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Trichosporin-B-III, an α-Aminoisobutyric Acid-Containing Peptide, Causes Ca^{2+}-Dependent Catecholamine Secretion from Adrenal Medullary Chromaffin Cells

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SUMMARY

We examined the effect of trichosporin-B-III, an α-aminoisobutyric acid-containing antibiotic peptide consisting of 19 amino acid residues and a phenylalaninol, on catecholamine secretion from cultured bovine adrenal chromaffin cells. Incubation of the cells with trichosporin-B-III (3–20 μM) caused an increase in the secretion of catecholamines. The secretion induced by trichosporin-B-III at low concentrations (3 and 5 μM) was completely dependent on external Ca^{2+}, whereas that induced by higher concentrations (10 and 20 μM) was partly independent of Ca^{2+}. Trichosporin-B-III at low concentration (5 μM) did not increase the release of lactate dehydrogenase, a marker enzyme of cytoplasm, from the cells. In contrast, the peptide at higher concentration (10 μM) increased the release of the enzyme. Trichosporin-B-III also caused both $^{45}Ca^{2+}$ influx into the cells and an increase in the intracellular free Ca^{2+} concentration. The increases in catecholamine secretion and $^{45}Ca^{2+}$ influx behaved similarly in relation to trichosporin-B-III concentration (3–10 μM). The time courses of the increases in secretion, $^{45}Ca^{2+}$ influx, and intracellular free Ca^{2+} concentration induced by trichosporin-B-III were also quite similar. Trichosporin-B-III-induced (at 5 μM) secretion was not affected by the elimination of Na^+ from the incubation medium or by the addition of tetrodotoxin, a blocker of highly selective voltage-dependent Na^+ channels, or hexamethonium, a blocker of nicotinic acetylcholine receptors. On the other hand, both diltiazem (2–200 μM) and nicardipine (1–200 μM), blockers of voltage-dependent Ca^{2+} channels, inhibited the secretion induced by trichosporin-B-III (5 μM) in a concentration-dependent manner. Trichosporin-B-III-induced (at 5 μM) secretion also was suppressed by the addition of Mn^{2+} (5 mM) to the medium. The diltiazem (20 μM) inhibition of trichosporin-B-III-induced (at 5 μM) secretion was reversed by increasing the external Ca^{2+} concentration. These results indicate that trichosporin-B-III causes the secretion of catecholamines from bovine adrenal chromaffin cells by two mechanisms, Ca^{2+} dependent and Ca^{2+} independent (only at high concentrations of trichosporin-B-III). Furthermore, these results strongly suggest that trichosporin-B-III, in Ca^{2+}-dependent secretion, activates endogenous voltage-dependent Ca^{2+} channels, or itself forms the channels in the membranes, and induces Ca^{2+} influx into the cells.

The TS-Bs, which have been isolated from the culture broth of the fungus *Trichoderma polysporum* (1, 2), comprise 11 kinds of antibiotic peptides with similar amino acid sequences. Their fungal peptides consist of 19 amino acid residues and an amino alcohol, phenylalaninol, as a protecting group of the carboxyl-terminal residue. Of these antibiotic peptides, TS-B-III is a mixture of four peptides (III_a-III_d) having the following sequences (with the structure Ac-Aib-Ala-Ala-Ala-X-Aib-Gln-Aib-Y-Aib-Gly-Leu-Aib-Pro-Val-Aib-Z-Gln-Gln-Pheol): III_a, X = Aib, Y = Leu, Z = Aib; III_b, X = Aib, Y = Ile, Z = Ala; III_c, X = Ala, Y = Ile, Z = Aib; and III_d, X = Aib, Y = Val, Z = Aib (1, 2). Thus, TS-B-III contains a high proportion of Aib, an unusual amino acid, and belongs to the class of Aib-containing fungal peptides like alamethicins (3), hypelcins (4), suzukacillins (5), trichotoxins (6), and antiamoebins (7). Aib-containing natural peptides have been reported to result in formation of voltage-gated ion channels (8–11), hemolysis (12), fusion of lipid vesicles (13), and uncoupling of oxidative phosphorylation in mitochondria (14, 15). All the Aib-containing peptides show such membrane-modifying properties; TS-B-III has been found to uncouple the oxidative phosphorylation in rat liver mitochondria (1).

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ABBREVIATIONS: TS-B, trichosporin-B; Aib, α-aminoisobutyric acid; KRH, Krebs-Ringer-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; fura-2, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

Bovine adrenal medullary chromaffin cells can secrete catecholamines via stimulation of the nicotinic receptor by a physiological secretagogue, acetylcholine; binding of acetylcholine to the receptor leads to depolarization of the cell membrane due to an influx of Na^+ through receptor-operated Na^+ channels, causes an influx of Ca^{2+} through voltage-dependent and/or receptor-operated Ca^{2+} channels, and results in catecholamine secretion by exocytosis (16–19). In addition to receptor-operated Na^+ channels, voltage-dependent Na^+ channels are also present in the membrane of adrenal chromaffin cells (19). Veratridine, an activator of voltage-dependent Na^+ channels, causes the secretion of catecholamines via Na^+ influx through the channels, which are antagonized by tetrodotoxin, a selective inhibitor of the channels (20). Thus, ion channels play highly important roles in catecholamine secretion, and chromaffin cells can serve as a useful model for studying receptor- and ion channel-associated catecholamine secretion in adrenergic neurons.

In this study, therefore, we examined the effect of TS-B-III on catecholamine secretion from chromaffin cells. The results show that TS-B-III causes an increase in external Ca^{2+} influx into the cells and that catecholamine secretion is induced. The mechanism by which TS-B-III causes Ca^{2+} influx is also investigated.

Experimental Procedures

Materials. TS-B-III was isolated from *T. polysporum*, and the structures were identified by Fujita *et al.* (1) and Iida *et al.* (2). The mean of the molecular weights of four peptides (TS-B-III$_a$-III$_d$) was used to determine the peptide concentration. Oxygenated KRH buffer (pH 7.4) was used as incubation medium and was composed of 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 5.6 mM glucose, and 0.5% bovine serum albumin. In Na^+-free medium, NaCl was replaced with an isotonic concentration of sucrose (270 mM) and Tris·HCl buffer (pH 7.4), which was used instead of HEPES. Ca^{2+}-free medium was prepared by the elimination of Ca^{2+} and the addition of 0.2 mM EGTA. Tissue culture instruments were obtained from the Falcon Plastics Co. (Cockeysville, MD). Eagle's minimum essential medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum, sodium pyruvate, NADH, and acetylcholine were obtained from Nacarai Tesque Inc. (Kyoto, Japan). $^{45}CaCl_2$ (0.5–2.0 Ci/mmol) was from Amersham International Ltd. (Arlington Heights, IL). Fura-2/acetoxymethyl ester was purchased from Dojin Laboratory (Kumamoto, Japan). Saponin, veratridine, diltiazem, and nicardipine were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest grade from commercial sources.

Primary cell culture. Bovine adrenal glands were kindly provided by the Center of Iwate Livestock Industry. Adrenal chromaffin cells were isolated by collagenase digestion, using a method described previously (21). The isolated cells were immediately suspended in Eagle's minimum essential medium, containing 10% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.3 μg/ml amphotericin B, and were plated on 35-mm dishes, at a density of 2×10^6 cells/dish. The cells were maintained at 37° in a CO_2 incubator (95% air/5% CO_2) and were used for experiments after 4 days of culturing. A total of 2×10^6 cells contained 174 ± 13 nmol of catecholamines as epinephrine and norepinephrine.

Measurements of catecholamine secretion and $^{45}Ca^{2+}$ influx. The cultured chromaffin cells were washed twice with KRH buffer and then preincubated with KRH buffer for 10 min at 37°. They were washed once more with prewarmed KRH buffer and incubated with or without TS-B-III or other test agents for 5 min (21), except as otherwise described below. The reaction was terminated by transferring the incubation medium to tubes in an ice-cold bath. The catecholamines secreted into the medium were extracted with 0.4 M perchloric acid and adsorbed to aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method (22), using a fluorescence spectrophotometer (650-10S; Hitachi, Tokyo, Japan) at an excitation wavelength of 420 nm and an emission wavelength of 540 nm. At these wavelengths, epinephrine and norepinephrine showed the same fluorescence intensity. The amount of catecholamines secreted from the cells was expressed as percentage of total cellular catecholamines.

After preincubation of the cells with KRH buffer for 10 min, the cells were incubated with $^{45}Ca^{2+}$ (1 μCi) in 1.0 ml of the medium, in the presence or absence of TS-B-III, at the times indicated in the figures. The medium was removed, and the cells were immediately cooled on ice and washed three times with 2.0 ml of ice-cold Ca^{2+}-free KRH buffer. The cells were scraped and solubilized in 1.0 ml of 10% Triton X-100. Radioactivity was determined by liquid scintillation counting (LSC-900; Aloka, Tokyo, Japan) (21). The Ca^{2+} influx was expressed as nanomoles of Ca^{2+} per 2×10^6 cells.

Measurement of lactate dehydrogenase activity in the medium. After incubation of the cells for 5 min with or without the test agents, in KRH buffer, the incubation medium was measured for lactate dehydrogenase (EC 1.1.1.27; pyruvate transhydrogenase:L-lactate, NAD^+ oxidoreductase) activity. The enzyme activity in the medium and the cell lysates solubilized by 2% Triton X-100 was determined by the method of Pesce *et al.* (23). The incubation mixture (3.0 ml), in a cuvette, contained 100 mM potassium phosphate buffer (pH 7.0), 330 μM sodium pyruvate, 120 μM NADH, and the medium or the cell lysates (0.5 ml). A decrease in absorbance due to the oxidation of NADH at 25° was monitored at a wavelength of 340 nm, using a spectrophotometer (Obest-30; Nihon Bunko, Tokyo, Japan). Enzyme activity was linear within the range of protein concentrations used here and is defined as nanomoles of NADH oxidized per minute. The activity of lactate dehydrogenase released from the cells was expressed as a percentage of total cellular content. The total activity of lactate dehydrogenase in the cells was 918 ± 23 nmol/min/2×10^6 cells.

Measurement of $[Ca^{2+}]_i$. The isolated cells were cultured for 4 days

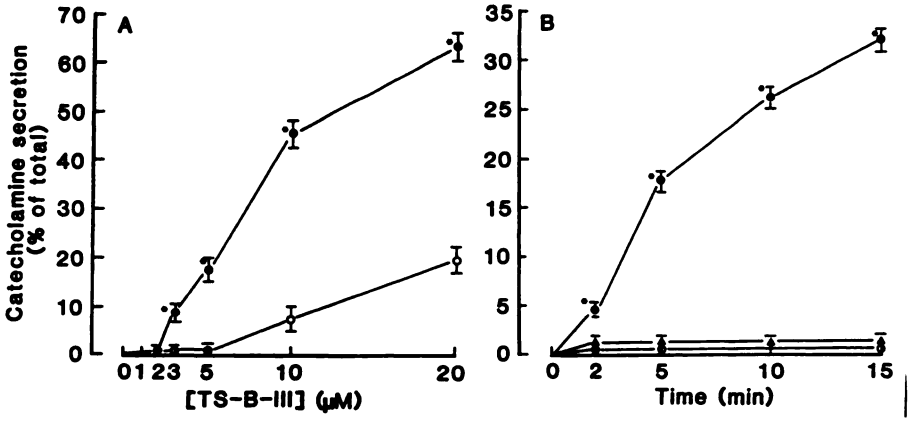

Fig. 1. Effect of TS-B-III on catecholamine secretion from adrenal chromaffin cells. A, The cells were incubated for 5 min at 37° with various concentrations of TS-B-III, in 2.6 mM Ca^{2+}-containing (●) or Ca^{2+}-free (plus 0.2 mM EGTA) medium (○). B, The cells were incubated with (●) or without (○) 5 μM TS-B-III in 2.6 mM Ca^{2+}-containing medium or with 5 μM TS-B-III in Ca^{2+}-free (plus 0.2 mM EGTA) medium (▲), for the times indicated. Catecholamines secreted from the cells were determined as described in Experimental Procedures. Catecholamine secretion is shown as a percentage of total cellular catecholamine content (174 ± 13 nmol). Data are means ± standard deviations from four experiments. *, $p < 0.001$, significantly different from control.

on coverslips cut to fit into the spectrofluorophotometer cuvette. The cultured cells on the coverslips were washed twice in serum-free culture medium and then incubated with 5 μM fura-2/acetoxymethyl ester in serum-free culture medium at 37°. After 40 min, the incubation medium was replaced with KRH buffer lacking bovine serum albumin. The coverslip with cells was washed three times with the serum-free KRH buffer and put in the cuvette. The cells in the cuvette were preincubated with the buffer at 37° for 10 min in the fluorescence meter, and then the test agents were added to the cuvette. Increases and decreases in the fluorescence induced by the fura-2-Ca^{2+} complex in the cells were simultaneously measured with a spectrofluorophotometer (CAF-100; Nihon Bunko), at an excitation wavelength of 340 nm and an emission wavelength of 500 nm and at an excitation wavelength of 380 nm and an emission wavelength of 500 nm, respectively. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (24).

Statistics. Statistical calculations were made according to the methods of Snedecor and Cochran (25). Differences were considered significant when p calculated by Student's t test was <0.05.

Results

Effect of TS-B-III on catecholamine secretion from chromaffin cells. When chromaffin cells were incubated for 5 min with TS-B-III in KRH buffer, catecholamines were secreted from the cells into the external medium (Fig.1A). TS-B-III caused the secretion of catecholamines from the cells in a concentration-dependent manner up to 20 μM, the highest concentration tested. A significant increase in secretion (9.2% of the total catecholamines in the cells, which corresponds to 16 nmol of catecholamines/2 × 10^6 cells) was observed with 3 μM TS-B-III. This amount of catecholamine secretion was comparable to that evoked by acetylcholine, a physiological secretagogue, at 30 μM, which was a half-maximal concentration. At 20 μM TS-B-III, the secretion reached 63.1%. The basal (spontaneous) secretion was 0.7% of the total catecholamines. When Ca^{2+} was eliminated from the external medium, the secretion induced by TS-B-III at concentrations of 3 and 5 μM was abolished. On the other hand, at higher concentrations (10 and 20 μM) of TS-B-III some secretion was observed even in the absence of external Ca^{2+}, although it was considerably less than with Ca^{2+} (Fig. 1A).

Fig. 1B shows the time courses of catecholamine secretion from the cells stimulated by TS-B-III at 5 μM, a low concentration. A significant increase in secretion (4.7% of the total catecholamines) was observed after 2 min of incubation with TS-B-III. The secretion induced by TS-B-III was approximately linear up to 5 min and continued to increase at a lower rate for at least 15 min. TS-B-III-induced (at 5 μM) secretion was completely dependent on the presence of external Ca^{2+}. The basal secretion was detectable after 2 min but did not change significantly during 15 min. There was little difference of potency in catecholamine secretion among the four peptides (III_a–III_d) in TS-B-III (data not shown).

Effect of TS-B-III on release of lactate dehydrogenase from the cells. The activity of lactate dehydrogenase, a cytoplasmic enzyme, in the incubation medium was measured to determine whether TS-B-III-induced catecholamine secretion represents only leakage of catecholamines from the cells. When the cells were incubated for 5 min with KRH buffer, 2.4% of the total activity of lactate dehydrogenase was released into the medium (Fig. 2). The exposure of the cells to TS-B-III at a low concentration (5 μM) did not affect the spontaneous release of lactate dehydrogenase. On the other hand, TS-B-III at a higher

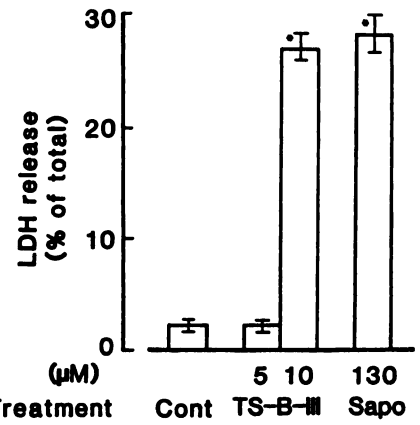

Fig. 2. Effects of TS-B-III and saponin (*Sapo*) on lactate dehydrogenase (*LDH*) release from the cells. The cells were incubated for 5 min at 37° with or without TS-B-III (5 or 10 μM) or saponin (130 μM), in KRH buffer. The activity of lactate dehydrogenase in the medium was determined as described in Experimental Procedures. Lactate dehydrogenase activity released was expressed as a percentage of total cellular content. Data are means ± standard deviations from four experiments. *, $p < 0.001$, significantly different from control.

concentration (10 μM) and saponin (130 μM), which was used as a detergent for disrupting the cell membranes, enhanced the release of the enzyme by 27.2 and 28.1%, respectively. These results indicate that TS-B-III at higher concentrations damages the plasma membrane.

Effect of TS-B-III on $^{45}Ca^{2+}$ influx into the cells. Catecholamine secretion from bovine adrenal chromaffin cells occurs by Ca^{2+} influx into the cells from the external medium (16, 18, 19). Fig. 3A shows the effects of different concentrations of TS-B-III on $^{45}Ca^{2+}$ influx into the cells. TS-B-III increased $^{45}Ca^{2+}$ influx in a concentration-dependent manner up to 10 μM. The significant Ca^{2+} influx that was observed with 3 μM TS-B-III amounted to 2.58 nmol/2 × 10^6 cells and peaked at 10 μM TS-B-III. We examined a correlation between the concentration-response curve for TS-B-III-induced catecholamine secretion (Fig. 1A) and that for TS-B-III-induced Ca^{2+} influx (Fig. 3A). As shown in Fig. 4A, there was a linear correlation between catecholamine secretion and Ca^{2+} influx ($r = 0.98$, $p < 0.005$). Thus, the concentration-response curves for TS-B-III-induced catecholamine secretion and $^{45}Ca^{2+}$ influx were quite similar, although 20 μM TS-B-III, compared with 10 μM TS-B-III, diminished $^{45}Ca^{2+}$ influx. This discrepancy between catecholamine secretion and $^{45}Ca^{2+}$ influx may be due to efflux of $^{45}Ca^{2+}$ from the cells damaged by TS-B-III at a higher concentration (20 μM).

$^{45}Ca^{2+}$ influx was dependent on the duration of exposure to TS-B-III (5 μM) (Fig. 3B). Enhancement of $^{45}Ca^{2+}$ influx by TS-B-III was detectable within 2 min and was approximately linear for up to 5 min of incubation. The $^{45}Ca^{2+}$ influx continued to increase at a lower rate for at least 15 min. As shown in Fig. 4B, the time courses of $^{45}Ca^{2+}$ influx and catecholamine secretion also correlated well ($r = 0.97$, $p < 0.005$).

Effects of external Ca^{2+} and Na^+ on Ca^{2+}-dependent catecholamine secretion induced by TS-B-III. Fig. 5 shows the effects of external Ca^{2+} concentrations on TS-B-III-induced (at 5 μM) catecholamine secretion. The secretion induced by TS-B-III was increased with increasing external Ca^{2+} concentrations from 0.16 to 10.4 mM. The secretion of catecholamines was detectable with 0.16 mM Ca^{2+} and attained a maximum at

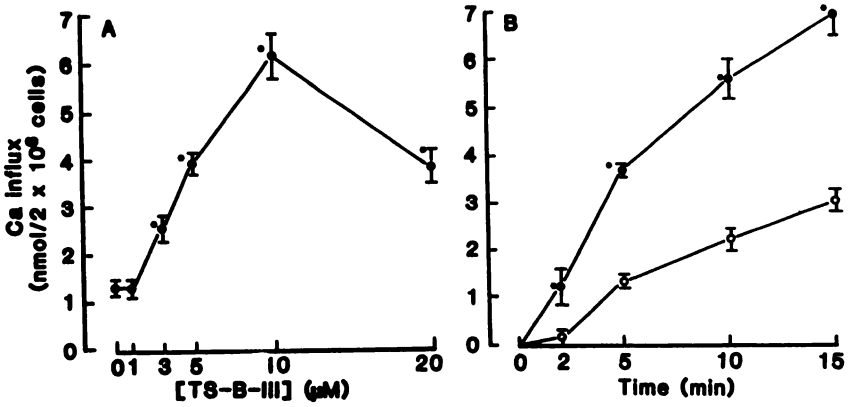

Fig. 3. Effect of TS-B-III on $^{45}Ca^{2+}$ influx into the cells. A, The cells were incubated for 5 min at 37° with various concentrations of TS-B-III, in KRH buffer containing 1 μCi of $^{45}CaCl_2$. B, The cells were incubated with (●) or without (○) 5 μM TS-B-III, in KRH buffer containing 1 μCi of $^{45}CaCl_2$, for the time indicated. Radioactivity in the cells was determined as described in Experimental Procedures. Data are means ± standard deviations from four experiments. *, $p < 0.02$, significantly different from control.

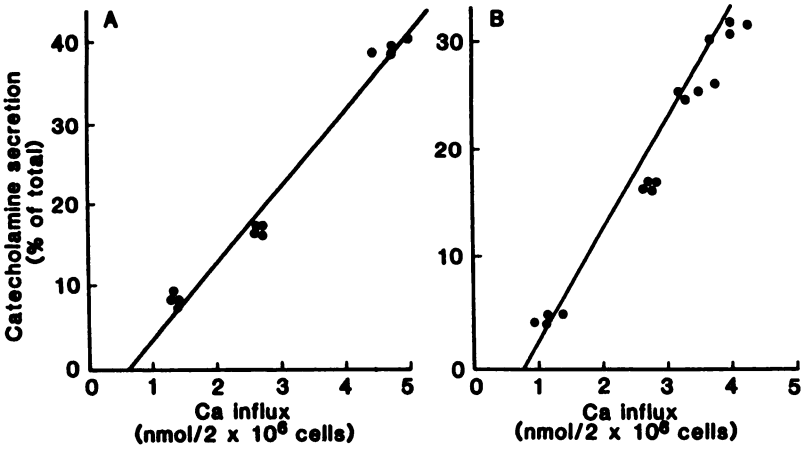

Fig. 4. A, Correlation between catecholamine secretion and $^{45}Ca^{2+}$ influx induced by different concentrations of TS-B-III. Ca^{2+}-free and basal values were subtracted from the data in Figs. 1A and 3A, respectively, excluding the data at 20 μM TS-B-III, and the values were plotted. Each *point* represents a value obtained from one culture dish. Regression equation is $y = 9.61x - 6.54$; $r = 0.98$, $t = 16.6$, and $p < 0.005$. B, Correlation between TS-B-III-induced catecholamine secretion and $^{45}Ca^{2+}$ influx time courses. Ca^{2+}-free and basal values were subtracted from the data in Figs. 1B and 3B, respectively, and the values were plotted. Each *point* represents a value obtained from one culture dish. Regression equation is $y = 9.89x - 8.27$; $t = 13.7$ and $p < 0.005$.

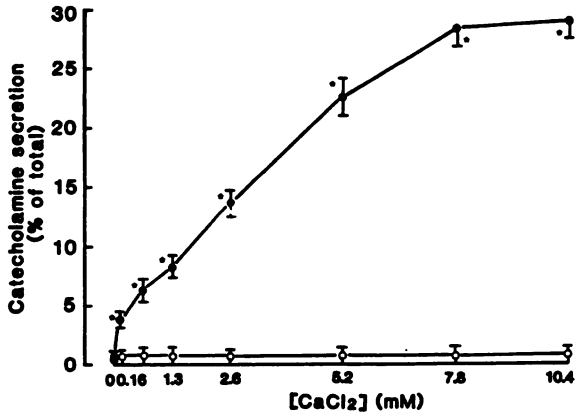

Fig. 5. Effects of various concentrations of external Ca^{2+} on catecholamine secretion induced by TS-B-III. The cells were incubated for 5 min at 37° with (●) or without (○) 5 μM TS-B-III, in Ca^{2+}-free (plus 0.2 mM EGTA) medium or various concentrations of Ca^{2+} medium. Catecholamines secreted from the cells were determined as described in Experimental Procedures. Data are means ± standard deviations from four experiments. *, $p < 0.01$, significantly different from control.

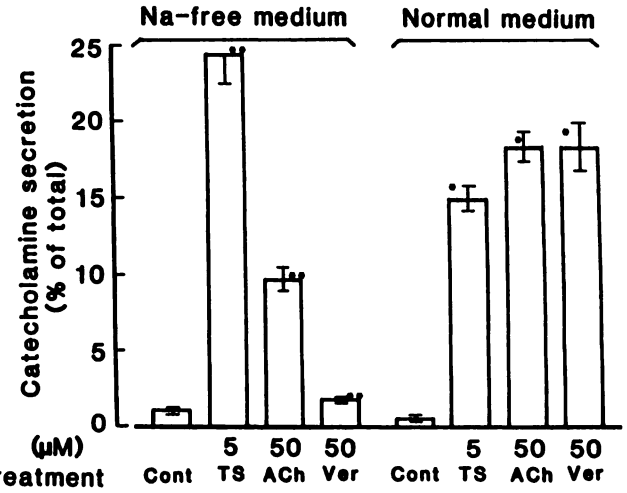

Fig. 6. Effects of external Na^+ on catecholamine secretion caused by TS-B-III (*TS*), acetylcholine (*ACh*), and veratridine (*Ver*). The cells were incubated for 5 min at 37° with 5 μM TS-B-III, 50 μM acetylcholine, or 50 μM veratridine, in Na^+-free sucrose or KRH (normal) medium. Catecholamines secreted from the cells were determined as described in Experimental Procedures. Data are means ± standard deviations from four experiments. *, $p < 0.005$, significantly different from control (*Cont*) in the normal medium; **, $p < 0.01$, significantly different from TS-B-III-, acetylcholine-, or veratridine-induced secretion in the normal medium.

approximately 7.8 mM Ca^{2+}. The basal secretion of catecholamines did not change significantly at external Ca^{2+} concentrations from 0 to 10.4 mM.

As shown in Fig. 6, in the absence of external Na^+, the secretion evoked by acetylcholine (50 μM) was greatly diminished, to 51% of the response in normal medium, and that evoked by veratridine (50 μM), a Na^+ ionophore that activates voltage-dependent Na^+ channels (20), was almost abolished. On the other hand, the secretion of catecholamines induced by TS-B-III (5 μM) was more enhanced in Na^+-free medium than in normal medium (Na^+-containing medium). The elimination of external Na^+ slightly increased the basal secretion.

Effects of Ca^{2+} channel blockers on TS-B-III-induced Ca^{2+}-dependent catecholamine secretion and increase in $[Ca^{2+}]_i$. Fig. 7 shows the effects of different concentrations of Ca^{2+} channel blockers [diltiazem and nicardipine, which block

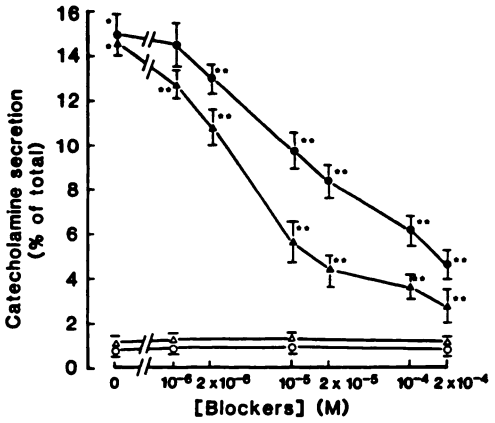

Fig. 7. Effects of diltiazem and nicardipine on catecholamine secretion induced by TS-B-III. The cells were preincubated for 10 min at 37° with various concentrations of diltiazem or nicardipine and then incubated for 5 min at 37° with various concentrations of diltiazem (●,○) or nicardipine (▲,△) in the presence (●, ▲) or absence (○, △) of 5 μM TS-B-III. Catecholamines secreted from the cells were determined as described in Experimental Procedures. Data are means ± standard deviations from four experiments. *, $p < 0.001$, significantly different from control; **, $p < 0.02$, significantly different from TS-B-III-induced secretion.

voltage-dependent Ca^{2+} channels (26–28)] on catecholamine secretion from chromaffin cells stimulated by TS-B-III (5 μM). Both diltiazem and nicardipine diminished TS-B-III-induced secretion in a concentration-dependent manner. The inhibitory effects of diltiazem and nicardipine were detectable with 2 and 1 μM, respectively, and about 50% inhibition was observed with 20 μM diltiazem and 5 μM nicardipine. The two blockers had no effect on the basal secretion.

Although the effects of Ca^{2+} channel blockers on TS-B-III-induced $^{45}Ca^{2+}$ influx into the cells were examined, the results of $^{45}Ca^{2+}$ influx were variable. TS-B-III-induced $^{45}Ca^{2+}$ influx was inhibited or stimulated by the blockers, at the same concentration, in experiments done several times on different days. Thus, no reproducible data were obtained. Therefore, we investigated the effects of TS-B-III on the change in $[Ca^{2+}]_i$ using fura-2. As shown in Fig. 8A, when the cells were incubated with TS-B-III (5 μM) $[Ca^{2+}]_i$ increased slowly, and the increase persisted for 8 min of incubation, the longest time tested. Omission of Ca^{2+} from the medium completely abolished the increase in $[Ca^{2+}]_i$ induced by TS-B-III (Fig. 8B), indicating that the TS-B-III-induced (at 5 μM) increase in $[Ca^{2+}]_i$ is caused by Ca^{2+} influx into the cells from the external medium. Diltiazem at 1 μM had little effect on the increase in $[Ca^{2+}]_i$ induced by TS-B-III (5 μM) but at 20 and 200 μM greatly reduced it (about 50 and 70% inhibition at 5 min of incubation) (Fig. 8, D–F). Nicardipine also inhibited the TS-B-III-induced increase in $[Ca^{2+}]_i$ more than diltiazem (data not shown). Acetylcholine (30 μM) and the Ca^{2+} ionophore ionomycin (1 μM) also resulted in an increase in $[Ca^{2+}]_i$ (Fig. 8, G and I). Diltiazem (200 μM) greatly suppressed the acetylcholine-induced increase in $[Ca^{2+}]_i$ but not the ionomycin-induced increase (Fig. 8, H and J). The stimulatory effect of TS-B-III (5 μM) on $[Ca^{2+}]_i$ was enhanced in Na^+-free medium more than in the normal medium (Fig. 8C), indicating that TS-B-III is able to cause Ca^{2+} influx into the cells even in the absence of external Na^+.

Effects of external Ca^{2+} concentrations on diltiazem inhibition of TS-B-III-induced Ca^{2+}-dependent catecholamine secretion. The inhibitory effects of diltiazem (20 μM) on TS-B-III-induced catecholamine secretion from chromaffin cells were examined under different concentrations of external Ca^{2+} (Fig. 9). Diltiazem (20 μM) inhibited the secretion by 52% in the presence of 2.6 mM Ca^{2+}. In the presence of 5.2 mM Ca^{2+} inhibition by diltiazem was 42%, and at 7.8 and 10.4 mM Ca^{2+} it was reduced to 34 and 17%, respectively. Thus, inhibition by diltiazem was overcome by increasing concentrations of external Ca^{2+}.

Properties of TS-B-III-induced Ca^{2+}-dependent catecholamine secretion. The properties of catecholamine secretion induced by TS-B-III (5 μM) were compared with those evoked by acetylcholine, ionomycin, and veratridine, using several inhibitors (Table 1). Hexamethonium (10 and 100 μM), a competitive blocker of nicotinic acetylcholine receptor (29), greatly antagonized the secretion of catecholamines evoked by acetylcholine (30 μM), but not by TS-B-III (5 μM). TS-B-III- and acetylcholine-induced secretion were inhibited by diltiazem (20 and 200 μM), but ionomycin-induced (at 1 μM) secretion was little affected by diltiazem. Tetrodotoxin (100 nM), a highly selective inhibitor of voltage-dependent Na^+ channels (20), almost abolished veratridine-induced secretion, whereas it did not suppress TS-B-III- and acetylcholine-induced secretion. Polyvalent cations (such as Co^{2+}, La^{3+}, Mn^{2+}, and Cd^{2+}) are well known to block Ca^{2+} currents via voltage-dependent Ca^{2+} channels in many excitable cells (28). The incubation of the cells with 5 mM Mn^{2+} greatly inhibited TS-B-III-, veratridine-, or ionomycin-induced catecholamine secretion. These inhibitors had little effect on basal secretion in nonstimulated cells.

Discussion

The data presented here show that TS-B-III alone (without the addition of other secretagogues) caused secretion of catecholamines from cultured bovine adrenal chromaffin cells.

Ca^{2+}-dependent and Ca^{2+}-independent secretion produced by TS-B-III. Incubation of chromaffin cells with TS-B-III for 5 min at 37° caused secretion of catecholamines from the cells. The increase in secretion was dependent on the concentration of TS-B-III (3–20 μM) (Fig. 1A). The secretion induced by TS-B-III at low concentrations (3 and 5 μM) was completely abolished by eliminating Ca^{2+} from incubation medium, but some secretion induced by TS-B-III at higher concentrations (10 and 20 μM) was observed (Fig. 1A). Thus, TS-B-III induces catecholamine secretion in two distinct ways, which are dependent on and independent of the presence of external Ca^{2+}. The Ca^{2+}-independent secretion of catecholamines is probably due simply to leakage from the cell or chromaffin granule membrane damaged by TS-B-III at the higher concentrations, because TS-B-III (10 μM) enhanced the release of lactate dehydrogenase from the cells (Fig. 2). On the other hand, TS-B-III at a low concentration (5 μM) had no effect on lactate dehydrogenase release (Fig. 2), indicating that the membranes are not damaged.

Mechanism of Ca^{2+}-dependent secretion. It is well known that Ca^{2+} influx into the cells and the consequent increase in $[Ca^{2+}]_i$ are indispensable for the exocytotic secretion of catecholamines from bovine adrenal chromaffin cells evoked by secretagogues such as acetylcholine, high K^+ medium, and veratridine (16–19, 30, 31).

TS-B-III (5 μM) increased the secretion in an external Ca^{2+} concentration-dependent manner (Fig. 5). Furthermore, TS-B-III increased both the influx of $^{45}Ca^{2+}$ and $[Ca^{2+}]_i$ (Figs. 3 and 8A). The time courses of the increases in catecholamine secre-

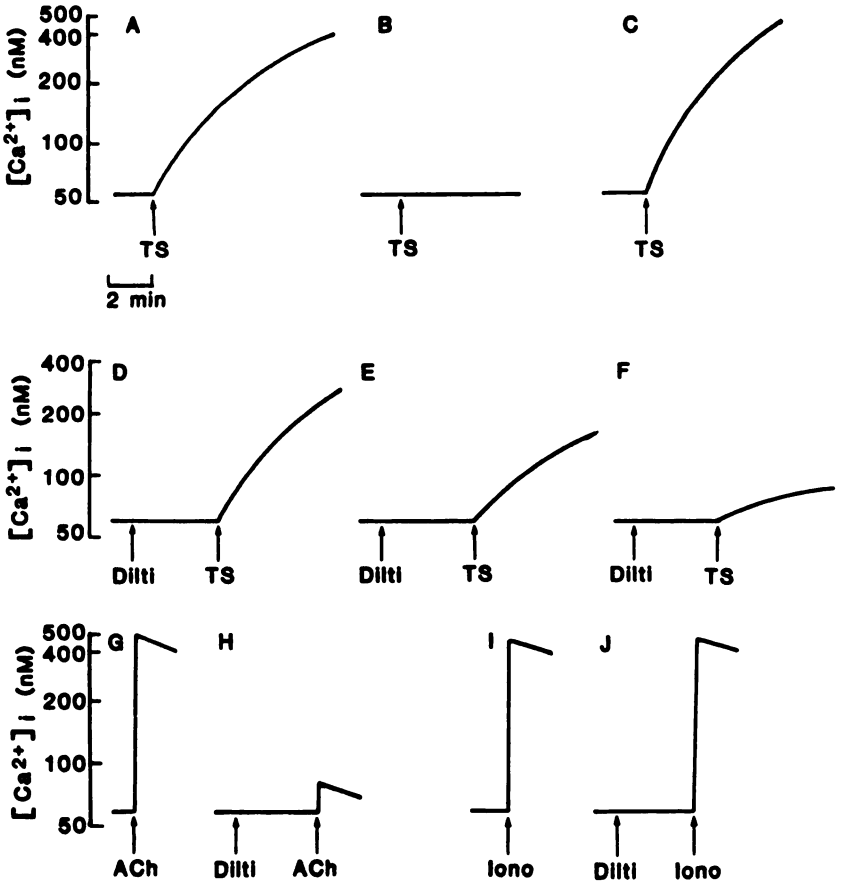

Fig. 8. Effect of diltiazem (*Dilti*) on TS-B-III (*TS*)-, acetylcholine (*ACh*)-, or ionomycin (*Iono*)-induced increase in $[Ca^{2+}]_i$. The fura-2-loaded cells in the cuvette were preincubated for 10 min at 37° in the fluorescence meter, and then TS-B-III (5 μM) (A–F), acetylcholine (30 μM) (G and H), ionomycin (1 μM) (I and J), or diltiazem (1 μM, D; 20 μM, E; and 200 μM, F, H, and J) was added. The cells were incubated in 2.6 mM Ca^{2+}-containing (A and D–J), Ca^{2+}-free (plus 0.2 mM EGTA) (B), or Na^+-free (C) KRH buffer. The fluorescence was recorded before and after the addition of test agents. $[Ca^{2+}]_i$ was measured as described in Experimental Procedures. The change in $[Ca^{2+}]_i$ obtained by the calculation is shown. Data are from a representative sample of at least four experiments.

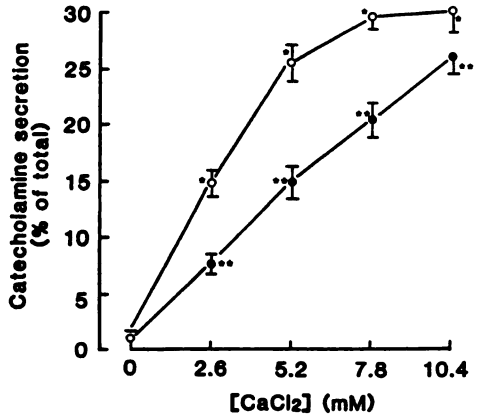

Fig. 9. Effects of various concentrations of external Ca^{2+} on diltiazem inhibition of TS-B-III-induced catecholamine secretion. The cells were preincubated for 10 min at 37°, with or without 20 μM diltiazem, and then the untreated and diltiazem-treated cells were incubated for 5 min at 37° with 5 μM TS-B-III (○) or 5 μM TS-B-III plus 20 μM diltiazem (●), respectively, with various concentrations of external Ca^{2+}. Catecholamines secreted from the cells were determined as described in Experimental Procedures. Data are means ± standard deviations from four experiments. *, $p < 0.001$, significantly different from TS-B-III-induced secretion in Ca^{2+}-free medium; **, $p < 0.001$, significantly different from TS-B-III-induced secretion in various concentrations of Ca^{2+} medium.

tion, $^{45}Ca^{2+}$ influx, and $[Ca^{2+}]_i$ were quite similar (Figs. 1B, 3B, 4B, and 8A). The concentration-response curves for increases in catecholamine secretion and $^{45}Ca^{2+}$ influx also correlated well (Figs. 1A, 3A, and 4A). These results suggest that the stimulation of Ca^{2+}-dependent catecholamine secretion by TS-B-III is mediated via an increased influx of Ca^{2+} into the cells and a subsequent increase in $[Ca^{2+}]_i$.

Mechanism of Ca^{2+} influx by TS-B-III. In adrenal chromaffin cells, an accumulation of Na^+ in the cells by the influx of Na^+ through voltage-dependent or nicotinic acetylcholine receptor-operated Na^+ channels depolarizes the cell membrane and results in the activation of voltage-dependent Ca^{2+} channels (19). Therefore, TS-B-III might stimulate the influx of Na^+ into the cells and result in the influx of Ca^{2+}. In Na^+-free medium, however, increases in secretion and $[Ca^{2+}]_i$ were not suppressed but rather enhanced (Figs. 6 and 8C). Furthermore, the secretion of catecholamines stimulated by TS-B-III was antagonized neither by hexamethonium, a blocker of nicotinic acetylcholine receptor (29), nor by tetrodotoxin, an inhibitor of voltage-dependent Na^+ channels (20) (Table 1). These results strongly suggest that TS-B-III causes the direct influx of Ca^{2+} into the cells but not the indirect influx of Ca^{2+}, which is mediated via the influx of Na^+ or the stimulation of nicotinic acetylcholine receptor.

TS-B-III-induced secretion of catecholamines was inhibited by blockers of voltage-dependent Ca^{2+} channels, diltiazem and nicardipine (Fig. 7), and by a divalent cation, Mn^{2+} (Table 1). In the same manner, the increase in $[Ca^{2+}]_i$ induced by TS-B-III was also inhibited by diltiazem (Fig. 8, E and F) and nicardipine (data not shown). On the other hand, although the increase in catecholamine secretion induced by ionomycin, a Ca^{2+} ionophore, was blocked by Mn^{2+}, increases in both secretion and $[Ca^{2+}]_i$ by ionomycin were not suppressed by diltiazem (Fig. 8, I and J; Table 1). Therefore, the entry pathway of external Ca^{2+} into the cells induced by TS-B-III is closely

TABLE 1

Effects of several inhibitors on catecholamine secretion evoked by TS-B-III, acetylcholine (ACh), ionomycin (Iono), and veratridine (Ver)

The cells were preincubated for 10 min at 37 ° with hexamethonium (10 or 100 μM), diltiazem (20 or 200 μM), or tetrodotoxin (100 nM) and then incubated for 5 min with each of the aforementioned agents, or $MnCl_2$ (5 mM), and various secretagogues (5 μM TS-B-III, 30 μM acetylcholine, 1 μM ionomycin, or 20 μM veratridine). Catecholamines secreted from the cells were determined as described in Experimental Procedures. Basal values were subtracted from the data, and secretagogue-induced responses were assigned values of 100%. Percentages of catecholamine secretion induced by various secretagogues were as follows: TS-B-III, 15.14 ± 0.4; acetylcholine, 16.39 ± 0.22; ionomycin, 5.11 ± 0.19; and veratridine, 12.83 ± 1.03. Basal secretion was 0.80 ± 0.11%. Each value represents the mean of four experiments. ND, not determined.

Treatment	Catecholamine secretion			
	TS-B-III (5 μM)	ACh (30 μM)	Iono (1 μM)	Ver (20 μM)
	%			
None	100	100	100	100
Hexamethonium				
10 μM	102.7	56.3*	ND	ND
100 μM	99.6	19.8*	ND	ND
Diltiazem				
20 μM	48.8*	10.0*	111.1	ND
200 μM	28.5*	6.3*	98.0	ND
Tetrodotoxin, 100 nM	128.0	96.5	ND	12.4*
$MnCl_2$, 5 mM	6.8*	ND	12.8*	8.1*

* $p < 0.01$, significantly different from secretagogues-induced secretion.

associated with voltage-dependent Ca^{2+} channels, and it is distinct from a Ca^{2+}-ionophoretic pathway like that provided by ionomycin. This view is supported by the fact that diltiazem inhibition of TS-B-III-induced secretion was almost overcome by an increase in external Ca^{2+} concentration (Fig. 9), because it is well known that the inhibitory effects of Ca^{2+} channel blockers are competitively antagonized by external Ca^{2+} (26).

Finally, two possible mechanisms by which TS-B-III causes Ca^{2+} influx via voltage-dependent Ca^{2+} channels are as follows: 1) TS-B-III directly activates endogenous voltage-dependent Ca^{2+} channels on the cell membrane or 2) TS-B-III itself forms pores similar to voltage-dependent Ca^{2+} channels in the membrane. During the preparation of this paper, Artalejo *et al.* (32) reported that alamethicin, isolated from *Trichoderma viride* (3), enhances the secretion of catecholamines from perfused cat adrenal glands and that the transient secretion pattern produced by alamethicin resembles that caused by nicotine or high potassium stimulation of adrenal glands but not that caused by Ca^{2+} ionophores (A23187, X537A, or ionomycin). Alamethicin, a linear Aib-containing peptide that consists of 19 amino acid residues with L-phenylalaninol as a protecting group of its carboxyl-terminal residue, is very similar to the primary structure of TS-B-III. It has been shown that alamethicin can transport ions across artificial lipid membranes by forming voltage-gated ion channels (pores) in electrophysiological experiments (8, 9). From studies of the crystal structure of alamethicin, it has been inferred that its oligomer spontaneously inserts into lipid membranes and produces the ion channels (pores) by an applied voltage (9). Furthermore, it has been suggested that alamethicin increases Ca^{2+} permeability of sarcoplasmic reticulum vesicles (33) or myelinated nerve membranes (34). Moreover, other Aib-containing peptides (suzukacillin and trichotoxin) have also been reported to form voltage-gated ion channels (10, 11). Therefore, TS-B-III may be a Ca^{2+} channel-forming ionophore that is sensitive to blockers of voltage-dependent Ca^{2+} channels, rather than an activator of endogenous voltage-dependent Ca^{2+} channels, in bovine adrenal chromaffin cells. However, we cannot rule out the possibility that TS-B-III activates endogeneous voltage-dependent Ca^{2+} channels. Further studies on the mechanism of Ca^{2+} influx produced by TS-B-III are now in progress.

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