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Pathway for Ca^{2+} influx into cells by trichosporin-B-VIa, an α-aminoisobutyric acid-containing peptide, from the fungus *Trichoderma polysporum*

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Abstract

Trichosporin (TS) -B-VIa, a fungal α-aminoisobutyric acid (Aib) -containing peptide consisting of 19 amino acid residues and a phenylalaninol, produced both $^{45}Ca^{2+}$ influx into bovine adrenal chromaffin cells and catecholamine secretion from the cells. The secretion induced by TS-B-VIa at lower concentrations (2–5 μM) was completely dependent on the external Ca^{2+}, while that induced by TS-B-VIa at higher concentrations (10–30 μM) was partly independent of the Ca^{2+}. The concentration-response curves (2–5 μM) for the TS-B-VIa-induced Ca^{2+} influx and secretion correlated well. The TS-B-VIa (at 5 μM) -induced secretion was not antagonized by diltiazem, a blocker of L-type voltage-sensitive Ca^{2+} channels. The treatment of fura-2-loaded C_6 glioma cells with TS-B-VIa (2–5 μM) led to an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner but the stimulatory effects of TS-B-VIa on $[Ca^{2+}]_i$ were only slightly observed in Ca^{2+}-free medium, indicating that TS-B-VIa causes Ca^{2+} influx from the external medium into the C_6 cells. The TS-B-VIa-induced increase in $[Ca^{2+}]_i$ in the C_6 cells was not antagonized by diltiazem and by SK&F 96365, a novel blocker of receptor-mediated Ca^{2+} entry. High K^+ increased neither $[Ca^{2+}]_i$ in the C_6 cells nor Mn^{2+} influx into the cells, while TS-B-VIa increased Mn^{2+} influx. Also in other non-excitable cells, bovine platelets, similar results were obtained. These results strongly suggest that the mechanism of Ca^{2+} influx by TS-B-VIa at the lower concentrations is distinct from the event of Ca^{2+} influx through receptor-operated or L-type voltage-sensitive Ca^{2+} channels in both excitable cells (the chromaffin cells) and non-excitable cells (the C_6 cells and the platelets) and that TS-B-VIa per se may form Ca^{2+}-permeable ion channels in biological membranes. On the other hand, the peptide at the higher concentrations seems to damage cell membranes.

Keywords: Calcium ion influx; Trichosporin-B; Adrenal chromaffin cell; Glioma C_6 cell; Bovine platelet

1. Introduction

TS-Bs are 11 kinds of fungal peptides with similar amino acid sequences isolated from the culture broth of *Trichoderma polysporum* and consist of 19 amino acid residues and an amino alcohol, phenylalaninol (Pheol), as a protecting group of the carboxyl-terminal residue [1,2]. As a representative example, TS-B-III, which is a mixture of four peptides (IIIa-IIId), has the following sequences; Ac-Aib-Ala-Ala-Ala-X-Aib-Gln-Aib-Y-Aib-Gly-Leu-Aib-Pro-Val-Aib-Z-Gln-Gln-Pheol: IIIa, X = Aib, Y = Leu, Z = Aib; IIIb, X = Aib, Y = ILe, Z = Ala; IIIc, X = Ala, Y = ILe, Z = Aib; and IIId, X = Aib, Y = Val, Z = Aib [1,2]. Thus, TS-Bs contain a high proportion of Aib, an unusual hydrophobic amino acid, belonging to the class of Aib-containing fungal peptides which include alamethicins [3], hypelcins [4], suzukacillins [5], trichotoxins [6], and antiamoebins [7]. The Aib-containing peptides show membrane-modifying actions, formation of voltage-gated ion

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

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channels in artificial membranes [8–11], hemolysis [12], fusion of lipid vesicles [13], and uncoupling of oxidative phosphorylation in mitochondria [14], etc. TS-Bs have also been found to uncouple the oxidative phosphorylation in rat liver mitochondria [15].

We previously reported that TS-B-III at lower concentrations (3–5 μM) causes Ca^{2+} influx and Ca^{2+}-dependent catecholamine secretion in bovine adrenal chromaffin cells, while TS-B-III at higher concentrations (10–20 μM) impairs the cells such that catecholamines leak from the cells [16]. TS-B-III (at the lower concentrations) -induced Ca^{2+} influx and secretion have been similarly observed in Na^+-free medium and have been antagonized by the blockers of L-type voltage-sensitive Ca^{2+} channels. Accordingly, we have proposed a possible mechanism that TS-B-III at the lower concentrations activates endogenous L-type voltage-sensitive Ca^{2+} channels and/or itself forms channels in the cell membranes and consequently induces Ca^{2+} influx into the cells.

Bovine adrenal chromaffin cells have various ion channels (such as receptor-operated Na^+ and Ca^{2+} channels [17,18] and voltage-sensitive Na^+ and Ca^{2+} channels [18,19], which complicate further analysis of the mechanism of TS-B effects. In this study, therefore, we investigated whether TS-B can cause Ca^{2+} influx into non-excitable cells, C_6 glioma cells and bovine platelets which are regarded as not having voltage-sensitive Ca^{2+} channels: we also tried to elucidate the mechanism of TS-B-induced Ca^{2+} influx into the bovine adrenal chromaffin cells. We used TS-B-VIa (Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Ile-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol) instead of TS-B-III as a source of TS-Bs, because the synthesis of TS-B-VIa is relatively easier and the potencies of TS-B-VIa in stimulating Ca^{2+} influx and catecholamine secretion are much stronger than those of TS-B-III in bovine adrenal chromaffin cells.

2. Materials and methods

2.1. Materials

Oxygenated Krebs-Ringer-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (KRH buffer) (pH 7.4) was used as an 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.

TS-B-IIIa and TS-B-VIa were isolated from *Trichoderma polysporum* and their structures were identified according to Fujita et al. [4] and Iida et al. [2]. Further, TS-B-VIa was synthesized and supplied [20]. Tissue culture instruments were obtained from the Falcon Plastics Co. (Cockeysville, MD, USA). Eagle's minimum essential medium and Dulbecco's modified Eagle's medium were from Nissui Seiyaku (Tokyo, Japan). Calf serum, acetylcholine, and $MnCl_2$ 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, USA). Fura-2 acetoxymethyl ether was from Dojindo Laboratories (Kumamoto, Japan). SK&F 96365 was from BIOMOL Research Lab., Inc. (Plymouth Meeting, PA, USA). All other chemicals were of the highest grade available from commercial sources.

2.2. Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands were kindly provided by the Center of Iwate Livestock Industry. Adrenal chromaffin cells were prepared by the method of collagenase digestion as described elsewhere [16]. The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum and antibiotics (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. The cells were cultured at 37°C in a CO_2 incubator (95% air/5% CO_2) for four days. A total of 2×10^6 cells contained 37.1 ± 1.2 μg ($n = 8$) of catecholamines as epinephrine and norepinephrine.

2.3. Measurements of $^{45}Ca^{2+}$ influx into the chromaffin cells and catecholamine secretion from the cells

The chromaffin cells were washed twice with KRH buffer and then preincubated with KRH buffer for 10 min at 37°C. The cells were washed once more with prewarmed KRH buffer and incubated with $^{45}Ca^{2+}$ (1 μCi) in 1.0 ml of the medium, in the presence or absence of TS-B-VIa for 10 min. 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 using a liquid scintillation counter (LSC-900; Aloka, Tokyo, Japan) [16]. The Ca^{2+} influx was expressed as nanomoles of Ca^{2+} per 2×10^6 cells.

After preincubation of the cells with KRH buffer for 10 min, the cells were incubated with or without TS-B-VIa or TS-B-III for 10 min. 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 on aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method [21], 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 a percentage of total cellular catecholamines.

2.4. Preparations of C_6 glioma cells and bovine platelets

C_6 glioma cells were purchased from Dainippon Pharmaceutical Ltd. (Osaka, Japan). The C_6 cells were seeded in a culture flask (150 cm^2 surface area) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a CO_2 incubator (95% air/5% CO_2).

Platelet-rich plasma was prepared from bovine blood by the modification of the method by Kitagawa et al. [22]. Briefly, bovine blood was obtained from the carotid artery and was immediately mixed with 10% of ACD (acid-citrate-dextrose) anticoagulant solution (122 mM glucose, 74.8 mM sodium citrate and 38.1 mM citric acid). The plasma was centrifuged at $300 \times g$ for 13 min and the platelet-rich fraction was collected. The platelets were washed twice and suspended with Ca^{2+}-free (+1 mM EGTA) KRH buffer.

2.5. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

To directly monitor the Ca^{2+} influx into cells, the use of isotope $^{45}Ca^{2+}$ is better than that of fura 2, an indicator of intracellular free Ca^{2+} concentration. However, in the experiment of $^{45}Ca^{2+}$ uptake to the platelets, we could not obtain good results because of the aggregation of platelets during the experiments. Therefore, we used fura 2 instead of $^{45}Ca^{2+}$ for the platelets and also for the C_6 glioma cells.

After culturing of C_6 cells, the cells were washed with Dulbecco's modified Eagle's medium and incubated with 5 μM fura 2-acetoxymethyl ether in the culture medium at room temperature for 60 min. They were centrifuged at $700 \times g$ for 5 min and washed twice with KRH buffer. The cells at a density of 2×10^6 cells/ml were preincubated with Ca^{2+}-free (+0.5 mM EGTA) KRH buffer or KRH buffer in the cuvette of the spectrofluorometer at 37°C for 10 min, and 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 spectrofluorometer (CAF-100, Nihon Bunko, Tokyo, Japan) 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. [23].

The platelets were incubated with 1 μM fura-2-acetoxymethyl ester in Ca^{2+}-free (+1 mM EGTA) KRH buffer at 37°C for 30 min, centrifuged at $1000 \times g$ for 10 min, and washed twice with Ca^{2+}-free KRH buffer. The platelets were preincubated with Ca^{2+}-free or normal KRH buffer in the cuvette of the spectrofluorometer at 37°C for 10 min, and then the test agents were added to the cuvette. The change in $[Ca^{2+}]_i$ was measured by the method described above.

The isolated chromaffin cells were cultured for 4 days on coverslips cut to fit into the spectrofluorometer cuvette. The cultured cells on the coverslips were washed twice with the culture medium and then incubated with 5 μM fura-2 acetoxymethyl ester in the culture medium at 37°C. After 40 min of incubation, the medium was replaced with KRH buffer. The coverslip with the cells was washed three times with KRH buffer and placed in the cuvette. The cells in the cuvette were preincubated with the buffer at 37°C

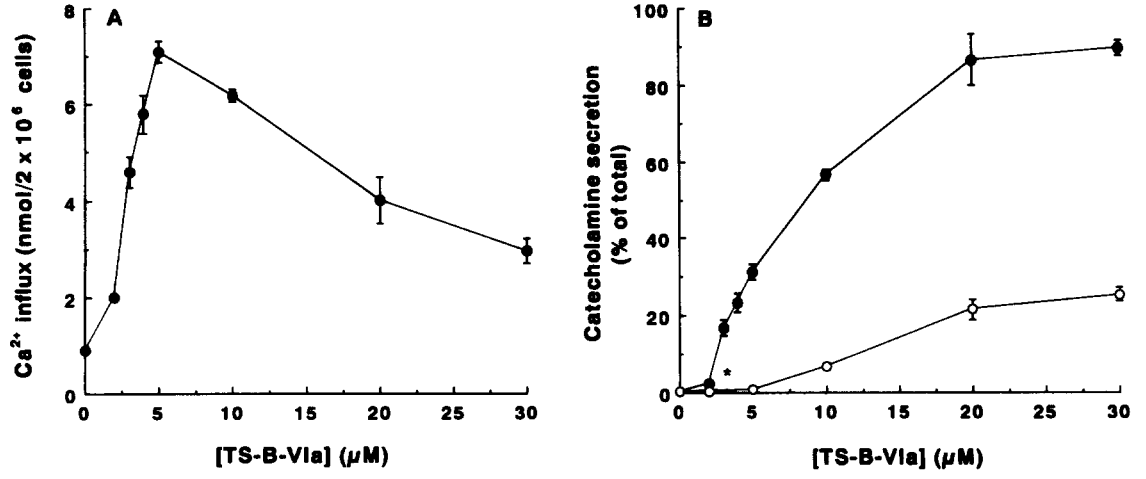

Fig. 1. Effects of TS-B-VIa on Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells. The chromaffin cells were preincubated with KRH buffer for 10 min at 37°C. (A) The cells were incubated for 10 min at 37°C with various concentrations of TS-B-VIa in KRH buffer containing 1 μCi of $^{45}CaCl_2$. The radioactivity in the cells was determined as described in Section 2. Data are means ± S.E. from four experiments. (B) The cells were incubated for 10 min at 37°C with various concentrations of TS-B-VIa in 2.6 mM Ca^{2+}-containing (filled circle) or Ca^{2+}-free (+0.5 mM EGTA) medium (open circle). Catecholamines secreted from the cells were determined as described in Section 2. Data are means ± S.E. from four experiments. * $P < 0.01$, significantly different from TS-B-VIa-induced secretion in Ca^{2+}-free medium.

for 10 min, and then the test agents were added. The change in $[Ca^{2+}]_i$ was measured by the method described above.

2.6. Measurement of Mn^{2+} influx into cells

The fura-2-loaded C_6 cells were preincubated with Ca^{2+} and Mg^{2+}-free KRH buffer for 10 min at 37°C in the cuvette of spectrofluorometer. Mn^{2+} (1 mM) and the test agents were added to the cuvette. The quenching of fluorescence induced by fura-2-Mn^{2+} was measured at an excitation wavelength of 340 nm and at an emission wavelength of 500 nm.

2.7. Statistics

Statistical calculations were done according to the methods of Snedecor and Cochran [24]. Differences were considered significant when P calculated by Student's t-test was < 0.05.

3. Results

3.1. Effects of TS-B-VIa on Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells

First, to determine whether TS-B-VIa affects chromaffin cells in the same manner as TS-B-III, used in the previous study [16], we examined the effects of TS-B-VIa on Ca^{2+} influx and catecholamine secretion. When bovine adrenal chromaffin cells were incubated with TS-B-VIa (2–30 μM) for 10 min, an increase in the $^{45}Ca^{2+}$ influx from the external medium occurred (Fig. 1A). The increase in the $^{45}Ca^{2+}$ influx was observed with 2 μM TS-B-VIa and was maximal at 5 μM TS-B-VIa, but gradually diminished at concentrations above 10 μM.

TS-B-VIa increased the secretion of catecholamines from the cells depending on its concentrations in normal (Ca^{2+}-containing) KRH buffer (Fig. 1B); the TS-B-VIa-induced secretion was easily detected at 2 μM, and at 5 μM TS-B-VIa, the secretion was 31% of the total catecholamines in the cells, which corresponds to 11.5 μg or 62.8 nmol of catecholamines/2×10^6 cells. The secretion was maximal at 20 μM TS-B-VIa, which induced 87% secretion. On the other hand, in Ca^{2+}-free medium, TS-B-VIa up to 5 μM did not produce secretion from the cells, but over 10 μM it caused the secretion of catecholamines. This indicates that the secretion by TS-B-VIa at lower concentrations (below 5 μM) is completely dependent on the external Ca^{2+}, and at higher concentrations (over 10 μM), the secretion is partly independent of the external Ca^{2+}. When the chromaffin cells were incubated with 2–5 μM TS-B-VIa for 10 min, no increase in the activity of lactate dehydrogenase, a cytoplasmic enzyme, in the incubation medium was observed, compared with the control, indicating that the cell membranes are not impaired by TS-B-VIa at the lower concentrations. The Ca^{2+}-independent part of the secretion is probably due to a simple leakage of catecholamines from the cells and the granules damaged by the fungal peptide, as previously described for TS-B-III [16]. In the range of 2–5 μM (the lower concentrations) TS-B-VIa, we observed a linear correlation between Ca^{2+} influx and catecholamine secretion (Regression equation is $y = 5.63x - 4.49$; $r = 0.999$, $t = 32.7$, and $P < 0.001$) (data not shown).

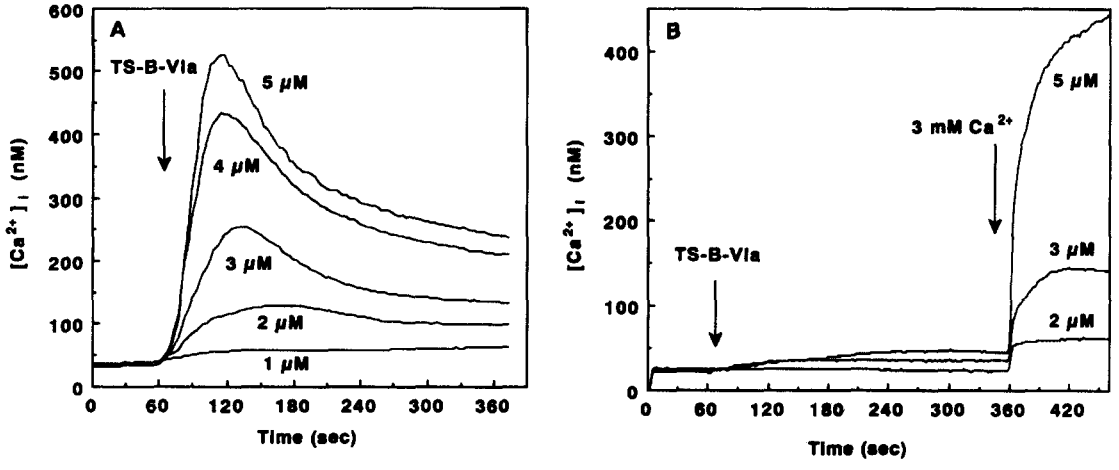

Fig. 2. Effect of TS-B-VIa on $[Ca^{2+}]_i$ in C_6 glioma cells. The fura-2-loaded C_6 cells were preincubated with normal (Ca^{2+}-containing) (A) or Ca^{2+}-free (+0.5 mM EGTA) KRH buffer (B) for 10 min at 37°C. (A) Various concentrations (1–5 μM; final concentrations) of TS-B-VIa were added to the cell suspension. (B) TS-B-VIa (2, 3 or 5 μM) was added and after 5 min, Ca^{2+} (3 mM) was added. The fluorescence was recorded before and after the addition of the test agents. The change in $[Ca^{2+}]_i$ obtained by the calculation is shown from the latter part of preincubation in each figure. Data are from a representative sample of at least four experiments.

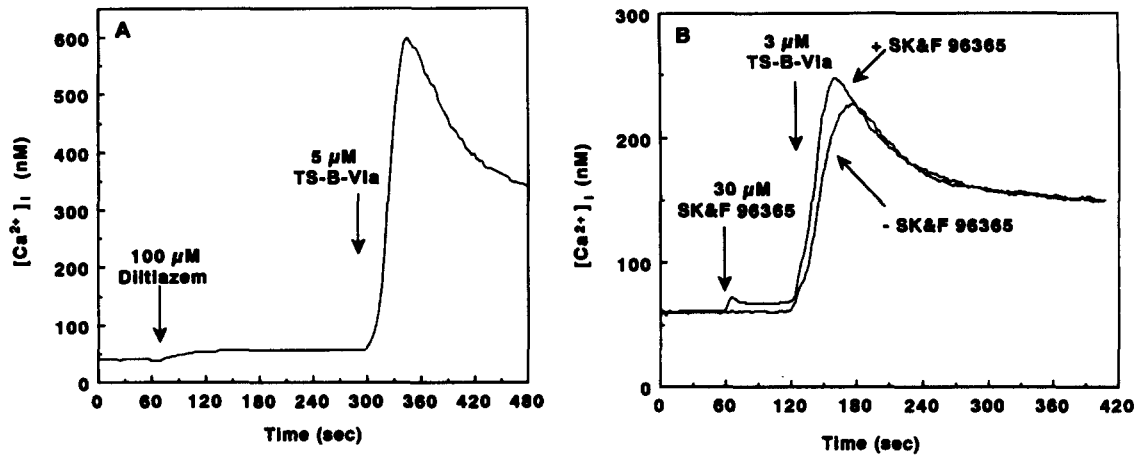

Fig. 3. Effects of diltiazem and SK&F 96365 on TS-B-VIa-induced increase in $[Ca^{2+}]_i$ in C_6 glioma cells. The fura-2-loaded C_6 cells were preincubated with KRH buffer for 10 min at 37°C. (A) The cells were incubated with diltiazem (100 μM) for 4 min, and TS-B-VIa (5 μM) was then added. (B) The cells were incubated with or without SK&F 96365 (30 μM) for 1 min, and then TS-B-VIa (3 μM) was added. The fluorescence was recorded before and after the addition of the test agents. The change in $[Ca^{2+}]_i$ obtained by the calculation is shown from the latter part of preincubation in each figure. Data are from a representative sample of at least four experiments.

3.2. Effects of TS-B-VIa, endothelin-1, and high K^+ on $[Ca^{2+}]_i$ in C_6 glioma cells

When TS-B-VIa (1–5 μM) was added to the suspension of fura-2-loaded C_6 glioma cells in KRH buffer, an increase in $[Ca^{2+}]_i$ was observed (Fig. 2A). This increase in $[Ca^{2+}]_i$ induced by TS-B-VIa relied on increasing TS-B-VIa concentrations (1–5 μM). On the contrary, in Ca^{2+}-free (+1.25 mM EGTA) KRH buffer, the addition of TS-B-VIa to the C_6 cells led to only a slight increase in $[Ca^{2+}]_i$. The addition of 3 mM Ca^{2+} markedly increased the $[Ca^{2+}]_i$, depending on TS-B-VIa concentrations (2–5 μM) (Fig. 2B). The increases in $[Ca^{2+}]_i$ were comparable to those observed in the normal KRH buffer (Fig. 2A), indicating that the increased $[Ca^{2+}]_i$ is nearly all attributable to an influx of Ca^{2+} from the external medium. The slight increase in fluorescence in Ca^{2+}-free KRH buffer may be due to Ca^{2+} release from the intracellular storage sites or due to an interaction of TS-B-VIa with fura-2 slightly leaking out from the cells. After the treatment of the C_6 cells with diltiazem (100 μM), a blocker of L-type voltage-sensitive Ca^{2+} channels, the addition of TS-B-VIa (5 μM) caused an increase in $[Ca^{2+}]_i$ (Fig. 3A) comparable to that in the non-treated cells (Fig. 2A).

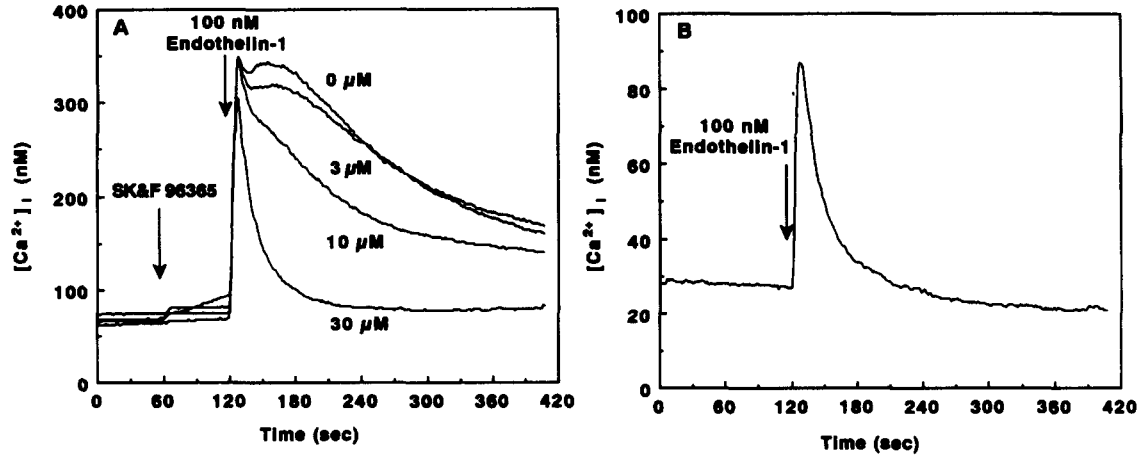

Fig. 4. Effect of endothelin-1 on $[Ca^{2+}]_i$ in C_6 glioma cells. The fura-2-loaded C_6 cells were preincubated with normal (Ca^{2+}-containing) (A) or Ca^{2+}-free (+0.5 mM EGTA) KRH buffer (B) for 10 min at 37°C. (A) SK&F 96365 (0–30 μM) was added to the suspension and after 1 min, endothelin-1 (100 nM) was further added. (B) Endothelin-1 (100 nM) was added. The fluorescence was recorded before and after the addition of the test agents. The change in $[Ca^{2+}]_i$ obtained by the calculation is shown from the latter part of preincubation in each figure. Data are from a representative sample of at least four experiments.

Pretreatment of the C_6 cells with SK&F 96365 (30 μM), a blocker of receptor-mediated calcium entry [25], did not affect the TS-B-VIa-induced increase in $[Ca^{2+}]_i$ (Fig. 3B).

Next, we examined the effects of endothelin-1, which binds the receptor and reveals the biological activities [26], on $[Ca^{2+}]_i$ in the C_6 cells. Endothelin-1 (100 nM) caused a rapid and a sustained increase in $[Ca^{2+}]_i$ in the C_6 cells (Fig. 4A), and the rapid increase was also observed in Ca^{2+}-free medium, although it was much less than that in the normal medium (Fig. 4B). The preincubation of the cells with SK&F 96365 led to a reduction of the sustained increase in $[Ca^{2+}]_i$ induced by endothelin-1 (100 nM) but not the rapid increase (Fig. 4A) in a concentration-dependent manner (3–30 μM).

It is widely known that stimulation of excitable cells by high K^+ medium depolarizes the cell membranes and results in an influx of Ca^{2+} into the cells through voltage-sensitive Ca^{2+} channels activated by the depolarization [27]. The exposure of the C_6 cells to high K^+ (50 mM) medium did not change $[Ca^{2+}]_i$. On the other hand, high K^+ increased $[Ca^{2+}]_i$ in bovine adrenal chromaffin cells in the normal medium but not in Ca^{2+}-free medium (data not shown).

3.3. Effects of TS-B-VIa and high K^+ on Mn^{2+} influx into C_6 glioma cells

We further compared the effect of TS-B-VIa with that of high K^+ on Mn^{2+} influx into the C_6 cells. As shown in Fig. 5A, the addition of 1 mM Mn^{2+} to the suspension of fura-2 loaded C_6 cells in Ca^{2+}-free KRH medium caused a drastic, fast decrease and then a slow decrease in the fluorescence emitted from fura-2-Mn^{2+} complex (quenching), indicating that Mn^{2+} spontaneously enters the cells (basal Mn^{2+} influx). The exposure of the cells to TS-B-VIa (5 μM) after Mn^{2+} addition further produced a great quenching (Fig. 5B). This indicates that TS-B-VIa strongly enhanced the basal Mn^{2+} influx into the cells. On the other hand, high K^+ (70 mM) stimulation of the cells had no effect on the basal Mn^{2+} influx into the C_6 cells (Fig. 5C).

3.4. Effect of TS-B-VIa on $[Ca^{2+}]_i$ in bovine platelets

An incubation of fura-2-loaded bovine platelets with TS-B-VIa (1–2 μM) in Ca^{2+}-free (+1 mM EGTA) KRH medium did not affect $[Ca^{2+}]_i$, while TS-B-VIa at higher concentrations (3–5 μM) only slightly increased $[Ca^{2+}]_i$ (Fig. 6A). The addition of 4.4 mM Ca^{2+} to the medium resulted in increases in $[Ca^{2+}]_i$ depending on the TS-B-VIa concentrations (1–5 μM) (Fig. 6A), indicating that the increase in $[Ca^{2+}]_i$ by TS-B-VIa is caused by an influx of the external Ca^{2+}.

The pretreatment of the platelets with diltiazem (100 μM) did not affect the TS-B-VIa (at 4 μM) -induced increase in $[Ca^{2+}]_i$ (Fig. 6B). On the other hand, high K^+ (56 mM) failed to induce the increase in $[Ca^{2+}]_i$ but after the treatment of the platelets with high K^+, TS-B-VIa (4 μM) maintained the stimulatory effect on $[Ca^{2+}]_i$ (Fig. 6C). SK&F 96365 (30 μM) did not affect the TS-B-VIa-induced increase in $[Ca^{2+}]_i$ (data not shown).

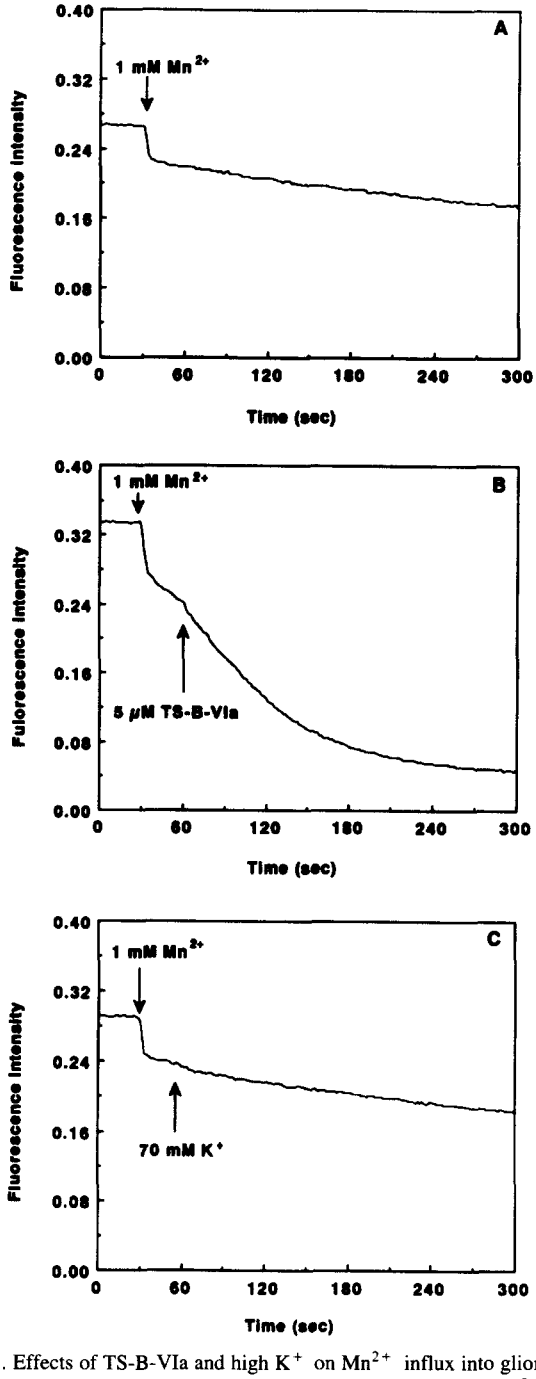

Fig. 5. Effects of TS-B-VIa and high K^+ on Mn^{2+} influx into glioma C_6 cells. The fura-2-loaded C_6 cells were preincubated with Ca^{2+} and Mg^{2+}-free KRH buffer for 10 min at 37°C, then Mn^{2+} (1 mM) (A, B and C) was added, and after 30 s, TS-B-VIa (5 μM) (B) or high K^+ (70 mM) (C) was further added into the cell suspension. The fluorescence was recorded before and after the addition of the test agents. The quenching of the fluorescence by Mn^{2+} influx into the cells is shown as decreases in fluorescence intensity and is shown from the latter part of preincubation in each figure. Data are from a representative sample of at least four experiments.

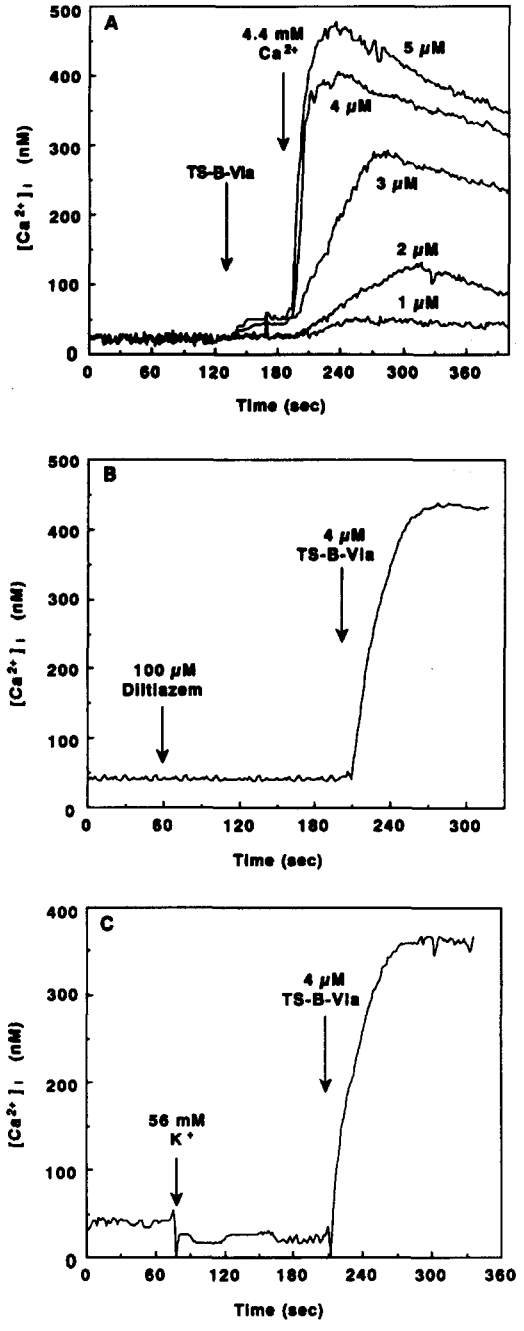

Fig. 6. Effects of TS-B-VIa and high K^+ on $[Ca^{2+}]_i$ in bovine platelets. The fura-2-loaded platelets were preincubated with Ca^{2+}-free (+1 mM EGTA) (A) or the normal KRH buffer (B and C) for 10 min at 37°C. (A) TS-B-VIa (1–5 μM) was added into the cell suspension, and after 1 min, Ca^{2+} (4.4 mM) was further added. (B) Diltiazem (100 μM) was added, and after 150 s, TS-B-VIa (4 μM) was added. (C) High K^+ (56 mM) was added, and after 130 s, TS-B-VIa (4 μM) was further added. The fluorescence was recorded before and after the addition of the test agents. The change in $[Ca^{2+}]_i$ obtained by the calculation is shown from the latter part of preincubation in each figure. Data are from a representative sample of at least four experiments.

3.5. *Effects of diltiazem on TS-B-VIa-induced catecholamine secretion*

In the presence of voltage-sensitive Ca^{2+} channel blockers, the results of $^{45}Ca^{2+}$ influx by TS-Bs were variable in the chromaffin cells. TS-Bs-induced $^{45}Ca^{2+}$ influx was inhibited or stimulated by the blockers in experiments done several times on different days. Thus, no reproducible data were obtained, as previously reported [16]. An influx of Ca^{2+} into the cells is essential for triggering the secretion of catecholamines. Thus, the secretion practically reflects the Ca^{2+} influx in the chromaffin cells. Therefore, we examined the effect of diltiazem on catecholamine secretion from the chromaffin cells instead of Ca^{2+} influx into the cells, induced by TS-B-IIIa or TS-B-VIa. The potency of the TS-B-VIa (at 5 μM) -induced secretion (28%) was stronger than that of the TS-B-III (at 5 μM) -induced secretion (20%) (Table 1). Diltiazem (50 μM) inhibited the TS-B-IIIa (at 5 μM) -induced secretion by 44%, whereas it had no effect on the TS-B-VIa (at 5 μM) -induced secretion (Table 1).

Table 1
Effects of diltiazem on the secretion of catecholamines from the chromaffin cells induced by TS-B-IIIa and TS-B-VIa

Treatment	Catecholamine secretion (% of total)		Inhibition (%)
	-	Diltiazem (50 μM)	
None	1.1±0.3	1.1±0.2	-
TS-B-IIIa	20.1±0.4	11.8±0.4	44
TS-B-VIa	27.9±0.8	26.7±0.9	0

The chromaffin cells were preincubated with KRH buffer in the presence or absence of 50 μM diltiazem for 10 min at 37°C and then were incubated for 10 min at 37°C with or without 5 μM TS-B-IIIa or TS-B-VIa in the presence or absence of 50 μM diltiazem. Catecholamines secreted from the cells were determined as described in Section 2. Data are means±S.E. from four experiments.

4. Discussion

Our previous studies showed that, in bovine adrenal chromaffin cells, TS-B-III at low concentrations (3–5 μM) caused Ca^{2+} influx and Ca^{2+}-dependent catecholamine secretion, which were antagonized by diltiazem or nicardipine, blockers of L-type voltage-sensitive Ca^{2+} channels. The adrenal chromaffin cells have L-type voltage-sensitive Ca^{2+} channels identified by electrophysiological and pharmacological properties [18,19,28]. Therefore, we proposed two mechanisms for the TS-B-III-induced Ca^{2+} influx: (1) TS-B-III acts as an activator of voltage-sensitive Ca^{2+} channels and/or (2) it per se forms Ca^{2+}-permeable ion channels sensitive to the blocker of voltage-sensitive Ca^{2+} channels in the cell membranes and results in Ca^{2+} influx into the cells [16]. In this study, our results strongly indicate that TS-B-VIa at low concentrations (2–5 μM), which has an amino acid sequence similar to TS-B-III, is a Ca^{2+}-permeable ionophore, which may form ion channels permeable to Ca^{2+} in biological membranes.

In bovine adrenal chromaffin cells, TS-B-VIa at the lower concentrations (2–5 μM) produced Ca^{2+} influx into

the cells and Ca^{2+}-dependent secretion of catecholamines from the cells (Fig. 1A,B). The concentration-response curves for the TS-B-VIa-induced $^{45}Ca^{2+}$ influx and secretion correlated well, indicating that TS-B-VIa at the lower concentrations causes Ca^{2+} influx and consequently results in the secretion of catecholamines in the chromaffin cells. On the other hand, TS-B-VIa at higher concentrations (10–30 μM) seems to impair the cells. Thus, this behavior of TS-B-VIa is similar to that of TS-B-III [16].

In C_6 glioma cells which are known as non-excitable cells, until now, there has been no report showing the presence of voltage-sensitive Ca^{2+} channels. In this study, high K^+ produced neither Ca^{2+} influx (data not shown) nor Mn^{2+} influx into the cells (Fig. 5C), indicating that voltage-sensitive Ca^{2+} channels activated by high K^+ are absent in the C_6 cells. On the contrary, TS-B-VIa produced not only Ca^{2+} influx in a concentration-dependent manner (at low concentrations of 2–5 μM) (Fig. 2A,B), which is similar to that for Ca^{2+} influx and catecholamine secretion in adrenal chromaffin cells (Fig. 1A,B), but also Mn^{2+} influx into the C_6 cells (Fig. 5B), and its Ca^{2+} influx was not antagonized by diltiazem (Fig. 3A). On the other hand, endothelin-1 caused a rapid and a sustained increase in $[Ca^{2+}]_i$ (two phases) in the C_6 cells (Fig. 4A,B). It has been reported that the rapid increase in $[Ca^{2+}]_i$ is due to the Ca^{2+} release from the intracellular Ca^{2+} store sites and the sustained increase is due to Ca^{2+} influx through the receptor-operated Ca^{2+} channels from the external medium [26]. In fact, SK&F 96365 (3–30 μM), a blocker of receptor-mediated Ca^{2+} entry [25], inhibited only the latter phase Ca^{2+} influx induced by endothelin-1 (Fig. 4A). However, SK&F 96365 (30 μM) did not affect TS-B-VIa-induced Ca^{2+} influx into the C_6 cells (Fig. 3B). These results strongly indicate that TS-B-VIa allows Ca^{2+} to enter the C_6 cells by a mechanism distinct from that of Ca^{2+} influx through voltage-sensitive or receptor-operated Ca^{2+} channels and that TS-B-VIa acts as a Ca^{2+}-permeable ionophore in non-excitable cells. This is also confirmed by the following results using other non-excitable cells, bovine platelets, which gave no response to high K^+ stimulation (Fig. 6C); (1) TS-B-VIa caused Ca^{2+} influx into the platelets in a concentration-dependent manner (2–5 μM) (Fig. 6A) similar to that in the C_6 cells (Fig. 2A) or the chromaffin cells (Fig. 1A), (2) the TS-B-VIa-induced Ca^{2+} influx was not antagonized by diltiazem (Fig. 6B) and SK&F 96365 (data not shown).

In the chromaffin cells, the secretion induced by TS-B-VIa was not antagonized by diltiazem, contrary to our prediction, while that induced by TS-B-IIIa as well as TS-B-III [16] was inhibited by it (Table 1). In excitable cells as well as non-excitable cells, therefore, TS-B-VIa probably acts as a Ca^{2+}-permeable ionophore, which produces Ca^{2+} influx regardless of the endogenous Ca^{2+} channels. On the other hand, the mechanism of TS-B-III-induced Ca^{2+} influx into the chromaffin cells remains unsolved. However, on the basis of the TS-B-VIa mechanism, it is anticipated that TS-B-III is also an ionophore susceptible to L-type voltage-sensitive Ca^{2+} channel blockers rather than activating the channels. We need to further investigate the TS-B-III mechanism.

The Aib-containing natural peptide, alamethicin, isolated from *Trichoderma viride* [3], has been reported to enhance the secretion of catecholamines from perfused cat adrenal glands [29]. Alamethicin, which consists of 19 amino acid residues with L-phenylalaninol as a protecting group of its carboxyl-terminal residue, is very similar to the primary structures of TS-Bs. It can transport ions across artificial lipid membranes by forming ion channels (pores) due to an applied voltage (voltage-gated ion channels) in electrophysiological experiments [8]. It has been inferred from its crystal structure analysis that an oligomer of alamethicin inserts into lipid membranes and forms a pore for ions due to a charging voltage [9]. We have also observed that TS-B-VIa induces voltage-dependent conductance in KCl medium in planar lipid bilayers [30]. This indicates that TS-B-VIa also forms voltage-gated ion channels in the artificial membranes. Further, based on the measurement of the single-channel activity of TS-B-VIa in the lipid bilayers, TS-B-VIa has been estimated to form an ion channel which is a bundle of four to nine peptide monomers (the probability of six monomers is highest based on the results of macroscopic current-voltage properties of the peptide, and the pore size is approx. a diameter of 10 Å in the case of six peptide monomers if calculated by the theory of Sansom [31]) [32], when the channel was regarded as a cylindrical electrolyte-filled pore in a bilayer [31]. Therefore, also in biomembranes, it is possible that the monomers of TS-B-VIa accumulate, insert into the cell membranes and produce ion channels.

The Ca^{2+} influx into the C_6 glioma cells and the bovine platelets decreased with time (Figs. 2 and 6A). The formation of TS-B-VIa channels in the lipid bilayer is dependent on the applied voltage, and the channels are formed above the critical voltage while their formation is prevented below the critical voltage [30]. Because the plasma membranes in the C_6 glioma cells and the bovine platelets polarize, it is presumed that TS-B-VIa forms ion channels in the membranes and ion influxes occur through the channels as described above. The ion influxes decrease the polarization of the membranes. Consequently, the channel formation and the subsequent Ca^{2+} influx would be suppressed, and the cytosolic free Ca^{2+} would be diminished with time by the uptake to the intracellular store sites of Ca^{2+} and the Ca^{2+} efflux from the cells.

In the artificial lipid membranes, TS-B-VIa has induced K^+ [30], Na^+ and Ca^{2+} fluxes (unpublished data) across the membranes, while in the chromaffin cells, TS-B-III [16] and TS-B-VIa (unpublished data) have induced Ca^{2+} influx into the cells in Na^+-free medium to an extent similar to that in the normal (Na^+-containing) medium. Further, if TS-B-VIa induces a Na^+ influx sufficient to depolarize the cell membranes and secondarily results in

Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in the chromaffin cells, the TS-B-VIa-induced secretion should be inhibited by voltage-sensitive Ca^{2+} channel blockers. However, it was not inhibited by diltiazem (Table 1). TS-B-VIa also produced influxes of another divalent cation, Mn^{2+}, as well as Ca^{2+} in the C_6 glioma cells (Fig. 5B). In biomembranes, therefore, TS-Bs may allow divalent ions rather than monovalent ions to enter the cells. Further studies of the ion selectivity of the pores formed by TS-Bs are now in progress.

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