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The inhibitory effects of N^2-dansyl-L-arginine-4-*t*-butylpiperidine amide (TI 233) on contraction of vascular and intestinal smooth muscle

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1 Effects of N^2-dansyl-L-arginine-4-*t*-butylpiperidine amide (TI 233) on the contractions in vascular and intestinal smooth muscles were examined.

2 High K-induced sustained contractions in the smooth muscles were inhibited by TI 233 with an IC_{50} of 2.1×10^{-5} M for rabbit aorta and 3.6×10^{-6} M for guinea-pig taenia coli in a solution containing 1.5 mM Ca. Initial transient contraction induced by K in taenia coli was less sensitive to the inhibitory effect of TI 233. When the Ca concentration in the medium was decreased to 0.3 mM, the concentration-inhibition curves for TI 233 shifted to the left in both aorta and taenia coli. Increasing the Ca concentration to 7.5 mM shifted the curve to the right in the aorta. TI 233 also inhibited the noradrenaline-induced contraction in the aorta ($IC_{50} = 2.1 \times 10^{-5}$ M).

3 In a hypoxic solution without added glucose, the inhibitory effect of TI 233 on the K-induced contraction in aorta was augmented. In the presence of high concentrations (40 mM) of glucose in hypoxia, TI 233 did not inhibit the noradrenaline-induced contraction of the aorta. Hypoxia and a high concentration of glucose also decreased the inhibitory effect of TI 233 on the K-induced contraction in taenia coli.

4 TI 233 inhibited the K-induced increase in cellular Ca content measured by a modified lanthanum method.

5 TI 233 decreased oxygen consumption and ATP content of resting and K-stimulated aorta and taenia coli.

6 It was concluded that TI 233 inhibits the vascular and intestinal smooth muscle contraction by a Ca antagonistic action and also by inhibition of aerobic metabolism.

Introduction

N^2-dansyl-L-arginine-4-*t*-butylpiperidine amide (TI 233) is a thrombin inhibitor (Okamoto, Hijikata, Kinjyo, Kikumoto, Ohkubo, Tonomura & Tamao, 1975) which also has a 5-hydroxytryptamine (5-HT) antagonistic action (Ho, Nakao & Shibata, 1980) and a calmodulin-antagonistic action (Hidaka, Yamaki, Naka, Tanaka, Hayashi & Kobayashi, 1980). Further, TI 233 inhibits both tension development and ^{45}Ca uptake in smooth muscle preparations (Karaki, Murakami, Nakagawa, Ozaki & Urakawa, 1982a). However, the mechanism of the inhibitory effect of TI 233 on smooth muscle is not fully understood and this has been the purpose of this paper.

Methods

Preparations

Two muscle preparations were used: (1) male New Zealand rabbits weighing 2.0 to 2.5 kg were killed by an air embolism under pentobarbitone anaesthesia. The thoracic aorta was rapidly removed and cut into a spiral strip 3 to 4 mm wide (Furchgott, 1960). The adventitial layer was then separated from the media-intimal layer as described by Karaki & Urakawa (1977) and muscle strips of 4 to 8 mm long were prepared. (2) Male guinea-pigs weighing 250 to 300 g were killed by a blow on the neck and a section of taenia coli 5 to 10 mm in length was removed. Each

muscle strip was weighed, attached to a holder under a resting tension of 1 g for aorta and 0.2 g for taenia coli and equilibrated in the bathing solution for 60 to 90 min before starting the experiments. Cellular Ca content, ATP content and oxygen consumption of the muscle strips are expressed in terms of their initial weight, which ranged from 5 to 10 mg.

Solutions

The normal bathing solution contained (mM) NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5 (Karaki, Suzuki & Urakawa, 1981); the concentration of $CaCl_2$ was changed either to 0.3 mM or 7.5 mM and the concentration of glucose was also changed to either 0 mM or 40 mM in some experiments. High K solutions were made either by increasing the concentration of KCl to 45.4 mM (hyperosmotic 45.4 mM K) or by substituting 60 mM Na in the normal solution with equimolar K (isosmotic 65.4 mM K). These solutions were aerated with 95% O_2 and 5% CO_2 mixture at 37°C (pH 7.4), or in some experiments, with 95% N_2 and 5% CO_2 mixture in order to produce hypoxia.

Tension and cellular Ca content

Muscle tension was recorded isometrically with a force-displacement transducer connected to a Nihon Kohden polygraph (Japan). The concentration of TI 233 producing half-maximal inhibition (IC_{50}) was determined graphically from log concentration-inhibition curves. The La-inaccessible Ca fraction was determined as described by Karaki and Weiss (1979); cellular Ca content measured by this method represents the largest part of cellular exchangeable Ca (Karaki & Weiss, 1980). Briefly, muscle strips were incubated in solutions containing added ^{45}Ca (0.4–1.6 μCi ml^{-1}) for 30 min. After incubation with ^{45}Ca, the strips were washed for 60 min in an ice-cold solution containing $LaCl_3$ 73.8 mM, glucose 5.5 mM and Tris(hydroxymethyl)aminomethane 24 mM. This solution was adjusted to pH 6.8–6.9 at 0.5°C with 1 N maleic acid. After the La-wash period, tissues were removed from the holders, blotted, placed in scintillation vials and solubilized overnight at 50°C with 0.4 ml solubilizer (Soluene 350, Packard, USA or Lumasolve, Lumac Systems AG, Switzerland) and 0.01 ml demineralized water. The solubilized samples were then neutralized by the addition of 0.1 ml of

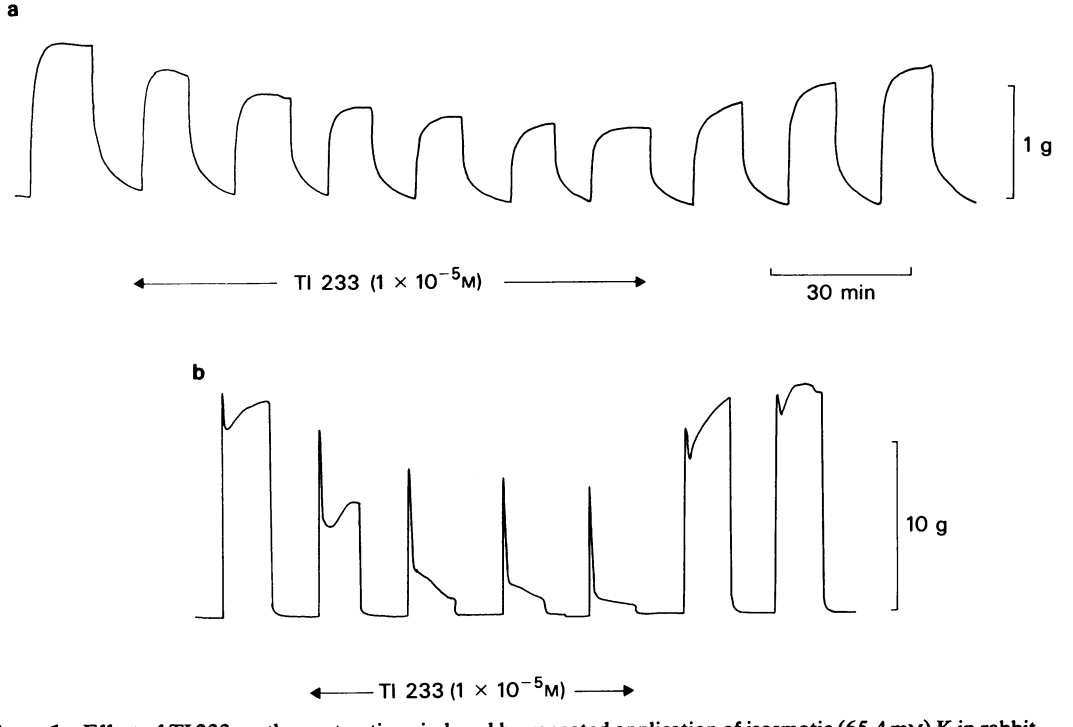

Figure 1 Effect of TI 233 on the contractions induced by repeated application of isosmotic (65.4 mM) K in rabbit aorta (a) and hyperosmotic (45.4 mM) K in guinea-pig taenia coli (b). High K was applied for 10 min and the wash period was 15 min. Period of application of TI 233 is shown by arrows.

6 N HCl, 4 ml of the scintillation mixture (Instagel, Packard or Lumagel, Lumac Systems AG) was added to the neutralized sample and radioactivity was counted with Packard Tri-Carb 3380 liquid scintillation spectrometer.

ATP content

The ATP content of the muscle was measured as described by Strehler & McElroy (1957). After incubation in the chosen medium, muscle strips were boiled for 5 min in 2 ml water and then cooled to 0 °C. ATP in the boiled extract was measured with a Lumac Biometer M 1030 or M 2010 (Lumac B. V., Netherland) using ATP-luciferine-luciferase luminescence. The luciferine-luciferase mixture was obtained from Sigma (USA) or Lumac B. V.

Oxygen consumption

Oxygen consumption of the muscle was measured as described by Stephens, Kroeger & Wrogemann (1975) and Paul & Peterson (1975) using a Clark-type oxygen electrode (Rank Brothers, U.K.) attached to a 2 ml chamber containing approximately 200 mg smooth muscle at 37 °C.

Drugs and statistics

TI 233 was donated by Mitsubishi Kasei Co. Ltd, Japan. All data are expressed as the mean ± s.e.mean. Statistical significance was determined by Student's *t* test.

Results

Contractile response

Isosmotic 65.4 mM K and hyperosmotic 45.4 mM K induced sustained contractions in rabbit aorta and guinea-pig taenia coli, respectively. TI 233 inhibited the K-induced contractions in aorta and taenia coli as shown in Figure 1. In taenia coli, TI 233 inhibited the K-induced sustained contraction more strongly than the initial transient contraction. The effect of TI 233 was reversible and reproducible.

Concentration-inhibition curves obtained by the cumulative application of TI 233 are shown in Figure 2. In a normal solution containing 1.5 mM Ca, the IC_{50} values were $2.1 \pm 0.2 \times 10^{-5}$ M ($n = 4$) for aorta and $3.6 \pm 0.3 \times 10^{-6}$ M ($n = 10$) for taenia coli. When the concentration of external Ca was decreased to 0.3 mM, the concentration-inhibition curves for TI 233 shifted to the left in both aorta and taenia coli. Increase in the concentration of Ca to 7.5 mM shifted the concentration-inhibition curve to the right in aorta but there was no unequivocal shift in taenia coli (Figure 2).

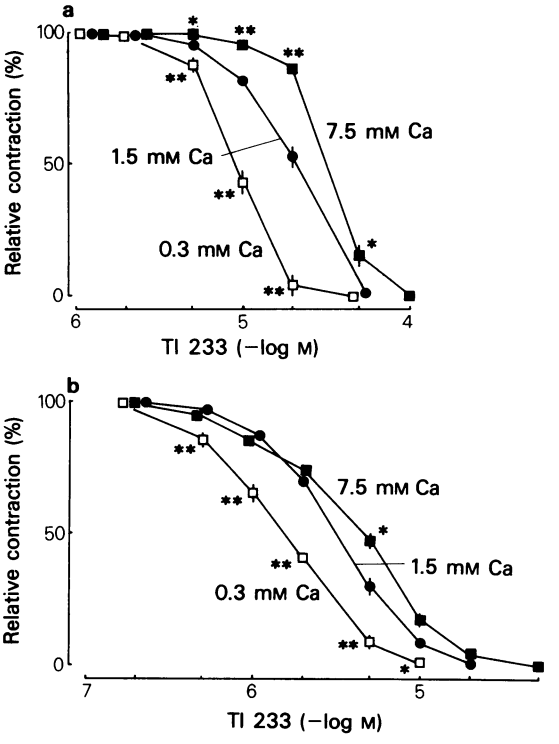

Figure 2 Effect of cumulative application of TI 233 on the K-induced sustained contraction in rabbit aorta (a) and guinea-pig taenia coli (b) in different Ca concentrations; (□), 0.3 mM; (●), 1.5 mM; (■) 7.5 mM. The sustained contraction induced by 65.4 mM K in aorta and the sustained contraction induced by 45.4 mM K in taenia coli were taken as control response (100%). Mean values for 4 to 10 experiments are given and s.e.mean is shown by vertical bars. * and **: significantly different ($P < 0.05$ and $P < 0.01$, respectively) from control (Ca = 1.5 mM).

TI 233 inhibited the contraction induced by 10^{-6} M noradrenaline in rabbit aorta ($IC_{50} = 2.1 \times 10^{-5}$ M, $n = 4$). In a solution containing 40 mM glucose and gassed with N_2 instead of O_2, 10^{-6} M noradrenaline induced a sustained contraction of approximately 60% of that in normal solution under normoxia (Namm & Zucker, 1973). TI 233 did not inhibit this noradrenaline-induced contraction supported by a high concentration of glucose under hypoxia (Figure 3). In taenia coli, hypoxia decreased the K-induced contractile tension which partially recovered on increasing the concentration of glucose to 40 mM (Nasu, Yui, Nakagawa & Ishida, 1982; Karaki, Suzuki, Ozaki, Urakawa & Ishida, 1982b). The effect of TI 233 was significantly less on this contraction supported by anaerobic metabolism (IC_{50} increased

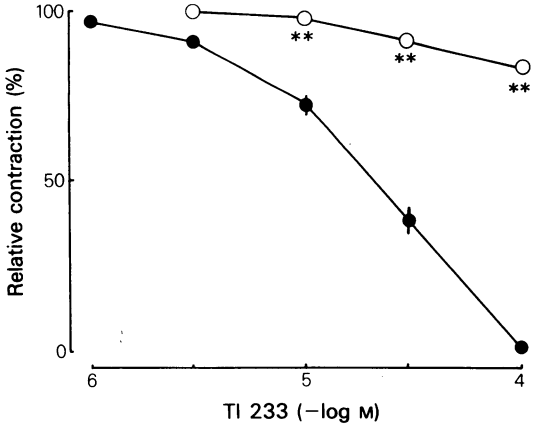

Figure 3 Effect of cumulative application of TI 233 on the noradrenaline (10^{-6} M)-induced sustained contraction in rabbit aorta in normal solution (●) or in the presence of 40 mM glucose under hypoxia (○). The sustained contractile tension induced by noradrenaline (10^{-6} M) was taken as 100% on the ordinate scale. Mean values for 4 experiments are given and s.e.mean is shown by vertical bars. **: significantly different ($P<0.01$) from control.

by 69%) (Figure 4). The sustained tension induced by K in the aorta was not affected by removal of glucose from the medium. In the absence of glucose, however, the inhibitory effect of TI 233 was significantly augmented (IC_{50} decreased by 33%) (Figure 5).

Oxygen consumption

As shown in Table 1, 3×10^{-5} M TI 233 significantly

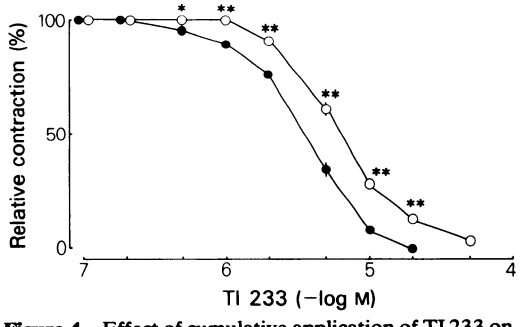

Figure 4 Effect of cumulative application of TI 233 on the K-induced sustained contraction in guinea-pig taenia coli in normal solution (●) or in the presence of 40 mM glucose under hypoxia (○). The sustained contraction induced by 45.4 mM K was taken as 100% on the ordinate scale. Mean values for 5 experiments are given and s.e.mean is shown by vertical bars. * and **: significantly different ($P<0.05$ and $P<0.01$, respectively) from control.

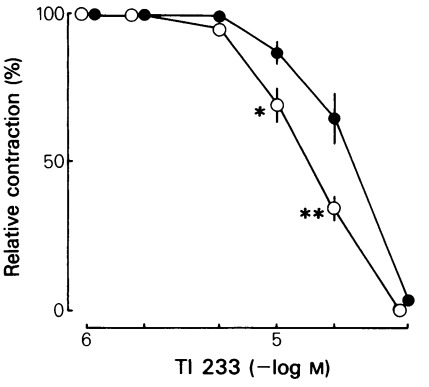

Figure 5 Effect of cumulative application of TI 233 on the K-induced sustained contraction in rabbit aorta in normal solution (●) or in glucose-free solution (○). The sustained contraction induced by 65.4 mM K was taken as 100%. Mean values for 6 experiments are given and s.e.mean is shown by vertical bars. * and **: significantly different ($P<0.05$ and $P<0.01$, respectively) from control.

decreased the resting oxygen consumption in aorta and taenia coli. This inhibitor also decreased the K-stimulated oxygen consumption to the resting level in taenia coli and aorta.

ATP content

As shown in Table 2, 3×10^{-5} M TI 233 significantly decreased the ATP content of aorta and taenia coli. In K-depolarized muscles, ATP content did not change significantly from resting levels. Addition of 3×10^{-5} M TI 233 to the depolarized muscles decreased the ATP content to a level significantly lower than the control.

Cellular Ca content

Table 3 shows the changes in cellular Ca content of

Table 1 Effect of TI 233 (3×10^{-5} M) on the rate of oxygen consumption in rabbit aorta and guinea-pig taenia coli

Condition	Rate of O_2 consumption (μmol g^{-1} min^{-1})	
	aorta	taenia coli
Control	0.089 ± 0.006	0.452 ± 0.020
TI 233	0.049 ± 0.009**	0.325 ± 0.028*
K	0.235 ± 0.047*	0.652 ± 0.039**
K + TI 233	0.065 ± 0.009**	0.386 ± 0.090*

The mean values ± s.e. of 4 experiments are shown. * and **: significantly different ($P<0.05$, $P<0.01$ respectively) from control.

Table 2 Effect of TI 233 on the ATP content in rabbit aorta and guinea-pig taenia coli

Condition	ATP content (μmol g^{-1})	
	aorta	taenia coli
Control	0.932 ± 0.009	2.48 ± 0.07
TI 233	0.736 ± 0.061**	1.80 ± 0.16**
K	0.909 ± 0.037	2.13 ± 0.21
K + TI 233	0.570 ± 0.046**	1.75 ± 0.14**

Muscle preparations were incubated in normal or high K solution with or without TI 233 (3×10^{-5} M) for 30 min. The mean values ± s.e.mean of 6 experiments are shown. **: significantly different ($P < 0.01$) from control.

Table 3 Effect of TI 233 on cellular Ca content in rabbit aorta

Condition	Ca content (nmol g^{-1})	
Control	140.1 ± 3.3††	(6)
K	249.5 ± 17.8**	(6)
TI 233 5×10^{-5} M	129.1 ± 3.2*,††	(6)
TI 233 5×10^{-6} M + K	244.3 ± 20.3**	(6)
TI 233 1×10^{-5} M + K	249.1 ± 19.8**	(6)
TI 233 2×10^{-5} M + K	193.8 ± 16.2**,†	(6)
TI 233 5×10^{-5} M + K	179.9 ± 13.5*,††	(6)
TI 233 2×10^{-4} M + K	124.6 ± 3.3**,††	(9)

Muscle strips were loaded with ^{45}Ca for 30 min in normal or high K solution with or without TI 233. The means ± s.e.mean of 6 to 9 experiments are shown. * and **: significantly different ($P < 0.05$ and $P < 0.01$, respectively) from control. † and ††: significantly different ($P < 0.05$ and $P < 0.01$, respectively) from K-stimulated muscles.

the muscles obtained by a modified lanthanum method. In rabbit aorta, 5×10^{-5} M TI 233 decreased resting Ca content. During the K-induced contraction, cellular Ca content significantly increased and TI 233 showed a concentration-dependent inhibition of the K-induced increase; the IC_{50} for inhibiting the cellular Ca content was approximately 2×10^{-5} M. In taenia coli, 3×10^{-5} M TI 233 did not change the resting Ca content while the K-induced increase in Ca content was decreased to the resting level by the same concentration of TI 233.

Discussion

The inhibitory effect of TI 233 on K-induced contraction was competitively antagonized by external Ca in rabbit aorta. Also in taenia coli, the decrease in Ca concentration shifted the concentration-inhibition curve to the left although the increase in Ca concentration had only an equivocal effect. In aorta, the K-induced increase in cellular Ca content was decreased by TI 233 and the IC_{50} values for contraction (2.1×10^{-5} M) and for Ca content (2×10^{-5} M) were identical. Also in taenia coli, TI 233 inhibited the K-induced increase in cellular Ca content. These results suggest that at least a part of the inhibitory effect of TI 233 is attributable to the Ca antagonistic effect. Organic Ca antagonists like verapamil and D600 are, however, known to have little effect on the noradrenaline-induced contraction in rabbit aorta (Golenhofen & Weston, 1976; Ito, Karaki & Urakawa, 1979). Therefore, the inhibitory effect of TI 233 is not solely attributable to the Ca antagonistic effect.

Since mitochondrial inhibitors are known to inhibit the aortic contraction induced by adrenoceptor agonists (Detar & Bohr, 1968; Namm & Zucker, 1973; Karaki & Weiss, 1981) and also the K-induced sustained contraction in taenia coli (Pfaffman, Urakawa & Holland, 1965), we examined the effects of TI 233 on the muscles under different metabolic conditions. Inhibitory effects of hypoxia on the noradrenaline-induced contraction in aorta and the K-induced contraction in taenia coli were partially reversed by raising the concentration of external glucose, possibly because the addition of glucose stimulated anaerobic glycolysis (Namm & Zucker, 1973; Nasu *et al.*, 1982; Karaki *et al.*, 1982b). In this anaerobic condition, TI 233 did not inhibit the noradrenaline-induced contraction in aorta and had less inhibitory effect on the K-induced contraction in taenia coli. These results suggest that the inhibitory effects of TI 233 on the noradrenaline-induced contraction in aorta and a part of the effect on the K-induced contraction in taenia coli are attributable to the inhibition of aerobic metabolism. The inhibitory effect of mitochondrial inhibitors on smooth muscle contraction is not antagonized by external Ca (unpublished observation) and this may be the reason why the increase in Ca concentration did not antagonize substantially the effect of TI 233 on the K-induced contraction in taenia coli.

The K-induced contraction in aorta is rarely inhibited by mitochondrial inhibitors (Karaki & Weiss, 1981; Karaki *et al.*, 1982b) or by glucose-free solution (Coe, Detar & Bohr, 1968). In the absence of glucose, however, hypoxia strongly inhibited the K-induced contraction in aorta. Similarly, TI 233 more strongly inhibited the K-induced contraction in aorta in the absence of glucose. This result also suggests that TI 233 affects the smooth muscle contraction by inhibiting aerobic metabolism. Inhibition of the rate of oxygen consumption and decrease in the ATP content induced by TI 233 in both aorta and taenia coli strongly support the above suggestion.

TI 233 is reported to interact with calmodulin and thus inhibit myosin light chain kinase and Ca-dependent ATPase of chicken gizzard actomyosin with an IC_{50} of approximately 1×10^{-5} M (Hidaka *et al.*, 1980). In saponin-treated or chemically 'skinned' smooth muscle of taenia coli (Sparrow, Mrwa, Hoffmann & Rüegg, 1981) and chicken gizzard (Kerrick, Hoar, Cassidy, Bolles & Malencik, 1981), calmodulin inhibitors inhibited contraction. These results suggest the possibility that the inhibitory effect of TI 233 on intact smooth muscle is also due to a calmodulin-antagonistic action. However, this is not likely since the inhibitory effect of calmodulin-antagonists is reversed only by the addition of calmodulin but not by the addition of Ca (Hidaka *et al.*, 1980) while the inhibitory effect of TI 233 on K-induced contraction was modified by Ca (Figure 1).

In conclusion, TI 233 seems to inhibit the K-induced contraction in rabbit aorta and guinea-pig taenia coli mainly by a Ca-antagonistic effect. On the other hand, impairment of the noradrenaline-induced contraction in aorta and a part of K-induced contraction in taenia coli may be due to mitochondrial inhibition by TI 233.

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