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European Journal of Pharmacology 350 (1998) 59–65

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Vasoconstriction induced by zooxanthellatoxin-B, a polyoxygenated long-chain product from a marine alga

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Received 20 November 1997; revised 9 March 1998; accepted 11 March 1998

Abstract

We found that zooxanthellatoxin-B from a symbiotic marine alga, *Symbiodinium* sp., caused a concentration-dependent contraction of the rabbit isolated aorta at concentrations of 10^{-7}–10^{-5} M. Verapamil (10^{-6} M) and mefenamic acid (10^{-5} M) significantly attenuated the contractile response to zooxanthellatoxin-B at lower concentrations (10^{-7}–10^{-6} M) but not at higher concentrations (3×10^{-6}–10^{-5}M). The response to zooxanthellatoxin-B was partly inhibited by phentolamine (10^{-6} M), whereas it was potentiated by ouabain (10^{-5} M). Tetrodotoxin (10^{-6} M), methysergide (10^{-6} M), chlorpheniramine (10^{-6} M) or indomethacin (3×10^{-6} M), however, did not affect it. The zooxanthellatoxin-B-induced contraction was abolished by incubation in Ca^{2+}-free solution. The contractile response increased in a concentration-dependent fashion with Ca^{2+} (0.03 and 10 mM) or Sr^{2+} (0.10 and 10 mM). After treatment with verapamil (10^{-6} or 5×10^{-6} M), the concentration–contractile response curves for Ca^{2+} and Sr^{2+} in the presence of zooxanthellatoxin-B were shifted to the right in parallel. $MgCl_2$ (10 mM) shifted the concentration–response curve for Ca^{2+} more markedly than did verapamil. Zooxanthellatoxin-B increased tissue Na^+ and reduced tissue K^+ contents in the aorta, suggesting that zooxanthellatoxin-B increases Na^+ and K^+ permeability across the plasma membrane. These results suggest that the zooxanthellatoxin-B-induced contraction of the aorta is caused mainly by a direct action on smooth muscle, i.e., an increase in Ca^{2+} permeability that occurs at least partly through voltage-sensitive Ca^{2+} channels as well as through nonselective cation channels in the cell membrane of smooth muscle. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Zooxanthellatoxin-B; Contraction; Aorta, rabbit; Ca^{2+} channel; Smooth muscle

1. Introduction

A number of physiologically active substances have been isolated from marine organisms and exert a potent action on various mammalian cells through a unique mechanism (Ohizumi, 1997). For example, maitotoxin, the largest organic compound (molecular mass 3424 Da) ever known except for polysaccharides or peptides (Murata et al., 1992), causes the release of neurotransmitter from pheochromocytoma cells, or muscle contraction in a Ca^{2+}-dependent manner (Takahashi et al., 1982, 1983; Ohizumi and Yasumoto, 1983, Ohizumi et al., 1983). Since Ca^{2+} is involved in many regulatory processes of cellular functions, maitotoxin has been extensively used as a tool for examining Ca^{2+}-dependent cellular mechanisms (Kobayashi et al., 1987a,b; Gusovsky et al., 1988; Bernard et al., 1988; Watanabe et al., 1993). Another compound, palytoxin (molecular mass 2676 Da) isolated from marine coelenterates (Klein et al., 1982; Ko et al., 1982; Fujioka et al., 1982), also causes potent contraction of various smooth muscles (Ito et al., 1977; Ohizumi and Shibata, 1980; Ishida et al., 1985).

Recently, novel polyoxygenated long-chain compounds, named zooxanthellatoxin-A and -B, were isolated from a symbiotic marine alga *Symbiodinium* sp. as potent vasoconstrictor substances (Nakamura et al., 1993a) and their chemical structures were fully determined (Asari et al., 1993; Nakamura et al., 1993b, 1995). They were characterized as relatively large molecules (molecular mass about

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2900 Da) containing a large number of oxygen atoms and olefinic carbons (Asari et al., 1993). Zooxanthellatoxin-A has been shown to cause aggregation of rabbit platelets, accompanied by an increase in intracellular Ca^{2+} concentration (Rho et al., 1995) and to enhance tyrosine phosphorylation of p42 mitogen-activated protein kinase (Rho et al., 1997). On the other hand, the action of zooxanthellatoxin-B has not been investigated extensively. Thus, the present study attempted to define the mode of vasoconstrictive action of zooxanthellatoxin-B using the rabbit isolated aorta. This is the first report suggesting that zooxanthellatoxin-B augments the cation permeability of the plasma membrane of vascular smooth muscle.

2. Materials and methods

2.1. Mechanical response

The experiment on mechanical response was done as previously described(Ohizumi and Yasimoto, 1982). Male rabbits (Japanese white rabbits weighing 2.5–4.0 kg) were killed by bleeding from the carotid artery after anesthesia with pentobarbital (Tokyo Chemical Industry, Tokyo, Japan, 50 mg/kg i.m.), and the thoracic aorta was excised and cut into helical strips. The strips were suspended in a 10-ml organ bath containing a Krebs–Ringer bicarbonate solution (37°C) of the following composition (mM): NaCl 120, KCl 4.8; $CaCl_2$ 1.2, $MgSO_4$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose 5.8 at pH 7.4 (aerated with 95% O_2 and 5% CO_2). Ca^{2+}-free solution was prepared by omitting $CaCl_2$ in the solution in the presence or absence of ethyleneglycol-*bis*(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM). Na^+-free solution was prepared by replacing NaCl with choline chloride (120 mM) and omitting $NaHCO_3$ and adding 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (20 mM) and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) (pH 7.4). The Na^+-free solution was bubbled with 100% O_2.

The animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government, and the treatment of animals was approved by the Animal Welfare Committee of the Faculty of Pharmaceutical Sciences, Tohoku University.

2.2. Na^+, K^+-ATPase assay

Na^+, K^+-ATPase (0.3 units/mg protein) isolated from porcine cerebral cortex was purchased from Sigma Chemical (St. Louis, MO, USA). The enzyme reaction was carried out at 37°C in 0.5 ml reaction mixture containing (mM): NaCl, 100; KCl, 20; $MgCl_2$, 5; ATP, 1 and Tris–HCl, 50 (pH 7.4). ATPase activity was measured from the amount of inorganic phosphate released from ATP during a 10-min incubation. The amount of inorganic phosphate was determined by the method of Martin and Doty (1949).

2.3. Tissue Na^+ and K^+ contents

Tissue Na^+ and K^+ content of the rabbit isolated aorta was determined as reported previously (Ohizumi et al., 1982), using a modification of the method of Casteels and Kuriyama (1965). Endothelium-denuded helical strips about 20 mg each were made. After incubation with various test solutions, the strip was blotted and placed in a quartz tube. Then the strip was ashed by incubation in 0.5 ml of a mixture containing equal amounts of HNO_3 (61%) and $HClO_4$ (60%) at 60°C for 1 h and subsequent heating at 180°C overnight. The ashed sample was dissolved in a solution containing CsCl (1000 ppm) and 0.01 N HCl. The amount of Na^+ or K^+ was determined using an atomic absorption spectrophotometer (Varian, AA40, Australia).

2.4. Purification of zooxanthellatoxin-B

The cultured dinoflagellate (*Symbiodinium* sp.) was extracted with 70% ethanol and the ethanol extract was dissolved in water and extracted with ethyl acetate followed by *n*-butanol. The *n*-butanol extract was chromatographed on a polystyrene column (MCI gel CHP-20P, 75–150 mm, Mitsubishi Chemical, Tokyo, Japan), a DEAE Sephadex A-25 (1/30 M phosphate buffer, pH 6.9) and was separated by reversed phase high performance liquid chromatography (HPLC) (YMC Pack D-ODS-5, 2 cm f × 5 cm, YMC, Kyoto, Japan) to give zooxanthellatoxin-B (Fig. 1).

2.5. Statistical analysis

Three to ten preparations were used for each experiment. Results of the experiments are expressed as means

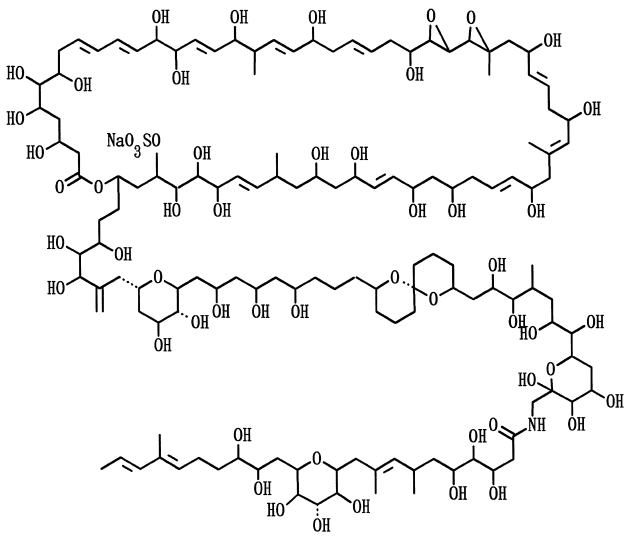

Fig. 1. Chemical structure of zooxanthellatoxin-B isolated from a symbiotic marine alga *Symbiodinium* sp. Zooxanthellatoxin-B is characterized by the presence of a diepoxide, two conjugated dienes, a macrolactone ring, a sulfate ester and an exomethylene.

± S.E.M. Student's *t*-test was used for statistical analysis of the results.

2.6. Materials

The following drugs were used: L-noradrenaline hydrogen tartrate (Wako Pure Chemicals, Osaka, Japan), DL-chlorpheniramine maleate (Wako), tetrodotoxin (Sigma), indomethacin (Wako), phentolamine hydrochloride (Sigma), verapamil hydrochloride (Sigma), mefenamic acid (Sigma), ouabain octahydrate (Aldrich Chemical, St. Louis, MO, USA), methysergide (Sandoz Pharmaceuticals, Basel, Switzerland), Tris–ATP (Sigma), atropine sulphate (Sigma) and choline chloride (Sigma).

3. Results

3.1. Mechanical response

Zooxanthellatoxin-B at concentrations above 10^{-7} M caused a sustained contraction of the aorta. Fig. 2A shows the concentration–response curve for zooxanthellatoxin-B in the presence or absence of phentolamine (10^{-6} M). The contractile responses were obtained with a single dosage method and each response was normalized against the maximum contractile response of the aorta to noradrenaline (10^{-6} M). The contractile response increased as the zooxanthellatoxin-B concentration was increased from 10^{-7} to 10^{-5} M. The maximum response to zooxanthellatoxin-B (10^{-5} M) was nearly equivalent to the response to noradrenaline (10^{-6} M) and EC_{50} for zooxanthellatoxin-B was approximately 10^{-6} M. Phentolamine (10^{-6} M) unsurmountably inhibited the contractile responses to zooxanthellatoxin-B at concentrations higher than 10^{-6} M and significantly ($P < 0.05$) decreased the maximum response to zooxanthellatoxin-B (10^{-5} M) by approximately 30%. The response to zooxanthellatoxin-B (10^{-6} M) was not significantly affected by treatment with methysergide (10^{-6} M), chlorpheniramine (10^{-6} M), tetrodotoxin (10^{-6} M), indomethacin (3×10^{-6} M) or atropine (10^{-6} M) (data not shown).

Verapamil (10^{-6} M) significantly attenuated the contractile response to zooxanthellatoxin-B at lower concentrations (10^{-7}–10^{-6} M), but responses to zooxanthellatoxin-B 3×10^{-6} and 10^{-5} M were not significantly different from the control (Fig. 2B). When the aorta was incubated in the Ca^{2+}-free solution containing 1 mM EGTA, the contractile response to zooxanthellatoxin-B (10^{-6} M) was abolished (data not shown). In the nominally Ca^{2+}-free solution without EGTA, zooxanthellatoxin-B (10^{-6} M) elicited a small contraction, about 10–20% of the control response to noradrenaline (10^{-6} M), as shown in Fig. 3. Then, the cumulative addition of Ca^{2+} to the nominally Ca^{2+}-free solution in the presence of zooxanthellatoxin-B (10^{-6} M) caused further contraction of the aorta in a concentration-dependent manner (Fig. 3A). Verapamil (10^{-6} M) elicited a rightward shift of the Ca^{2+}-

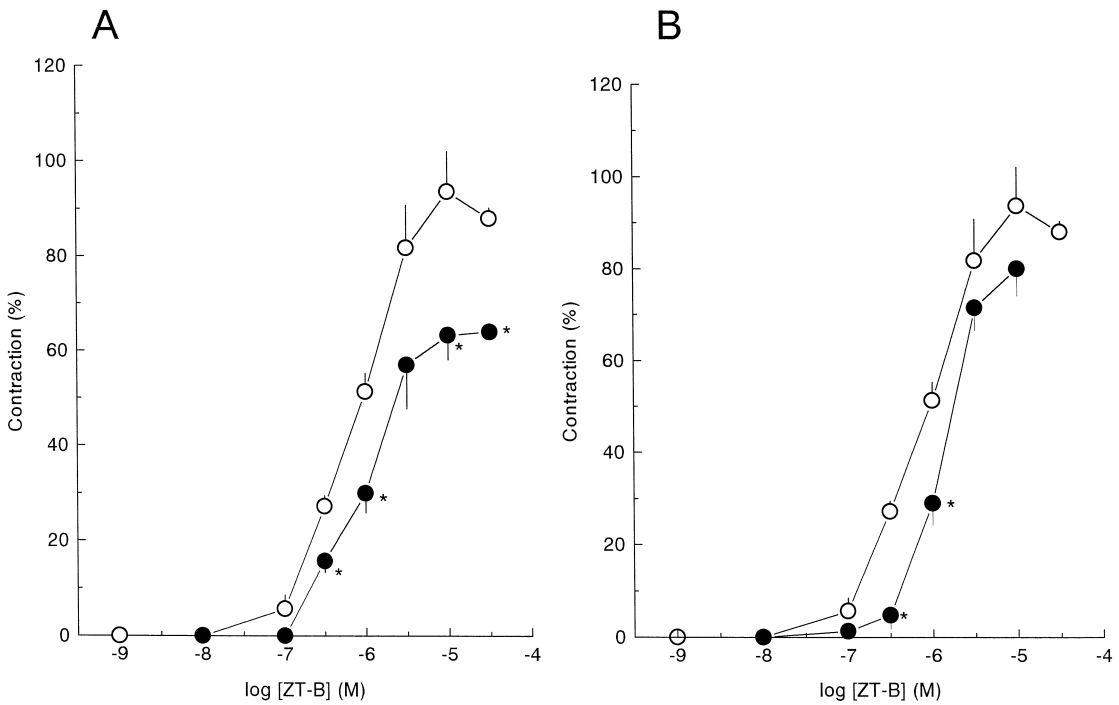

Fig. 2. The concentration–contractile response curve for zooxanthellatoxin-B (ZT-B) in rabbit isolated aorta in the presence (●) or absence (○) of phentolamine (10^{-6} M) (A) or verapamil (10^{-6} M) (B). Phentolamine (10^{-6} M) or verapamil (10^{-6} M) was added 15 min before application of zooxanthellatoxin-B. The maximum response to noradrenaline (10^{-6} M) is expressed as 100%. Vertical lines indicate S.E.M. ($n = 3$–10). * Significantly different from control: $P < 0.05$.

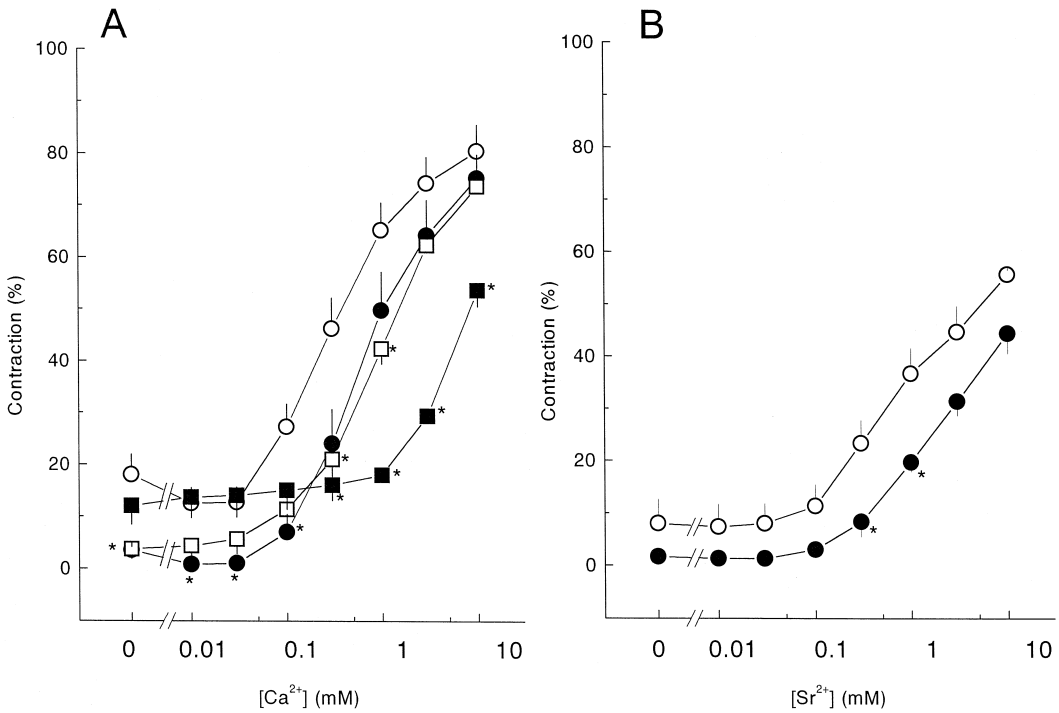

Fig. 3. Effects of varying the concentration of external Ca^{2+} (A) or Sr^{2+} (B) on the contractile response of rabbit aorta to zooxanthellatoxin-B in the presence or absence of Ca^{2+}-channel antagonists. Aorta was incubated in the Ca^{2+}-free solution for 60 min before the cumulative application of $CaCl_2$ or $SrCl_2$. Verapamil (10^{-6} and 5×10^{-6} M) or $MgCl_2$ (10 mM) and zooxanthellatoxin-B (10^{-6} M) were added 45 and 30 min before the application of $CaCl_2$ or $SrCl_2$, respectively. ○, control; ●, verapamil (10^{-6} M); □, verapamil (5×10^{-6} M); ■, $MgCl_2$. The maximum response to noradrenaline (10^{-6} M) is expressed as 100%. Vertical lines indicate S.E.M. ($n = 3$–4). ∗Significantly different from control: $P < 0.05$.

concentration–response curve and increasing the verapamil concentration to 5×10^{-6} M induced the same degree of rightward shift as did verapamil 10^{-6} M (Fig. 3A), suggesting that the maximal effective concentration of verapamil was lower than 10^{-6} M. Treatment with a high concentration (10 mM) of Mg^{2+} apparently caused a parallel shift of the Ca^{2+}-concentration–response curve to the right and the shift was much greater than that elicited by verapamil (Fig. 3A). The cumulative addition of Sr^{2+} (0.1–10 mM) to the nominally Ca^{2+}-free solution in the presence of zooxanthellatoxin-B (10^{-6} M) also caused a concentration-dependent contraction of the aorta (Fig. 3B). Verapamil (10^{-6} M) elicited a similar parallel shift of the Sr^{2+}-concentration–response curve to the right as observed in the addition of Ca^{2+}. Using the concentration of 10^{-6} M, pA_2 values, the negative logarithm of the affinity (Van Rossum, 1963), were estimated to be 6.21 ± 0.044 ($n = 4$) and 6.34 ± 0.022 ($n = 3$) for responses to Ca^{2+} and Sr^{2+}, respectively. These values were apparently smaller than that (7.58) reported for the response to K^+ depolarization of rabbit aorta (Shümann et al., 1975) since the concentration 10^{-6} M used may be much higher than that eliciting the maximum effect.

In the Na^+-free solution, zooxanthellatoxin-B (10^{-6} M) was still able to cause a marked contraction of the aorta (Fig. 4). Successive additions of verapamil (10^{-6} M) partly inhibited the contraction induced by zooxanthellatoxin-B in the Na^+-free solution as well as in the normal solution.

The effect of mefenamic acid, a nonselective cation channel inhibitor (Gögelein et al., 1990; Jung et al., 1992),

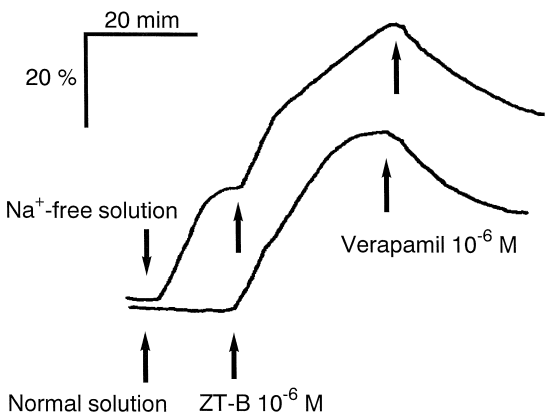

Fig. 4. Zooxanthellatoxin-B (ZT-B)-induced contraction of rabbit aorta in the presence or absence of Na^+. These traces show the contraction induced by zooxanthellatoxin-B (10^{-6} M) in the Na^+-free solution (upper trace) and in a normal solution (lower trace). Phentolamine (10^{-6} M) and atropine (10^{-6} M) were added 30 min before application of zooxanthellatoxin-B (10^{-6} M). Verapamil (10^{-6} M) was added when zooxanthellatoxin-B-induced contraction reached its maximum. The contractile response to noradrenaline (10^{-6} M) is expressed as 100%.

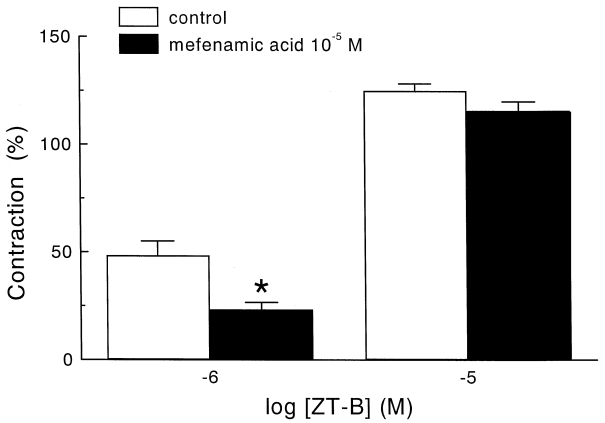

Fig. 5. Effects of mefenamic acid (10^{-5} M) on the zooxanthellatoxin-B (ZT-B)-induced contraction of rabbit aorta. Mefenamic acid was administered 15 min before application of zooxanthellatoxin-B (10^{-6} or 10^{-5} M). Tensions 45 min after zooxanthellatoxin-B application were measured. Vertical bars represent ±S.E.M. ($n=4$). ∗Significantly different from control: $P<0.01$.

on the contractile responses of the aorta to zooxanthellatoxin-B was investigated (Fig. 5). Mefenamic acid (10^{-5} M) was added 15 min before the application of zooxanthellatoxin-B (10^{-6} or 10^{-5} M). In the presence of mefenamic acid (10^{-5} M), a near half-maximum contractile response of the aorta to 10^{-6} M zooxanthellatoxin-B was inhibited by approximately 50% of the control. The contractile response to 10^{-5} M zooxanthellatoxin-B, which caused the maximum contraction, was not apparently inhibited by the presence of mefenamic acid. These results suggest that zooxanthellatoxin-B elicits contraction of the aorta partly due to the activation of nonselective cation channels.

Ouabain, a Na^+, K^+-ATPase inhibitor, significantly potentiated the contractile response to zooxanthellatoxin-B

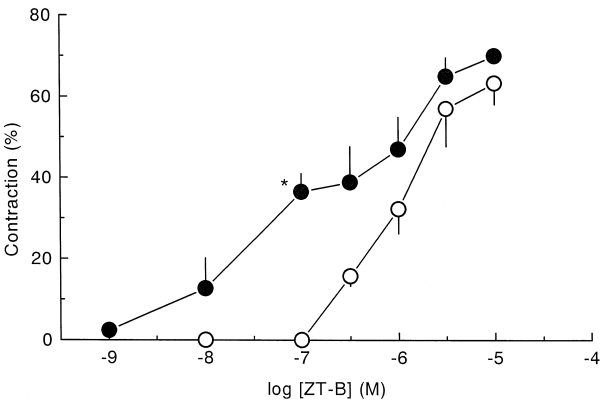

Fig. 6. Effects of ouabain on the contractile response of rabbit aorta to zooxanthellatoxin-B (ZT-B) in the presence of phentolamine (10^{-6} M). Ouabain (10^{-5} M) and phentolamine (10^{-6} M) were added 15 min before application of zooxanthellatoxin-B. ○, phentolamine; ●, phentolamine + ouabain. The maximum response to noradrenaline (10^{-6} M) is expressed as 100%. Vertical lines indicate S.E.M. ($n=3$–7). ∗Significantly different from control: $P<0.05$.

Table 1
Effects of zooxanthellatoxin-B on the tissue Na^+ and K^+ content of the rabbit isolated aorta

Time (min)	mmol/kg wet weight[a]	
	Na^+ content	K^+ content
0	136.1 ± 3.81	72.5 ± 1.77
15	146.7 ± 1.22	46.1 ± 3.61[b]
30	164.5 ± 1.81[b]	37.6 ± 2.57[b]
45	172.2 ± 2.66[b]	38.0 ± 4.94[b]
60	172.9 ± 5.42[b]	22.8 ± 3.54[b]

Zooxanthellatoxin-B (10^{-5} M) was administered at time 0.
[a]Each value is expressed as the mean ± S.E.M. ($n=4$).
[b]Significantly different from control (values at 0 time): $P<0.05$.

especially at the lower concentrations of 10^{-8}–3×10^{-7} M in the presence of phentolamine (10^{-6} M) (Fig. 6).

We also tested whether zooxanthellatoxin-B would interfered with noradrenaline-induced contractions of the aorta. Zooxanthellatoxin-B (3×10^{-8} M) itself did not affect the tension of the aorta and did not interfere with noradrenaline (10^{-9}–3×10^{-6} M)-induced contractions of the aorta (data not shown).

3.2. Na^+ and K^+ contents

Zooxanthellatoxin-B (10^{-5} M) produced a significant elevation of the Na^+ content and a significant reduction of the K^+ content of the aorta (Table 1). When the muscle was incubated with zooxanthellatoxin-B for 60 min, the Na^+ content increased by 30% and the K^+ content decreased by 70% of control. The time course of the contractile response to zooxanthellatoxin-B was much steeper than that of the increase of the Na^+ content.

3.2.1. Na^+, K^+-ATPase

As shown in Table 2, ouabain (10^{-6} or 10^{-5} M) significantly inhibited the Na^+- and K^+-stimulated ATPase activity by 28.42 and 75.96%, respectively, whereas zooxanthellatoxin-B (10^{-7}–10^{-4} M) did not affect the activity significantly.

Table 2
Effects of zooxanthellatoxin-B and ouabain on the activity of Na^+, K^+-ATPase prepared from porcine cerebral cortex

Drug	Concentration (M)	Relative activity (%)[a]
Ouabain	10^{-6}	71.6 ± 2.66
	10^{-5}	24.0 ± 1.48[b]
Zooxanthellatoxin-b	10^{-7}	111.2 ± 6.26
	10^{-6}	110.3 ± 2.73
	10^{-5}	110.6 ± 3.20
	10^{-4}	110.4 ± 3.43

The enzyme reaction was carried out at 37°C for 10 min. The activity was measured from the amount of inorganic phosphate released from ATP.
[a]Each value is expressed as the mean ± S.E.M. ($n=3$).
[b]Significantly different from control: $P<0.05$.

4. Discussion

Zooxanthellatoxin-B caused a sustained contraction of the rabbit isolated aorta at concentrations above 10^{-7} M. Among antagonists tested against adrenoceptors, 5-HT receptors, histamine receptors and acetylcholine receptors or a cyclooxygenase inhibitor, only phentolamine, an α-adrenoceptor antagonist, partly inhibited the contractile response of the aorta to zooxanthellatoxin-B. Therefore, it is suggested that the zooxanthellatoxin-B-induced contraction is mainly due to a direct action on smooth muscle and partly to an indirect action on adrenergic nerves via the release of noradrenaline.

The contractile response to zooxanthellatoxin-B was abolished by incubation of the aorta in the Ca^{2+}-free solution containing EGTA. In the nominally Ca^{2+}-free solution, however, a small contraction was still observed in the presence of zooxanthellatoxin-B. Furthermore, the zooxanthellatoxin-B-induced contraction increased in a linear fashion with increasing concentrations of external Ca^{2+} or Sr^{2+}, a Ca^{2+} substitute (Boullin, 1967; Kirpekar and Misu, 1967). These results suggest that the contractile response of the aorta to zooxanthellatoxin-B is highly dependent on external Ca^{2+}.

In the normal solution, verapamil significantly inhibited the contractile response to lower concentrations (10^{-7}–10^{-6} M), but not to higher concentrations (more than 3×10^{-6} M) of zooxanthellatoxin-B. The concentration–response curve for Ca^{2+} or Sr^{2+} in the presence of zooxanthellatoxin-B was shifted toward approximately 3 times higher concentrations by verapamil. On the other hand, Mg^{2+} (10 mM) caused a much greater rightward shift of the concentration–response curve for Ca^{2+} compared with that caused by verapamil. In vascular smooth muscle, high concentrations of external Mg^{2+} were reported to effectively counteract the action of external Ca^{2+} and to inhibit Ca^{2+} influx (Altura and Altura, 1981). Similarly to verapamil, mefenamic acid, a nonselective cation channel inhibitor, partly inhibited the contractile response to the lower concentration (10^{-6} M) of zooxanthellatoxin-B. Thus, these results suggest that zooxanthellatoxin-B elicits contraction of the rabbit aorta, presumably through at least two different kinds of Ca^{2+} influxes through voltage-dependent Ca^{2+} channels sensitive to verapamil and through other Ca^{2+}-entry pathways insensitive to it.

The present experiments further demonstrated that zooxanthellatoxin-B produced a significant elevation of the Na^+ content and a significant reduction of the K^+ content of the aorta. On the other hand, zooxanthellatoxin-B was able to elicit contraction of the aorta in the absence of external Na^+. Therefore, Na^+ entry into cytoplasm across the plasma membrane may not be directly related to the contractile response to zooxanthellatoxin-B. Instead, such a marked change in cation contents of the aorta may be indicative of depolarization of the plasma membrane as observed for the action of palytoxin on nerve and muscle cells (Dubois and Cohen, 1977; Muramatsu et al., 1984; Ito et al., 1985; Ecault and Sauviat, 1991). It is thus conceivable that zooxanthellatoxin-B elicits depolarization of the plasma membrane, leading to Ca^{2+} influx and to contraction of the aorta, although clear evidence for depolarization, obtained using an electrophysiological method is not yet available.

In contrast to palytoxin which was reported to inhibit Na^+, K^+-ATPase (Chhatwal et al., 1983; Ishida et al., 1983), zooxanthellatoxin-B apparently did not inhibit Na^+, K^+-ATPase activity. Furthermore, palytoxin-induced contraction of vascular smooth muscle was somewhat inhibited in the presence of ouabain (Ishida et al., 1985; Ozaki et al., 1984), but the presence of ouabain apparently potentiated the zooxanthellatoxin-B-induced contraction of the aorta through a mechanism directly related to Na^+, K^+-ATPase activity. Presumably, the ouabain-induced inhibition of the electrogenic Na^+ pump (Brading and Widdicombe, 1974) contributes to the contractile response of the aorta to zooxanthellatoxin-B due to the additional depolarization in the presence of ouabain.

Acknowledgements

We wish to thank Drs. Y. Shigeta and T. Kaneko at the Central Institute, Mitsubishi Chemical, for the use of the atomic absorption spectrophotometer. We also thank Mrs. M. Okamoto of this Department for typing this manuscript. This work was supported in part by a grant of the Scientific Research Fund (No. 05454567) to Y. Ohizumi from the Ministry of Education, Science, Sports and Culture of Japan.

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