effect of TMB-8 on catecholamine release is mainly due to its effect on the process of Ca^{2+} uptake.

Figure 3 shows the differential actions of TMB-8 and verapamil on the carbamylcholine-evoked catecholamine release in the presence of various concentrations of extracellular Ca^{2+}. Verapamil (3 μM), a well-known Ca^{2+} channel blocker [11], inhibited carbamylcholine-evoked catecholamine release significantly. The inhibitory effect of verapamil was overcome by an increase in extracellular Ca^{2+} concentration, indicating that the inhibitory effect of verapamil is competitively antagonized by Ca^{2+}. The results were consistent with previous reports [8]. On the contrary, the inhibitory effect of TMB-8 was not overcome by Ca^{2+}.

In order to investigate the type of inhibition caused by TMB-8, effects of changes in the carbamylcholine concentrations on the inhibitory effects of TMB-8 were studied. As shown in Fig. 4, the inhibitory effects of *d*-tubocurarine on the carbamylcholine-evoked catecholamine release and $^{45}Ca^{2+}$ uptake were overcome by an increase in the carbamylcholine concentrations, indicating that the inhibitory effects of *d*-tubocurarine are competitively antagonized by carbamylcholine. On the other hand, the inhibitory effects of TMB-8 were not overcome by an increase in the carbamylcholine concentrations, suggesting that the inhibitory effects of TMB-8 are not due to the competitive antagonism at the nicotinic receptor site.

Since TMB-8 blocked $^{45}Ca^{2+}$ uptake, the effects of TMB-8 and dantrolene sodium on the efflux of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$ preloaded cells were examined. As shown in Fig. 5, 0.3 mM carbamylcholine significantly increased the $^{45}Ca^{2+}$ efflux from the cells. Although TMB-8 (10 μM) did not affect the spontaneous $^{45}Ca^{2+}$ efflux, the carbamylcholine-evoked increase in $^{45}Ca^{2+}$ efflux was completely inhibited by this agent. On the contrary, dantrolene sodium showed no effect on either spontaneous or carbamylcholine-evoked $^{45}Ca^{2+}$ efflux.

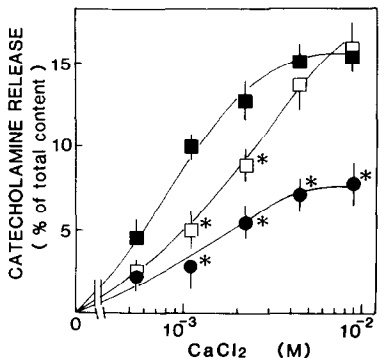

Fig. 3. Effects of various concentrations of Ca^{2+} on the inhibition of carbamylcholine-evoked catecholamine release by TMB-8 and verapamil. The cells were preincubated with TMB-8 (3 μM) or verapamil (3 μM) for 5 min. After preincubation, cells were stimulated by carbamylcholine (0.3 mM) for 2 min in the presence of various concentrations of Ca^{2+}. The spontaneous release (3.5% of the total cellular content) was subtracted from secretion data. The values represent the mean ± S.E. of triplicate determinations. Typical data obtained from two different cell preparations are presented. Symbols: (■), carbamylcholine alone; (●), + TMB-8; (□), + verapamil. *, $P < 0.01$ vs (■).

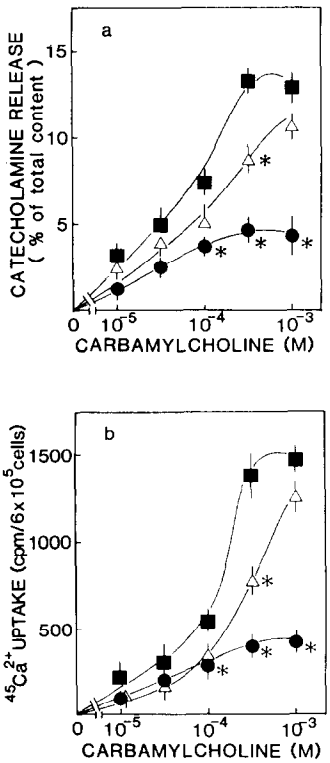

Fig. 4. Effects of changes in carbamylcholine concentrations on the inhibitory effects of TMB-8 and *d*-tubocurarine on carbamylcholine-evoked catecholamine release (a) and $^{45}Ca^{2+}$ uptake (b) in cultured adrenal chromaffin cells. (a) Cells were preincubated with TMB-8 (3 μM) or *d*-tubocurarine (0.75 μM) for 5 min. After preincubation, the cells were stimulated by various concentrations of carbamylcholine for 2 min. The spontaneous release (3.3% of the total cellular content) was subtracted from secretion data. (b) Cells were preincubated with TMB-8 (3 μM) or *d*-tubocurarine (0.75 μM) for 5 min. After preincubation, the cells were stimulated by various concentrations of carbamylcholine for 1 min. The basal $^{45}Ca^{2+}$ uptake (the mean value of triplicate determinations was 408 cpm/6×10^5 cells) was subtracted from the data. Each of these data represents the mean ± S.E. of triplicate determinations. Typical data obtained from two different cell preparations are presented. Symbols: (■), carbamylcholine alone; (●), + TMB-8; (△), + *d*-tubocurarine. *, $P < 0.01$ vs (■).

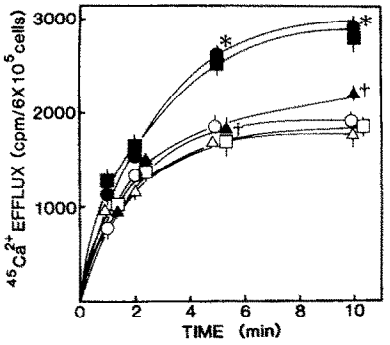

Fig. 5. Effects of TMB-8 and dantrolene sodium on $^{45}Ca^{2+}$-efflux from cultured adrenal chromaffin cells. The $^{45}Ca^{2+}$-loaded cells were incubated for the indicated time periods with carbamylcholine (0.3 mM) alone, carbamylcholine plus TMB-8 (10 μM) or carbamylcholine plus dantrolene sodium (10 μM). After incubation, the radioactivity released into the medium was determined. Solid and open symbols represent the carbamylcholine-evoked and basal $^{45}Ca^{2+}$ efflux, respectively. The values represent the mean ± S.E. of triplicate determinations. Typical data obtained from two different cell preparations are presented. Symbols: (○, ●), None; (△, ▲), + TMB-8; (□, ■), + dantrolene sodium. *, $P < 0.01$ vs (○). †, $P < 0.01$ vs (●).

DISCUSSION

According to Chiou and Malagodi, TMB-8 is a potent inhibitor of skeletal and smooth muscle contraction, and is capable of inhibiting caffeine-induced $^{45}Ca^{2+}$ release from skeletal muscle sarcoplasmic reticulum [12]. They suggested that TMB-8 exerts its inhibitory action by interfering with the release of Ca^{2+} from the intracellular store sites. Dantrolene sodium also has muscle relaxant properties and depresses excitation–contraction coupling without interfering with either neuromuscular transmission or conduction of the muscle action potential [13, 14]. In general, these compounds have been used as intracellular Ca^{2+} antagonists in various types of cells [12–17].

In this study, we examined the effects of these two compounds on catecholamine release, $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux in cultured adrenal chromaffin cells. Our present results clearly show that TMB-8 inhibits the carbamylcholine-evoked catecholamine release and $^{45}Ca^{2+}$ uptake. The concentrations of TMB-8 required to inhibit catecholamine release and $^{45}Ca^{2+}$ uptake were similar.

These results suggest that the inhibitory effects of TMB-8 on carbamylcholine-evoked catecholamine release are due to its inhibitory action on the process of Ca^{2+} uptake. TMB-8 also inhibited the carbamylcholine-stimulated $^{45}Ca^{2+}$ efflux (Fig. 5). In adrenal chromaffin cells, it has been reported that Ca^{2+} efflux is stimulated by muscarinic agents [18, 19]. Moreover, Tennes *et al.* reported that TMB-8 inhibits the binding of a muscarinic agonist ([^{3}H] QNB) to its receptor of mouse pancreas [20]. Therefore, it seems possible that the inhibitory effect of TMB-8 on the carbamylcholine-stimulated $^{45}Ca^{2+}$ efflux is due to the antagonism at the muscarinic receptor site. It seems possible that TMB-8 may also interact with the nicotinic receptor site. However, the inhibitory effects of TMB-8 on carbamylcholine-evoked catecholamine release and $^{45}Ca^{2+}$ uptake were not overcome by an increase in the carbamylcholine concentration, indicating that the inhibitory effects of TMB-8 are not due to the competitive antagonism at the nicotinic receptor site.

The inhibitory effects of TMB-8 were not antagonized by an increase in the extracellular Ca^{2+} concentration. The result clearly shows that the mechanism of inhibition of catecholamine release by TMB-8 is different from that by verapamil.

Although TMB-8 inhibits high K^+-evoked $^{45}Ca^{2+}$ uptake and catecholamine release by similar potency, the concentrations of TMB-8 required to inhibit high K^+-evoked events were higher than those required to inhibit the carbamylcholine-evoked events. It has been suggested that the voltage-sensitive Ca^{2+} channel and the acetylcholine receptor-coupled Ca^{2+} channel may play important roles for the entry of Ca^{2+} in adrenal chromaffin cells [21]. In line with this hypothesis, TMB-8 may block the Ca^{2+} uptake through the acetylcholine receptor-coupled Ca^{2+} channel with higher potency than the uptake through the voltage-sensitive Ca^{2+} channel.

On the other hand, dantrolene sodium did not show obvious inhibitory effects on catecholamine release and $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux in chromaffin cells. This result is consistent with the opinion that dantrolene sodium is an intracellular Ca^{2+} antagonist [13, 14].

It has been reported that caffeine (40 mM or above) and theophylline (10 mM) induce the catecholamine release from perfused bovine adrenal glands [22, 23] and also from rat adrenal slices in Ca^{2+} free medium [24], and it has been suggested that the catecholamine releasing effects of the above two methylxanthine derivatives were probably due to the Ca^{2+} mobilizing action of these agents from the intracellular Ca^{2+} store sites. In cultural adrenal chromaffin cells, however, theophylline (5 mM) failed to induce catecholamine release in Ca^{2+} free medium (data not shown). When we use the higher concentrations of theophylline (10 mM), the cell morphology is obviously altered (shrunken) by 3 min incubation. Therefore, we could not examine the effects of TMB-8 on the intracellular Ca^{2+}-mediated catecholamine release evoked by theophylline.

In summary, our present results clearly demonstrate that a putative intracellular Ca^{2+} antagonist, TMB-8, affects the transmembrane Ca^{2+} flux and/or the plasma membrane associated Ca^{2+} binding, and therefore inhibits the catecholamine release.

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