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EFFECTS OF CALMODULIN ANTAGONISTS ON TENSION AND CELLULAR CALCIUM CONTENT IN DEPOLARIZED VASCULAR AND INTESTINAL SMOOTH MUSCLES

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1 Several putative calmodulin antagonists have been examined for their inhibitory action on muscle tension and cellular Ca content in the K-depolarized vascular and intestinal smooth muscles.

2 The 65.4 mM K-induced sustained contraction in the media-intimal layer of rabbit aorta and the 45.4 mM K-induced sustained contraction in guinea-pig taenia coli were inhibited by the calmodulin antagonists, prenylamine, chlorpromazine, N^2-dansyl-L-arginine-4-*t*-butylpiperadine amide (No. 233), and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), and also by the organic Ca antagonists, verapamil and diltiazem.

3 The cellular Ca content in rabbit aorta and guinea-pig taenia coli as measured by a modified lanthanum technique increased in the high-K solutions. The increments were inhibited by these antagonists at concentrations similar to those required to inhibit the K-induced contractions. However, W-7 did not change (in aorta) or only slightly decreased (in taenia coli) the K-induced increase in the cellular Ca content.

4 A high concentration (2×10^{-4} M) of W-7 increased the resting cellular Ca content without increasing the muscle tension in aorta. The increment was inhibited by verapamil, sodium nitroprusside or hypoxia (N_2 aeration).

5 It is suggested that the inhibitory effects of prenylamine, chlorpromazine and No. 233 may be attributed mainly to the Ca antagonistic effect whereas W-7 may inhibit the process beyond the transmembrane Ca influx.

Introduction

It is suggested that calmodulin regulates smooth muscle contraction through the activation of myosin light chain kinase in the presence of Ca (Dabrowska, Sherry, Aromatorio & Hartshorne, 1978; Yagi, Yazawa, Kakiuchi, Ohshima & Uenishi, 1978). Calmodulin may also be involved in a Ca transporting process in erythrocyte (Gopinath & Vincenti, 1977; Jarret & Penniston, 1977) and heart sarcolemma (Caroni & Carafoli, 1981). In the smooth muscle microsomes prepared from canine trachea (Hogaboom & Fedan, 1981) and rat aorta (Godfraind, 1981), but not in the microsomes from porcine coronary artery (Wuytack & Casteels, 1980), adenosine 5′-triphosphate (ATP)-dependent Ca uptake was significantly increased by calmodulin. The calmodulin-regulated reactions are inhibited by compounds which selectively bind to calmodulin. Such compounds include prenylamine (Honda, Katsuki & Sakai, 1973), chlorpromazine (Levin & Weiss, 1976), N^2-dansyl-L-arginine-4-*t*-butylpiperidine amide (No. 233: Hidaka, Yamaki, Naka, Tanaka, Hayashi & Kobayashi, 1980) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7: Hidaka, Asano, Iwadare, Matsumoto, Totsuka & Aoki, 1978). Prenylamine (Fleckenstein, Grün, Tritthart & Byon, 1971), chlorpromazine (Shibata & Carrier, 1967), W-7 (Hidaka *et al.*, 1978) and No. 233 (Kanamori, Naka, Asano & Hidaka, 1981) are also reported to inhibit contractions in isolated smooth muscle preparations. The present study is concerned with the effects of these putative calmodulin antagonists on contractile function and cellular Ca content in the potassium-depolarized vascular and intestinal snooth muscle tissues.

Methods

Helical strips (3–4 mm wide) of thoracic aorta, isolated from male New Zealand white rabbits weighing 2.0 to 2.5 kg, were prepared as described by Furchgott (1960). The adventitial layer was removed from the media-intimal layer as described by Karaki & Urakawa (1977) to avoid the effects of endogenous catecholamines, and media-intimal strips approximately 10 mm or 5 mm in length were prepared for tension experiments or ^{45}Ca uptake experiments,

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respectively. Segments of taenia coli, approximately 15 mm in length for tension experiments or approximately 5 mm in length for ^{45}Ca uptake experiments, were removed from the caecum of male white guinea-pigs weighing 250 to 300 g. 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 for more than 60 min in normal solution of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 24.0, and glucose 5.5. The solution was continuously bubbled with a 95% O_2 and 5% CO_2 mixture at 37°C and pH 7.4 or in some experiments, with a 95% N_2 and 5% CO_2 mixture in order to produce hypoxia. Isosmotic 65.4 mM K solution was made by replacing 60 mM NaCl in the above solution with equimolar KCl. Hyperosmotic 45.4 mM K solution was made by increasing the KCl concentration to 45.4 mM in the above solution.

Isometric contraction of the muscle strips was recorded with a force displacement transducer connected to a polygraph (Nihon Kohden, Japan). When the high-K solution induced a sustained contraction, an antagonist was cumulatively applied to the muscle bath to obtain a concentration-inhibition curve. The concentration of the antagonist required to inhibit the sustained contraction by 50% (IC_{50}) was calculated from this curve.

Cellur Ca content was measured by a lanthanum technique (van Breemen & McNaughton, 1970), modified by Karaki & Weiss (1979). Muscle strips were incubated for 30 min in a solution containing a tracer amount of ^{45}Ca with or without a high concentration of K. An antagonist was added 30 min before and also during the ^{45}Ca incubation. After the incubation with ^{45}Ca, the muscle strips were washed for 60 min in an ice cold lanthanum solution. High concentration of lanthanum has been shown to remove effectively the extracellular ^{45}Ca, while the low temperature inhibits transmembrane ^{45}Ca fluxes and thus preserves intracellular ^{45}Ca (Karaki & Weiss, 1979; 1980). The lanthanum solution contained $LaCl_3$ 73.0 mM), glucose 5.5 mM and tris(hydroxymethyl)aminomethane 24 nM, adjusted to pH 6.8 at 0.5°C with 1N maleic acid (Karaki & Weiss, 1979). Then, the muscle strips were solubilized with Lumasolve (Lumac, Netherlands) and the radioactivity was counted in a Packard liquid scintillation spectrometer. Results of the experiments are given as nmol Ca/g initial wet weight of the tissue (Karaki & Weiss, 1980).

$^{45}CaCl_2$ (New England Nuclear, U.S.A.), W-7 (gift from Dr H. Hidaka, Mie University School of Medicine), N-(6-aminohexyl)-l-naphthalenesulphonamide (W-5: Riken Co. Ltd., Japan), No. 233 (Mitsubishi Kasei Co. Ltd., Japan), chlorpromazine (Sigma Chemicals, U.S.A.), prenylamine lactate (Hoechst Japan, Japan), verapamil (Eisai Co. Ltd., Japan), diltiazem (Tanabe Co. Ltd., Japan) and sodium nitroprusside (Wako Pure Chemicals, Japan) were used.

Statistical significance was determined by the Student's *t* test.

Results

Inhibition of K-induced contraction

The concentration-inhibition curves and the IC_{50} values for the calmodulin antagonists and the organic Ca antagonists on the K-induced contraction in rabbit aorta and guinea-pig taenia coli are shown in Figure 1 and Table 1.

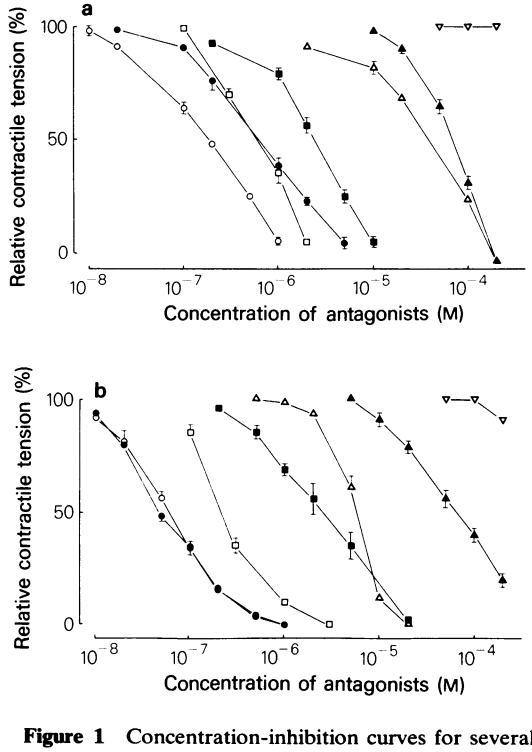

Figure 1 Concentration-inhibition curves for several antagonists in media-intimal layer of rabbit aorta (a) and guinea-pig taenia coli (b). The sustained contraction induced by 65.4 mM K (1.2 ± 0.1 g, $n = 42$) was taken as 100% for aorta and the sustained contraction induced by 45.4 mM K (9.5 ± 0.3 g, $n = 50$) was taken as 100% for taenia coli on the ordinate scale. Mean value was obtained from 4 to 10 experiments and s.e. is shown by vertical bars. (□) Prenylamine; (■) chlorpromazine; (△) No. 233; (▲) W-7; (▽) W-5; (○) verapamil; (●) diltiazem.

Table 1 IC_{50} values for several antagonists on K-induced contractions in rabbit aorta and guinea-pig taenia coli

Antagonists	*K-induced contraction in* *Rabbit aorta*	*Guinea-pig taenia coli*
Prenylamine	$6.4 \pm 0.8 \times 10^{-7}$ M	$1.2 \pm 0.2 \times 10^{-7}$ M
Chlorpromazine	$2.3 \pm 0.3 \times 10^{-6}$ M	$2.9 \pm 0.6 \times 10^{-6}$ M
No. 233	$4.7 \pm 0.3 \times 10^{-5}$ M	$5.8 \pm 0.3 \times 10^{-6}$ M
W-7	$6.9 \pm 0.8 \times 10^{-5}$ M	$7.6 \pm 1.1 \times 10^{-5}$ M
W-5	$> 2.0 \times 10^{-4}$ M	$> 2.0 \pm 10^{-4}$ M
Verapamil	$1.8 \pm 0.1 \times 10^{-7}$ M	$6.0 \pm 0.6 \times 10^{-8}$ M
Diltiazem	$6.4 \pm 0.5 \times 10^{-7}$ M	$5.2 \pm 0.5 \times 10^{-8}$ M

Mean ± s.e. for 4 to 10 experiments are shown.

In rabbit aorta, 65.4 mM K induced a sustained contraction averaging 1.2 ± 0.1 g ($n = 42$). The contraction was inhibited by all the antagonists examined except W-5. The organic Ca antagonists, verapamil and diltiazem, were the most potent inhibitors. Prenylamine was similar in potency to the organic Ca antagonists, and chlorapromazine was less effective. W-7 and No. 233 were the least effective inhibitors.

In guinea-pig taenia coli, 45.4 mM K induced a sustained contraction averaging 9.5 ± 0.3 g ($n = 50$). The contraction was inhibited by all the antagonists examined. Again, verapamil and diltiazem were the most effective and prenylamine less so. Chlorpomazine was less effective than prenylamine. No. 233, whis was the least effective in the aorta, was approximately 10 times more effective in taenia coli. W-7 was the least effective apart from W-5 which showed only a slight effect at a concentration of 2×10^{-4} M.

Effects on cellular Ca content

In Table 2, effects of calmodulin antagonists and organic Ca antagonists on the resting and the K-stimulated cellular Ca contents are shown. The concentrations of the antagonists to inhibit the K-induced contractions more than 90% were chosen from the concentration-inhibition curves in Figure 1. Since W-5 had very little, if any, inhibitory effect, the highest concentration (2×10^{-4} M) was used. In rabbit aorta, W-7 in a concentration of 2×10^{-4} M increased the resting cellular Ca content to a level similar to that of the K-stimulated level. The other antagonists, as well as W-7 at a concentration of 1×10^{-4} M, did not change the resting Ca content. In 65.4 mM K solution, the cellular Ca content increased significantly. Prenylamine, chloromazine, No. 233, verapamil and diltiazem decreased the cellular Ca content to the resting level; however, W-7 did not

Table 2 Effects of several antagonists on the cellular Ca content in normal and high K solutions in rabbit aorta and guinea-pig taenia coli

		Cellular Ca content (nmol/g wet tissue ± s.e.)			
		Rabbit aorta		*Guinea-pig taenia coli*	
Antagonists	*Conc* (M)	5.4 mM K	65.4 mM K	5.4 mM K	45.4 mM K
Control		255.2 ± 5.4	379.4 ± 14.2*	198.8 ± 10.7	468.0 ± 22.8*
Prenylamine	2×10^{-6}	259.4 ± 17.7	271.8 ± 15.8	184.8 ± 18.9	178.8 ± 10.5
Chlorpromazine	2×10^{-5}	263.3 ± 26.8	260.5 ± 12.7	233.7 ± 14.3	242.3 ± 19.2
No. 233	2×10^{-5}	not tested		197.2 ± 15.3	239.0 ± 28.2
	2×10^{-4}	261.4 ± 8.5	234.3 ± 6.0	not tested	
W-7	1×10^{-4}	270.3 ± 19.8	362.2 ± 17.7*	194.2 ± 14.4	392.2 × 21.5*
	2×10^{-4}	423.1 ± 53.7*	397.8 ± 22.2*	259.0 ± 6.5*	381.7 ± 42.9*
W-5	2×10^{-4}	278.0 ± 10.4	325.6 ± 12.6*	not tested	
Verapamil	1×10^{-6}	271.9 ± 17.7	260.0 ± 25.5	191.7 ± 11.4	196.1 ± 15.2
Diltiazem	5×10^{-6}	266.8 ± 20.0	278.4 ± 16.8	180.7 ± 23.9	205.2 ± 17.2

All muscles were incubated with ^{45}Ca for 30 min before a 60 min washout in lanthanum solution. Muscles were exposed to high concentration of K for the full ^{45}Ca incubation period and to the antagonists for an additional 30 min before ^{45}Ca incubation. *Significantly different ($P < 0.05$) from the control values in normal (5.4 mM K) solution. $n = 6$ to 18.

and W-5 decreased it only slightly. In taenia coli, only W-7 at a concentration of 2×10^{-4} M slightly, but significantly, increased the resting cellular Ca content. The K-induced increase in the cellular Ca content was inhibited by all the antagonists examined although W-7 showed only a slight effect.

Effects of W-7 on muscle tension and resting Ca content

Since a high concentration of W-7 increased the resting Ca content in smooth muscle preparations, the effects of W-7 were examined further. W-7, 2×10^{-4} M, slightly decreased the resting tension of the aorta during a 60 min incubation period. The same concentration of W-7 also inhibited the spontaneous contractions in taenia coli. The increase in the resting Ca content induced by W-7 in aorta was inhibited by verapamil, sodium nitroprusside or hypoxia (Table 3).

Discussion

Potassium-induced contraction in smooth muscle is the result of an increased Ca influx through a voltage-sensitive Ca channel, which is competitively and specifically inhibited by the organic Ca antagonists (Weiss, 1977; 1981). A part of the Ca entering the cell is taken up by mitochondria and this Ca fraction is detected as a K-induced increase in the cellular Ca content (Karaki & Weiss, 1981; Karaki, Suzuki, Ozaki, Urakawa & Ishida, 1982). Free Ca in the cell binds to leiotonin (Mikawa, Nonomura, Hirata, Ebashi & Kakiuchi, 1978) and causes an activation of the Mg-ATPase activity of actomyosin. Another possibility is that Ca binds to calmodulin, activates the myosin light chain kinase and allows the subsequent actin activation of the myosin ATPase activity, and thus the muscle contraction (for review see Stull & Sanford, 1981; Hartshorne & Mrwa, 1982). It has been reported that the calmodulin antagonists inhibit the Ca-dependent myosin light chain kinase as well as the cyclic nucleotide phosphodiesterase with an IC_{50} of approximately 5×10^{-5} M for prenylamine, chlorpromazine and W-7, approximately 1×10^{-5} M for No. 233 and approximately 2×10^{-4} M for W-5 (Hidaka *et al.*, 1980; Kanamori *et al.*, 1981).

In the present experiments, the organic Ca antagonists, verapamil and diltiazem, inhibited both the K-induced contraction and the increase in the cellular Ca content. Taenia coli was more susceptible to the organic antagonists than aorta. Prenylamine, orginally described as an organic Ca antagonist (Fleckenstein *et al.*, 1971), produced effects similar to verapamil and diltiazem. Chlorpromazine and No. 233 also inhibited the K-induced increases in muscle tension and Ca content. The inhibitory effect of chloropromazine on the K-induced contraction was partially antagonized by raising the external Ca concentration in rabbit aorta (Shibata & Carrier, 1967) and in guinea-pig ileal longitudinal smooth muscle (Chaturvedi, Landon & Sastry, 1978). The effect of No. 233 was similarly modified by changing the external Ca concentration in aorta and taenia coli (our unpublished observations). These and the present results suggest that these antagonists have a Ca antagonistic effect. However, both chlorpromazine and No. 233 seem to have additional effects since a typical organic Ca antagonist, verapamil, inhibits the K-induced contraction but not the noradrenaline-induced contraction in rabbit aorta (Ito, Karaki & Urakawa, 1977) while chlorpromazine and No. 233 inhibit the noradrenaline-induced contraction at concentrations similar to those needed to inhibit the K-induced contraction (Asano, Suzuki & Hidaka, 1982). Further, we found that No. 233 inhibits oxygen consumption in smooth muscle preparations (unpublished).

Although W-7, 2×10^{-4} M, decreased the resting tone of rabbit aorta, it increased the cellular Ca content. The increment was inhibited by verapamil, sodium nitroprusside or hypoxia in rabbit aorta. It has been reported that verapamil inhibits the

Table 3 Effects of verapamil, sodium nitroprusside and hypoxia on W-7-induced increse in the cellular Ca content in rabbit aorta

Conditions	*Cellular Ca content* (nmol/g wet tissue)
Control	264.2 ± 16.4
W-7 2×10^{-4} M	350.0 ± 15.8*
W-7 2×10^{-4} M + verapamil 2×10^{-6} M	243.2 ± 14.6
W-7 2×10^{-4} M + sodium nitroprusside 1×10^{-6} M	268.2 ± 22.6
W-7 2×10^{-4} M + hypoxia (N_2 aeration)	264.2 ± 11.2

All muscles were incubated with ^{45}Ca for 30 min prior to a 60 min washout in lanthanum solution. Muscles were exposed to W-7 for the full ^{45}Ca incubation period and to verapamil, sodium nitroprusside or hypoxia for an additional 30 min before ^{45}Ca incubation. *Significantly different ($P < 0.01$) from control. $n = 6$ each.

voltage-sensitive Ca channel, sodium nitroprusside inhibits the noradrenaline-stimulated inward Ca translocation and hypoxia inhibits the K-stimulated Ca accumulation by mitochondria in smooth muscle preparations (Karaki, Hester & Weiss, 1980; Karaki & Weiss, 1980; 1981; Weiss, 1981; Karaki *et al.*, 1982). A lower concentration (1×10^{-4} M) of W-7 did not have such an effect on the resting Ca content. The K-induced contraction was inhibited by $1-2 \times 10^{-4}$ M W-7 although the same concentration of W-7 did not change or only slightly decreased the K-induced increase in the cellular Ca content. These results suggest that the inhibitory effect of W-7 on the smooth muscle contraction is not attributable to the inhibition of transmembrane Ca influx.

In conclusion, it is suggested that prenylamine, chloromazine and No. 233 may inhibit smooth muscle contraction mainly by reducing the Ca influx, whereas W-7 may inhibit the process beyond the transmembrane Ca influx.

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