Arch. Pharm. Res. **15**(2), 115-125 (1992)

Influence of TMB-8 on Secretion of Catecholamines from the Perfused Rat Adrenal Glands

Dong-Yoon Lim, Chong-Dae Kim and Gi-Wan Ahn
College of Medicine, Chosun University, Kwang Ju 501-759, Korea
(Received January 20, 1992)

Abstract □ An attempt was made to investigate the effect of TMB-8 [3,4,5-trimethoxybenzoate-8 (N,N-diethylamino) octyl ester], which is known to be an inhibitor of intracellular Ca^{2+} release, on catecholamines (CA) secretion evoked by Ach, excess K^+, DMPP, McN-A-343 and caffeine from the isolated perfused rat adrenal glands and to clearify its mechanism of action.

The pretreatment with a low dose of TMB-8 (10 μM) for 20 min led to marked inhibition in CA secretion evoked by Ach (5.32 mM), excess K^+ (56 mM), DMPP (100 μM), McN-A-343 (100 μM) and BAY-K 8644 (10^{-5}M). Caffeine-induced CA secretion was similar to that of control only during the first periods (0-3 min) but thereafter marked inhibition in CA secretion evoked by caffeine was observed during the rest periods up to 30 min. The increased moderate concentration of TMB-8 (30 μM) caused the result similar to that of 10 μM TMB-8. However, in adrenal glands preloaded with a high dose of TMB-8 (100 μM), CA releases evoked by Ach, excess K^+, DMPP, McN-A-343 and caffeine were almost completely blocked by the drug.

These experimental data demonstrate that TMB-8 may inhibit cholinergic receptor-mediated and also depolarization-dependent CA secretion, suggesting that these TMB-8 effects seem to be mediated through inhibiting influx of extracellular calcium into the rat adrenal medullary chromaffin cells as well as reducing the release of calcium from intracellular sources.

Keywords □ TMB-8, catecholamines, adrenal gland, intracellular calcium

It has been shown that 3,4,5-trimethoxybenzoate 8-(N,N-diethylamino) octyl ester (TMB-8), a benzoic acid derivative, appears to act by preventing mobilization of calcium from intracellular stores without altering Ca^{2+} influx into stores[1-5]. TMB-8 is also known to inhibit caffeine-induced $^{45}Ca^{2+}$ release from, but not the uptake of $^{45}Ca^{2+}$ by, a sarcoplasmic reticulum preparation of skeletal muscle[2] and it was also found to inhibit the Ach-induced increase in the concentration of intracellular Ca^{2+} in isolated bovine adrenal medullary cells[6], carbamylcholine-induced secretion of catecholamines (CA) and $^{45}Ca^{2+}$ uptake in cultured bovine adrenal chromaffin cells[7].

Caffeine is suggested to mobilize intracellular Ca^{2+} in the adrenal medullary cells just as it does in muscle, because caffeine stimulates CA secretion from perfused bovine adrenal glands[8] and perfused rat adrenal glands[9] in the absence of extracellular Ca^{2+} as effectively as in the presence of Ca^{2+}. This secretory effect of CA evoked by caffeine is found to be inhibited by TMB-8 from the perfused cat adrenal gland in the absence of extracellular Ca^{2+}[10]. Some investigators reported that muscarinic, but not nicotinic, receptor activation caused CA secretion independent of extracellular Ca^{2+} in perfused adrenal glands of the cat[11] and guinea pig[12], suggesting that the presence of an intracellular Ca^{2+} pool is linked to a muscarinic receptor.

Recently, Misbahuddin and his colleagues (1985)[6] have suggested that in the bovine adrenal medullary cells stimulation of muscarinic Ach receptor causes

increase in intracellular calcium by mobilizing this ion from the intracellular pool and that protein kinase C is involved in "termination" or "down regulation" of this response. It was shown that TMB-8 decreased the ideal short-circuit current and increased active Na^+ and Cl^- absorption by increasing the mucosal-to-serosal Na^+ and Cl^- fluxes in rabbit ileum and did not alter cAMP-induced secretion, as judged by its lack of effect on the increase in short circuit current caused by 8-bromo-cAMP, and that it totally prevented the transport effect of carbachol, but did not inhibit the effects of secretion[13]. It is suggested that intracellular Ca^{2+} plays a role in regulation of basal ileal Na^+ and Cl^- transport but not in cAMP-induced secretion, and that there appears to be several pools of intracellular Ca^{2+} involved in neurohumoral effects on active electolyte transport[13]. Recently, TMB-8 has been shown to suppress the renal blood flow responses to angiotensin II and arg-angiotensin II or arg-vasopressin, where as it does not affect the renal blood flow response to BAY-K 8644. Accordingly, it is considered that vasoconstriction induced by angiotensin II or arg-vasopressin is mediated both by the influx of Ca^{2+} through calcium channels and the release of Ca^{2+} from TMB-8-sensitive Ca^{2+} pools in *in vivo* dog kidney[14]. Malagodi and Chiou (1974)[15] have reported that TMB-8 inhibits stimulation-induced responses in smooth muscle by interfering with the availability of Ca^{2+} for muscle contraction by blocking the Ca^{2+} release from intracellular bound stores.

In adrenal glomerulosa cells, although TMB-8 inhibits angiotensin II-stimulated aldosterone secretion almost completely, it does not suppress angiotensin II-induced $^{45}Ca^{2+}$ efflux from preloaded cells nor does it affect IP_3-induced calcium release from non-mitochondrial pool(s) in saponin-permeabilized cells. These findings indicate that TMB-8 inhibits aldosterone secretion without inhibiting mobilization of Ca^{2+} from an intracellular pool and that this inhibitory effect of it is due largely to an inhibition of plasma membrane calcium influx, but this drug also inhibits the activity of protein kinase C directly[16]. Moreover, recent evidences show that TMB-8 has no effect on renal vasoconstriction induced by the activation of voltage-dependent Ca^{2+} channels, and does not influence autoregulation of renal blood flow. Thus, Ca^{2+} release from intracellular stores does not appear to participate in autoregulatory process of renal blood flow[17].

Since there are many conflicting reports about the effect of TMB-8 in adrenal glands as well as in other preparations, the present experiment was designed to examine the influence of TMB-8 on CA secretion evoked by various secretogogues and to elucidate its mechanism of action, using isolated rat adrenal glands.

MATERIALS AND METHODS

Experimental animals

Mature male Sprague Dawley rats, weighing 180-250g, were anesthetized with ether. The adrenal gland was isolated by the method described previously[18]. The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by placing three hook retractors. The stomach, intestine and portions of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and canulations.

As shown in Fig. 1, cannula, used for perfusion of the adrenal gland(A), was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/m*l*) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite the entrace of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made on adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform on a leucite chamber. The chamber was continuously circulated with water heated at 37± 1℃(B).

Perfusion of adrenal gland

The isolated adrenal glands were perfused by means of a ISCO pump (WIZ Co.) at a rate of 0.4 m*l*/min. The perfusion was carried out with Krebs bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solu-

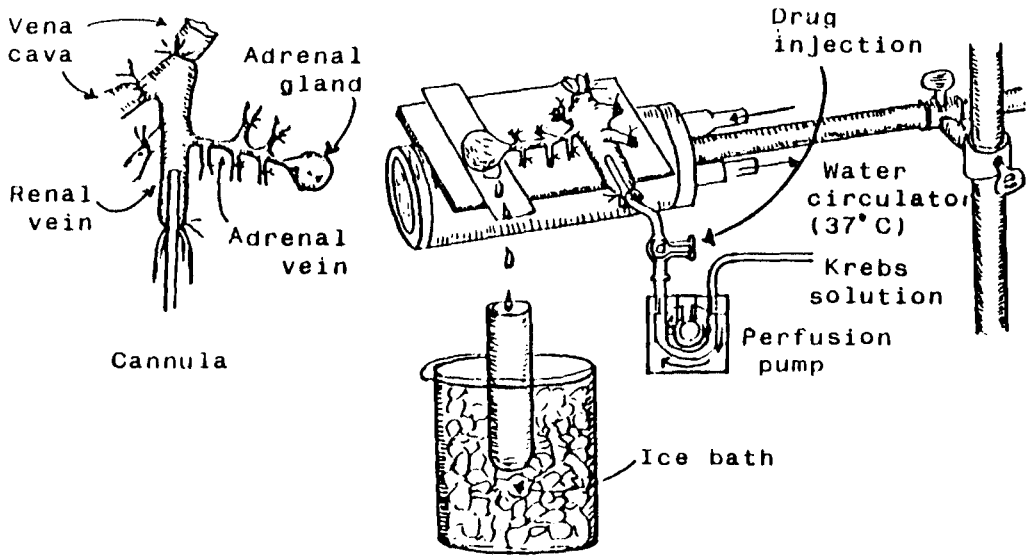

Fig. 1. Schematic drawing of the preparation used to study secretion of catecholamines in the isolated perfused rat adrenal gland.

tion was constantly bubbled with 95% O_2± 5% CO_2, and final pH of solution was maintained at 7.4± 0.5. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamines.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes and caffeine (0.3 mM) for 1 minute or a single injection of Ach (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock, and BAY-K 8644 (10^{-5}M) was also perfused for 4 min (Fig. 1).

In the preliminary experiments it was found that upon administration of the above drugs, secretory responses to Ach, KCl, McN-A-343 and BAY-K 8644 returned to preinjection level in about 4 min, but the responses to DMPP in 9 min. That to caffeine lasted more than 90 min. Generally, the adrenal glands perfusate was collected in chilled tubes. Details of the collection of samples are given in Result's section.

Collection of perfusate

As a rule, prior to each stimulation with cholinergic agonists perfusate samples were collected (4 min) to determine the spontaneous secretion of CA ("background sample"). Immediately after the collection of the "backgroud sample", collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Each perfusate was collected for 4 to 30 min. The amounts secreted in the "background sample" have been subtracted from those secreted from the "stimulated sample" to obtain the net secretion value of CA, which is shown in all of the figures. To study the effects of TMB-8 on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing TMB-8 for 20 min, then the perfusate was collected for a specific time period (background sample"), and then the medium was changed to the one containing the stimulating agent and the perfusates were collected for the same period as that for the "background sample".

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre (1962)[19] without the intermediated purfication alumina for the reasons described earlier[18], using fluorospectrophotometer (Shimadzu Co. Japan).

A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretogogues used in the

present work was high enough to obtain reading several fold greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

All data are presented as means with their standard errors, and the significance of differences were analyzed by Student's t-test using the computer system as previously described[20].

Drugs and their sources

The following drugs were used: 3,4,5-timethoxy benzoic acid-8-(diethylamino) octyl ester hydrochloride (TMB-8), acetylcholine chloride, 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, methyl-1,4-dihydro 2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K 8644) (Sigma Chemical Co., U.S.A.), (3-(m-chlorophenyl-carbamoyloxy) 2-butynyl trimetyl ammonium chloride [McN-A-343] (RBI, U.S.A.), caffeine citrated (Mallinckrodt Chemical Works, U.S. A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except BAY-K 8644. BAY-K 8644 was dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Influence of 10 μM TMB-8 on CA secretion evoked by Ach, excess K^+, McN-A-343 and BAY-K 8644 from the perfused rat adrenal glands

The spontaneous secretion of CA from the isolated perfused rat adrenal glands reached a steady state level about one hour after the perfusion with normal Krebs solution. When excess K^+ (56 mM) in a volume of 0.05 m*l* was injected into the perfusion stream via a three way stopcock, the amounts of CA secreted was 240.0± 22.26 μg for 4 min. However, after the preloading with 10 μM TMB-8 for 20 min, excess K^+-evoked CA secretion was markedly decreased to 113.4± 19.68 ($p<0.001$) for 4 min from 5 rat adrenal glands as shown Fig. 2.

Ach (5.32 mM)-evoked CA secretion following the perfusion with 10 μM TMB-8 for 20 min was greatly diminished to 34.7± 11.14 μg ($p<0.001$) as compared with the corresponding control secretion of 280.7± 33.04 μg for 4 min from 5 adrenal glands (Fig. 2.).

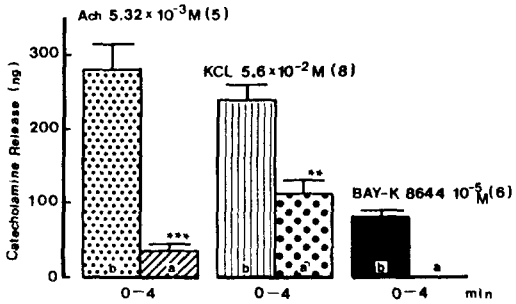

Fig. 2. Effect of 10 μM TMB-8 on secretion of catecholamines (CA) evoked by Ach, excess K^+ and BAY-K 8644 from the perfused rat adrenal glands.

CA secretion was evoked by single injection of Ach (5.32 mM) and excess K^+ (56 mM) or perfusion of BAY-A 8644 for 4 min after perfusion with normal Krebs for one hour and repeatedly by the same secretagogues after the pretreatment with TMB-8 (10 μM) for 20 min. Numerals in the parenthesis indicate number of experiments. Vertical bars represent S.E. of the mean. Ordinate: the amounts of CA secretion from the adrenal gland. Asbcissa: collecting time of perfusate. b and a indicate CA secretion evoked by secretogogues before (b) and after (a) pretreatment with TMB-8 (10 μM). **: $p<0.01$, ***: $p<0.001$.

CA secretion evoked by BAY-K 8644 (10^{-5}M), which is known to act as a calcium channel activator and to cause positive inotropy and vasoconstriction in isolated tissues and intact animals[21-23] was completely disappeared after the pretreatment with TMB-8 (10 μM) for 20 min but its control CA secretion before TMB-8 was 80.5± 7.50 μg for 4 min from 6 adrenal glands (Fig. 2).

Perfusion of adrenal glands with DMPP (100 μM) for 2 min, which is a selective nicotinic receptor in autonomic sympathetic ganglia, caused a rapid and great increase in CA secretion. As shown in Fig. 3 and Table I, DMPP-induced CA secretion before TMB-8 treatment was 245.0± 32.81(0-3 min) ng, 96.8± 11.45 (3-6 min) ng and 15.4± 2.16 (6-9 min) ng, respectively, while following the pre-

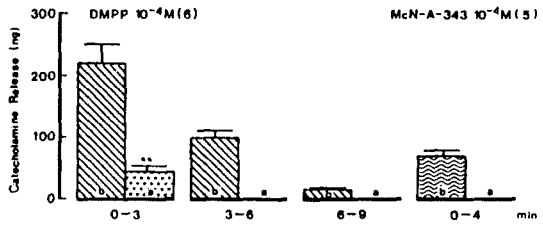

Fig. 3. Effect of 10 μM TMB-8 on CA secretion evoked by DMPP and McN-A-343 from the perfused rat adrenal glands.

DMPP (100 μM) and McN-A-343 (100 μM) were perfused into an adrenal vein before and after pretreatment with TMB-8 (10 μM) for 20 min, respectively. The perfusates for DMPP and McN-A-343 were collected for 9 and 4 min, respectively. There was no secretion of CA evoked by McN-A-343 in the presence of TMB-8 (10 μM). **: $p<0.01$.

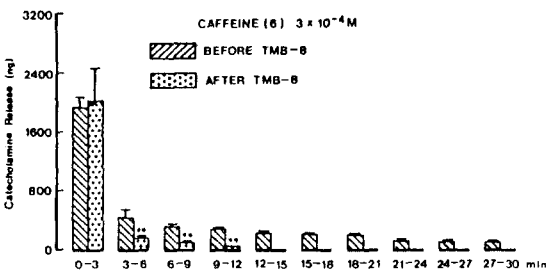

Fig. 4. Effect of 10 μM TMB-8 on caffeine-evoked CA secretion from the perfused rat adrenal glands.

Caffeine (0.3 mM) was perfused into an adrenal vein before and after pretreatment with TMB-8 (10 μM) for 20 min. The perfusate was collected for 30 min intervals. There was no secretion evoked by caffeine during 12-30 min periods after pretreatment with TMB-8. Other legends and methods are the same as in Figs. 2. and 3. **: $p<0.01$.

treatment with TMB-8 (10 μM), DMPP-evoked CA release was greatly reduced by 42.6± 8.15 (0-3 min, $p<0.01$) ng and completely disappeared during the 2nd and 3rd periods (3-9 min) from 6 rat adrenal glands.

McN-A-343 (100 μM), a selective M_1-muscarinic agonist[23], perfused into perfusion stream for 2 min produced the increase in CA secretion to 67.9± 11.6 ng for 4 min prior to perfusion with TMB-8. However, McN-A-343 (100 μM) did caused no secretion

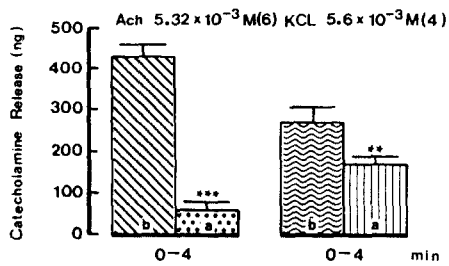

Fig. 5. Effect of 30 μM TMB-8 on CA release evoked by Ach or excess K^+ from the perfused rat adrenal glands.

Ach (5.32 mM) and KCl (56 mM) were infused into the perfusion in a volume of 0.05 ml before (b) and after (a) pretreatment with TMB-8 (30 μM). Other methods and legends are the same as in Fig. 2.

of CA in the presence of TMB (10 μM), as shown in Fig. 3.

Influence of 10 μM TMB-8 on CA secretion evoked by caffeine from the perfused rat adrenal gland

Caffeine is an alkaloid of methylxanthine derivatives, which is known to produce an increased CA secretion from the perfused cat adrenal glands[24,25], ox glands[26], the cultured bovine chromaffin cells[27,28] and the rat adrenal glands[9]. It is likely interesting to observe the effect of TMB-8 on caffeine-induced CA secretion.

In the present work, caffeine perfused into an adrenal vein produced a great and rapid increase in CA secretion which was maximal during the first periods (0-3 min of perfusate. The secretory effect of CA by caffeine lasted for more than 30 min as shown in Fig. 4. Caffeine (0.3 mM) perfused into the perfusion stream for 1 min enhanced greatly CA secretion to 1926.7± 158.23 (0-3 min) ng, 450.0± 143.05 (3-6 min) ng, 316.7± 24.31 (6-9 min) ng, 276.9 ± 19.19 (9-12 min) ng, 253.3± 17.60 (12-15 min) ng, 216.7± 22.36 (15-18 min) ng, 226.9± 24.31 (18-21 min) ng, 123.3± 23.48 (21-24 min) ng, 120.0± 26.33 (24-27 min) ng and 116.7± 24.39 (27-30 min) ng, respectively. However, after the preloading with 10 μM TMB-8 for 2 min, caffeine (0.3 mM)-evoked CA secretion was 2116.7± 449.45 (0-3 min, NS) ng without difference as compared with the corresponding control value but greatly decreased to 156.7± 9.74 (3-6 min, $p<0.01$) ng, 92.5± 14.05 (6-9 min, $p<$

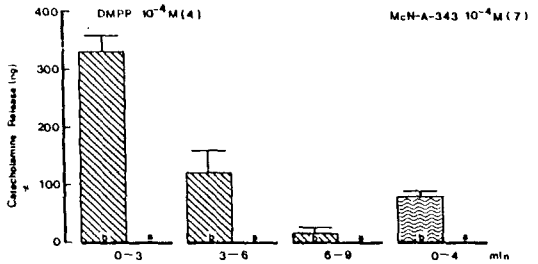

Fig. 6. Effect of 30 μM TMB on McN-A-343 and DMPP evoked CA secretion from the perfused rat glands.

There was no secretion evoked by McN-A-343 (100 μM) and DMPP (100 μM) after with TMB-8 (30 μM) for 20 min. Other legends and methods are as in Figs. 2 and 3.

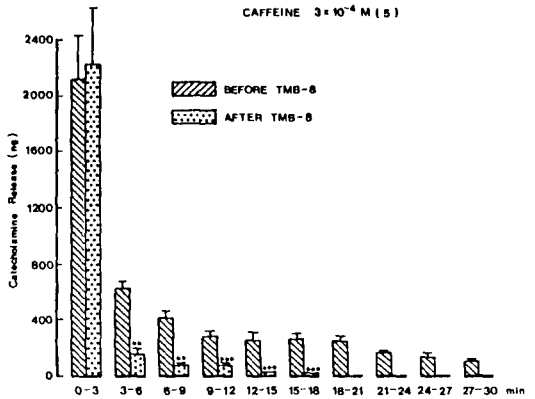

Fig. 7. Effect of 30 μM TMB-8 on caffeine (3×10^{-4}M)-induced CA secretion from the perfused rat adrenal glands.

Other legends and methods are the same as in Figs. 2 and 4. **: $p<0.01$, ***: $p<0.001$.

0.01) ng and 40.0± 11.73 (9-12 min, $p<0.01$) ng, respectively in comparison with the control value. In addition, there was no secretion of CA during 12-30 min periods following the pretreatment with TMB-8.

Influence of 30 μM TMB-8 on CA secretion evoked by Ach. excess K^+, DMPP, McN-A-343 and caffeine from the perfused rat adrenal glands

It was tried to examine the effect of more increased concentration of TMB-8 (30 μM) on various stimulation-evoked CA secretion from the rat adrenal glands.

In the present experiment, Ach (5.32 mM)- and excess K^+ (56 mM)-evoked CA releases prior to the preloading with TMB-8 (30 μM) were 428.9± 37.47 ng and 266.7± 37.21 ng for 4 min, respectively but following the perfusion of TMB-8 (30 μM) for 20 min their CA secretions were significantly depressed to 55.6± 18.6 ($n=6$, $p\pm0.001$) ng and 166.7 ± 18.48 ($n=4$, $p\pm0.01$) ng for 4 min, as compared with each corresponding control value was shown in Fig. 5.

DMPP (100 μM)-induced CA secretion was 330± 28.87 (0-3 min) ng, 117.6± 40.42 (3-6 min) ng and 15.1± 8.66 (6-9 min) ng, respectively before pretreatment with TMB-8 (30 μM), and McN-A-343 (100 μM)-evoked secretory effect of CA was also exerted to 76.3± 8.05 ng for 4 min before TMB-8 (30 μM). However, in the presence of 30 μM-TMB-8, McN-A-343 and DMPP induced secretory effects were clearly blocked.

Fig. 7 shows the effect of 30 μM TMB-8 on caffeine-induced CA release. Caffeine (0.3 mM) perfused into the perfusion stream for one min induced the marked CA secretion of 2106.3± 359.44 (0-3 min) ng, 618.2± 53.19 (3-6 min) ng, 403.5± 50.43 (6-9 min) ng, 273.5± 39.16 (9-12 min) ng, 290.9± 90.35 (12-15 min) ng, 260.1± 47.92 (15-18 min) ng, 253.6± 31.48 (18-21 min) ng, 160.5± 49.15 (21-24 min) ng, 131.2± 20.40 (24-27 min) ng and 109.8± 21.5 (27-30 min) ng, respectively. Perfusate for caffeine-induced CA release was collected only for 30 min at 3 min intervals through overall experiments. After preloaded with 30 μM TMB-8 for 20 min caffeine-induced CA release was 2272.5± 432.94 ng during first period (0-3 min) without statistical difference as compared with the corresponding control value. However, CA secretion during 3-18 min periods was greatly inhibited by 156.9± 46.82 (3-6 min, $p<0.001$) ng, 65.0± 12.96 (6-9 min, $p<0.01$) ng, 71.4± 16.33 (9-12 min, $p<0.001$) ng, 26.5± 5.40 (12-15 min, $p<0.001$) ng and 23.8± 4.95 (15-18 min, $p<0.001$) ng, respectively. Moreover, there was no release of CA during 18-30 min periods as shown in Fig. 7.

Influence of 100 μM TMB-8 on CA release evoked by Ach. excess K^+, DMPP, McN-A-343 and caffeine from the rat adrenal glands

In order to examine the does-dependent effect of TMB-8, 100 μM-concentration of TMB-8 as a

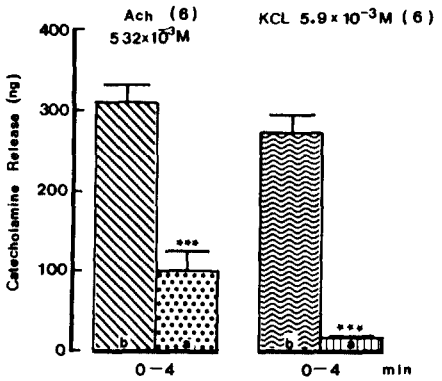

Fig. 8. Effect of 100 μM TMB-8 on Ach and K^+-evoked CA secretion from the perfused rat adrenal glands.
Other legends and methods are the same as in Fig. 2. ***: $p<0.001$.

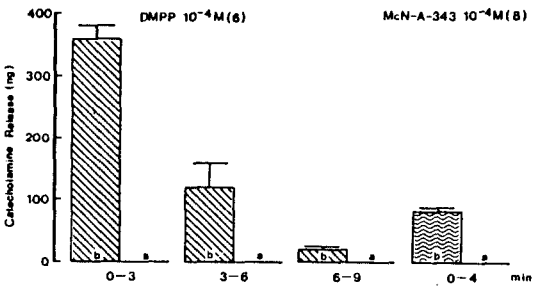

Fig. 9. Effect of 100 μM TMB-8 on CA secretion evoked by DMPP and McN-A-343 from the perfused rat adrenal glands.
Other legends and methods are the same as in Figs. 2 and 3.

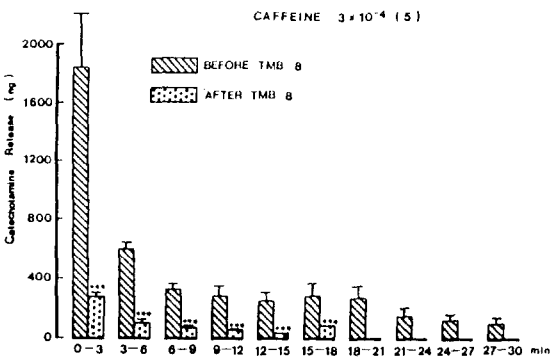

Fig. 10. Effect on 100 μM TMB-8 on caffeine-evoked CA secretion from the perfused rat adrenal glands.
Other methods and legends are the same as in Figs. 2 and 4.

maximal does in the present study was used. Prior to perfusion with 100 μM TMB-8 CA release evoked by a single injection of Ach (5.32 mM) and excess K^+ (56 mM) in a volume of 0.05 m*l* into the perfusion stream were 308.9± 21.34 ng and 271.1± 17.43 ng for 4 min, respectively, while they were markedly reduced to 17.78± 1.04 ($p<0.001$) ng and 142.2 ± 15.63 ($p<0.001$) ng for 4 min from 6 experiments after pretreatment with 100 μM TMB-8 as compared with each corresponding control secretion as shown in Fig. 8. DMPP (100 μM) and McN-A-343 (100 μM) perfused into an adrenal veir. for 2 min also increased CA secretion to 360.0± 41.15 (0-3 min, n= 6), 120.6± 12.65 (3-6 min, n=6) ng and 20.5± 5.17 (6-9 min, n=6) ng, and 79.4± 6.14 (0-4 min, n=8) ng, respectively. However, DMP as well as McN-A-343-evoked CA released were depicted in Fig. 9.

CA release evoked by caffeine (0-3 mM) infused into the perfusion stream for 1 min before TMB-8 (100 μM) was 184.0± 367.22 (0-3 min) ng, 582.5± 45.89 (3-6 min) ng, 371.5± 40.08 (6-9 min) ng, 262.5± 58.06 (9-12 min) ng, 240.0± 75.83 (12-15 min) ng, 282.5± 79.52 (15-18 min) ng, 262.5± 75.54 (18-21 min) ng, 155.0± 67.61 (21-24 min) ng, 115.0± 51.84 (24-27 min) ng and 97.5± 39.18 (27-30 min) ng, respectively as shown in Fig. 10. Interestingly, caffeine-induced CA secretion following the preloading with 100 μM TMB-8 for 20 min was greatly diminished to 272.5 ± 32.04 (0-3 min, $p<0.001$) ng even during the first period, 95.0± 24.06 (3-6 min, $p<0.001$) ng, 56.5± 12. 73 (6-9 min, $p<0.001$) ng, 50.6± 11.35 (9-12 min, $p<0.001$) ng and 25.0± 2.95 (12-15 min, $p<0.001$) ng, respectively, as compared with the corresponding control secretion. Moreover, there was no secretion of CA during 15-30 min periods after TMB-8 (100 μM) treatment. Caffeine-induced CA were obtained from 5 rat adrenal glands.

DISCUSSION

The present experimental results demonstrate that TMB-8 causes a dose-dependent inhibition of CA secretory responses evoked by Ach, excess K^+, DMPP, McN-A-343, BAY-K 8644 and caffeine in the isolated perfused rat adrenal glands. This effect of TMB-8 on various secretogogue-evoked CA secretions may be due to nonsepecific inhibition in

CA secretion from the adrenal gland in addition to be blockade of calcium release from intracellular pool.

In support of this idea, Yamada and his coworker (1988)[10] have reported that TMB-8 reduces CA output during resting state and blocks the secretory reponses to caffeine and acetylcholine almost completely, but reversibly. This is consistent with the result of the present work. Moreover, these findings confirm previous reports that muscarinic receptor activation causes an increase in CA secretion by releasing Ca^{2+} from intracellular pool[11,12] as caffeine does. In cultured bovine adrenal chromaffin cells, TMB-8 inhibited carbamylcholine-evoked CA secretion and $^{45}Ca^{2+}$ uptake[7]. In the present experiment it was found that TMB-8 blocked CA secretion evoked by DMPP, McN-A-343 and excess potassium, and even by AY-K 8644 in the presence of extracellular calcium in the perfused rat adrenal gland. These data suggest that TMB-8 functions as a Ca^{2+} channel blocker. Furthermore, Malagodi and Chiou (1974)[15] also introduced TMB-8 as a non-specific inhibitor of smooth muscle contration. TMB-8 was shown to inhibit Ach, norepinephrine-, excess potassium-and caffeine-evoked contraction. In a later subsequent study, Chiou and Malagodi (1975)[2] reported that TMB-8 attenuated caffeine-induced release of calcium from isolated sarcoplasmic reticulum preparations from skeletal muscle. More recently, TMB-8 is found to block greatly CA secretion evoked by caffeine in the perfused rat adrenal gland[9]. Previously, Yamada and his colleagues (1988)[10] have shown that caffeine is much more effective in releasing CA in the absence of extracellular Ca^{2+} than in its presence, but Lim and his coworkers (1991)[9] found that caffeine was almost equieffective in CA secretion both in the absence and in the presence of extracellular calcium, and that caffeine may cause CA secretion through the mobilization of calcium from an intracellular calcium pool in the chromaffin cells. Thus, the present finding that caffeine-evoked CA secretory effect was largely blocked by TMB-8 (100 μM) suggests that TMB-8 surely inhibits the calcium release from intracellular storage at least in the rat adrenal gland.

Wakade (1981)[18] has emphasized that chromaffin cells of the rat adrenal gland contain an intracellular store of calcium which participates in the secretion of CA as shown in the bovine adrenal gland[29].

Such a store may not be easily depleted by removal of extracellular calcium. Intracellular store of calcium has been shown to play some role in contraction of smooth muscle induced by norepinephrine or Ach in Ca^{2+} free medium[14,15,30,31].

Moreover, this fact is considerably supported by the finding that in the present work TMB-8 blocked CA secretion evoked by a M_1-muscarinic selective agonist McN-A-343. It is known that Ach and pilocarpine, but not nicotine, cause a partial increase in CA secretion from both guinea-pig adrenal glands[11] and perfused cat adrenal glands[12] with Ca^{2+}-free Lock solution. These responses to Ach and pilocarpine in the absence of extracellular calcium may be mediated by Ca^{2+} mobilized from an intracellular pool linked to muscarinic receptors.

Several groups have also found that the activation of muscarinic receptors increases cytoplasmic free Ca^{2+} in isolated bovine chromaffin cells, though there was no associated catecholamine secretion[32-34]. Recently, it has been demonstrated that muscarine-evoked CA secretion from perfused adrenal glands of the rat can occur in the absence of extracellular calcium[35]. In terms of the above findings, it is considered that neither voltage changes of chromaffin cell membrances nor extracellular Ca^{2+} are essential for muscarinic agonist-stimulated CA release from the rat adrenal gland as in the guinea-pig adrenal glands[12] as long as there is an intracellular source of calcium.

On the other hand, the present experimental data that TMB-8 inhibited DMPP-evoked CA secretio1n suggest that TMB-8 may possess Ca^{2+} entry blocking activity as aforementioned. In support of this evidence, it is well-known that activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} influx in both perfused rat adrenal glands[36] and isolated bovine adrenal chromaffin cells[37-39].

Moreover, in addition to its ability to block intracellular calcium release, TMB-8 greatly inhibited excess K^+-stimulated CA secretion in the present work. This observation is in agreement with previous reports, showing that TMB-8 suppresses calcium uptake in smooth muscle[2], adrenal meddula[7] and adrenal glomerulosa cells[36]. Likewise, the inhibition of excess K^+-evoked CA secretion by TMB-8 may be due to inhibition of calcium influx, because K^+-stimulated $^{45}Ca^{2+}$ influx in adrenal glo-

merulosa cells is known to be secondary to an increase in $^{45}Ca^{2+}$ influx and to be inhibited by removal of extracellular calcium[16].

Particulary, in the present study the fact that TMB-8 also blocked BAY-K 8644-evoked CA secretion from the perfused rat adrenal glands surely confirms the above evidence that TMB-8 has a calcium entry blocking activity in the rat adrenal gland. BAY-K 8644 is found to induce vasoconstriction by Ca^{2+} influx into the smooth muscle cells through voltage-dependent Ca^{2+} channels[41].

Thus, a major action of TMB-8 on cellular calcium metabolism may be to inhibit calcium influx at least at the concentration used in the present experiment.

Anyway, from the present experimental data it is felt that TMB-8 may inhibit calcium influx into the chromaffin cells as well as mobilization of calcium from an intracellular store into the cytosol.

Hence, more detailed interpretation of much published data obtained from using TMB-8 need to be deeply considered in the near future.

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