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Selective inhibition of myosin phosphorylation and tension of hyperplastic arteries by the kinase inhibitor HA1077

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Abstract

To examine possible alterations in myosin light chain phosphorylation in hyperplastic arteries, rabbit strips from right hyperplastic and left normal control carotid arteries were used for experiments 6 weeks after the ballooning procedure. When the hyperplastic artery was stimulated with various concentrations of K^+ (10, 20, 30, 40 and 60 mM), the maximal tension in response to each concentration was significantly higher ($P < 0.05$) than that in the control artery. The maximal extent of myosin light chain phosphorylation induced by 60 mM K^+ in the hyperplastic artery was also significantly higher than that in the control (55.1 ± 4.1 vs. $45.1 \pm 3.2\%$, mean $\pm$ S.D.). However, the $[Ca^{2+}]_i$ response to elevated K^+ in hyperplastic arteries was much the same as that in control arteries, when measured with fura-PE3. HA1077 (1-5-(isoquinolinesulfonyl)-homopiperazine), a protein kinase inhibitor, was about 3–5 times more effective in inhibiting the tension and myosin light chain phosphorylation induced by 60 mM K^+ in the hyperplastic artery than in the control artery. Nifedipine inhibited the tension and myosin light chain phosphorylation to the same extent in control and hyperplastic arteries. Thus, an alteration of the myosin light chain phosphorylation system, but not an alteration of Ca^{2+} mobilization, may be involved in the enhanced contraction of the hyperplastic artery. The enhanced phosphorylation of myosin light chain may be sensitive to HA1077.

Keywords: Intimal hyperplasia; Hyperreactivity; Myosin light chain phosphorylation; Protein kinase inhibitor; HA1077; $[Ca^{2+}]_i$

1. Introduction

Augmented vasoconstriction, which often occurs in regions within hyperplastic lesions, is one cause of vasospasm (Ginsburg et al., 1984; Bertrand et al., 1989). The accumulation of cholesterol in the membrane of smooth muscle cells may alter the properties of receptors and lead to arterial hyperreactivity (Henry and Yokoyama, 1980; Kawachi et al., 1984). However, vasospasm was found to occur in the regions of intimal hyperplasia in miniature pigs, with no apparent relation to the level of serum cholesterol (Egashira et al., 1986). This type of vasospasm cannot be explained simply by the accumulation of cholesterol in the membrane.

It was reported that, following denudation of the endothelium, smooth muscle cells in the region of intimal hyperplasia were morphologically and biochemically different from those present in normal media (Kocher et al., 1984; Mosse et al., 1985). In smooth muscle cells located in the region of intimal hyperplasia, changes were noted in contractile elements such as the actin isoform (Kocher et al., 1984), intermediate filament (Gabbiani et al., 1982), caldesmon isoform (Glukhova et al., 1988) and myosin heavy chain isoform (Kuro-o et al., 1991). However, these changes cannot directly explain the enhanced responsiveness of the hyperplastic artery. Contraction of smooth muscle cells in response to an appropriate agonist is mediated by a transient increase in the concentration of intracellular free Ca^{2+} (Morgan and Morgan, 1984; Himpens and Somlyo, 1988). A well-defined molecular target of this Ca^{2+} message is the calmodulin-myosin light chain kinase pathway (Hai and Murphy, 1983; Kamm and Stull, 1989). Myosin light chain phosphorylation is one of the most important mechanisms involved in the contraction of smooth muscle. We reported that augmented vasoconstriction in the hyperplastic artery fol-

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lowing endothelial denudation was induced by various types of agonists and was accompanied by an enhanced and sustained phosphorylation of myosin light chain (Seto et al., 1993).

HA1077 (1-5-(isoquinolinesulfonyl)-homopiperazine) (Morikawa et al., 1989), an inhibitor of protein kinases including myosin light chain kinase, inhibits myosin light chain phosphorylation and tension development induced by various agonists, as determined in experiments using rabbit aortic strips (Asano et al., 1987; Seto et al., 1991). HA1077 also produced vasodilation of arteries in the case of delayed cerebral vasospasm resulting from subarachnoid hemorrhage in dogs (Takayasu et al., 1986). We report here changes in $[Ca^{2+}]_i$ and myosin light chain phosphorylation patterns in hyperplastic arteries, determined using HA1077 and a Ca^{2+} channel blocker.

2. Materials and methods

2.1. Endothelial denudation

Twenty 10-week-old Japanese white rabbits were anesthetized with 25 mg/kg i.v. of sodium pentobarbital. Under aseptic surgical conditions, the right carotid artery was exposed and an arterial embolectomy catheter (12-060-2F/CV-1035, American Edwards Laboratories, Santa Ana, CA, USA) was inserted up to the proximal portion of the artery through a small longitudinal incision, as described elsewhere (Azuma et al., 1990; Seto et al., 1993). The intraluminal surface of the artery was then subjected to one pulse of ballooning. For the left carotid artery a sham operation was carried out and this artery served as a control.

2.2. Tissue preparation

The right and left carotid arteries, excised from the rabbits 6 weeks after this denudation, were placed in normal physiological salt solution (PSS). The normal PSS was composed of (in mM): NaCl, 115; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; and glucose, 10.0. After removal of fat and connective tissue, a 4-mm-wide transverse ring was cut off. Five pieces were prepared from one carotid artery. Each piece was cut in half, with one half being used for tension measurement (2-mm-wide transverse strip), and the other for light microscopy analysis. The intimal surface of the transverse strip was rubbed gently with a cotton swab to remove the endothelium. The removal of the endothelium was confirmed by the abolition of a relaxation response to acetylcholine in arteries precontracted with norepinephrine (Furchgott and Zawadzki, 1980).

2.3. Tension measurement and normalization

Each strip was mounted isometrically under 1.0 g tension in a 10 ml tissue bath (37°C) containing PSS (pH 7.4) aerated with 5% CO_2-95% O_2. Tension generation was monitored with an isometric transducer (Nihon Kohden, TB-611T) coupled to a polygraph recorder (Nihon Kohden, RM-6000). After a 90-min equilibration period, the strip contracted when we added 60 mM K^+ to the bath followed by immediate washing with normal PSS. This precontraction-relaxation procedure was repeated twice at 90-min intervals to obtain a stable response, and then a 90-min equilibration period was allowed. The strips were stimulated to contract (the fourth contraction) with the cumulative addition of K^+ (10, 20, 30, 40 and 60 mM) followed by washing with normal PSS. After a 50-min equilibration period, various concentrations of HA1077 (10–300 μM) or nifedipine (1–1000 nM) were added. After a 10-min incubation with the above inhibitors, the fifth contraction was induced by the cumulative addition of K^+ (10, 20, 30, 40 and 60 mM). Typical recordings of the response of strips (the fourth and fifth contraction) from the control left carotid artery (A) and the hyperplastic right carotid artery (B) are shown in Fig. 1. The degree of inhibition was expressed as a percentage of the fifth to the fourth contraction. The normalized tension was represented as the tension per cross-sectional area (g/cm^2) obtained by light microscopy analysis.

2.4. Anti-myosin light chain antibody

The myosin light chain of chicken gizzard was purified to apparent homogeneity, as described elsewhere (Hathaway and Haeberle, 1983). The anti-myosin light

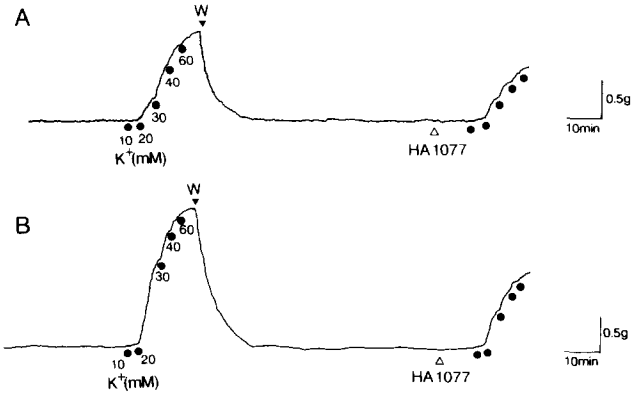

Fig. 1. Typical recordings of the contractile response of strips from control (A) and hyperplastic (B) arteries. The strips were stimulated to contract with the cumulative addition of K^+ (10, 20, 30, 40 and 60 mM), followed by washing with normal PSS. Various concentrations of HA1077 or nifedipine were added 10 min before stimulation with K^+.

chain IgG obtained from rabbits was purified by affinity chromatography on myosin light chain-coupled Sepharose 4B and the purified antibody had a high specificity for myosin light chain (Sasaki et al., 1990).

2.5. Measurement of myosin light chain phosphorylation

Strips mounted for isometric studies were frozen by immersion in acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol cooled with dry ice. Frozen tissues were washed twice with acetone containing 10 mM dithiothreitol to remove the trichloroacetic acid, dried and cut with surgical scissors into small pieces and homogenized for 2 min at 4°C, using a Potter Teflon homogenizer, in 35 μl of glycerol-polyacrylamide gel electrophoresis (PAGE) sample buffer that contained 20 mM tris(hydroxymethyl)aminoethane base-22 mM glycine (pH 8.6), 10 mM dithiothreitol, 8 M urea, and 0.1% bromphenol blue.

The urea-solubilized samples (2 μl) were subjected to glycerol-PAGE/immunoblot analysis, using the specific myosin light chain antibody (Hathaway and Haeberle, 1985; Persechini et al., 1986; Taylor and Stull, 1988). The region containing myosin light chain was visualized as dark blue bands, using a Vectastain ABC kit (Hsu et al., 1981). The validity of the myosin light chain phosphorylation assay system was demonstrated using myosin light chain-specific phosphatase (Yoshida and Yagi, 1988) purified from chicken gizzard as described by Seto et al. (1990). Densitometry of immunoblots and quantitation of absorbance peaks were performed with a Densitron PAN-FV (Jookoo, Tokyo, Japan) equipped with a recording integrator. The extent of myosin light chain phosphorylation is expressed as the percentage myosin light chain in the monophosphorylated form.

2.6. Measurement of cytoplasmic Ca^{2+} concentration

Cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the fluorescent Ca^{2+} indicator fura-PE3, as previously described (Naganobu and Ito, 1994). To measure $[Ca^{2+}]_i$, we used fura-PE3 instead of fura-2 as a fluorescent Ca^{2+} indicator, because this indicator hardly leaks from cells, as compared with fura-2 (Dr. H. Karaki, personal communication). The carotid arteries were excised from a rabbit 6 weeks after the ballooning procedures which was done by gently rubbing the intimal surface. Carotid strips were loaded with 5 μM fura-PE3/AM and 0.03% cremophore for 12–20 h. The fura-PE3-loaded carotid strips were held horizontally in a temperature-controlled organ bath constructed in a fluorimeter (CAF-100, JASCO, Japan). Fluorescence induced by 340 nm excitation and 380 nm excitation was monitored after stimulation with 60 mM K^+. At the end of the experiment, 10 μM ionomycin and then 10 mM EGTA were applied to get the maximum and minimum fluorescence, respectively. $[Ca^{2+}]_i$ was calculated, as described elsewhere (Grynkiewicz et al., 1985).

2.7. Light microscopy

Five pieces were prepared from strips of a carotid artery for light microscopy analysis. The specimens were fixed in Bouin's solution for 6 h and embedded in paraffin after dehydration and clearing. These sections were stained with hematoxylin-eosin and Elastica-Van Gieson (Weigert, 1898). Intimal hyperplasia of the cross-sections was evaluated by measuring the area of intima and media, at a final magnification of ×70, using a KP-90N planimeter (Uchida, Japan). The data are expressed as a percentage of intimal to medial area.

2.8. Compounds

HA1077 (1-5-(isoquinolinesulfonyl)-homopiperazine) was synthesized from 5-isoquinolinesulfonic acid (Morikawa et al., 1989). Nifedipine was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). The Vectastain ABC kit was from Vector Laboratories (Burlingame, CA, USA). Activated CH-Sepharose 4B was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Fura-PE3 was from Texas Fluorescence (Austin, TX, USA).

2.9. Statistics

Each set of data was expressed as a mean and SD. Student's t-test was used to determine the statistical difference of the means and a $P < 0.05$ value was considered to have statistical significance.

3. Results

Transverse sections of control and hyperplastic carotid arteries are shown in Fig. 2. Intimal hyperplasia was observed in the carotid arteries of all 20 rabbits. The degree of intimal hyperplasia (a percentage of intimal to medial area) was between 30 and 50%, and that of three different regions (distal, medial and proximal regions of identical arteries) was approximately the same; these arteries were used for the following experiments.

The concentration-response curves for tension development elicited by K^+ in hyperplastic and control arteries are shown in Fig. 3. The curve for the hyperplastic artery shifted upward and exhibited significantly reduced ED_{50} values (19.3 ± 1.0 mM vs. 27.5 ± 1.2 mM). The extent of submaximal tension of the hyper-

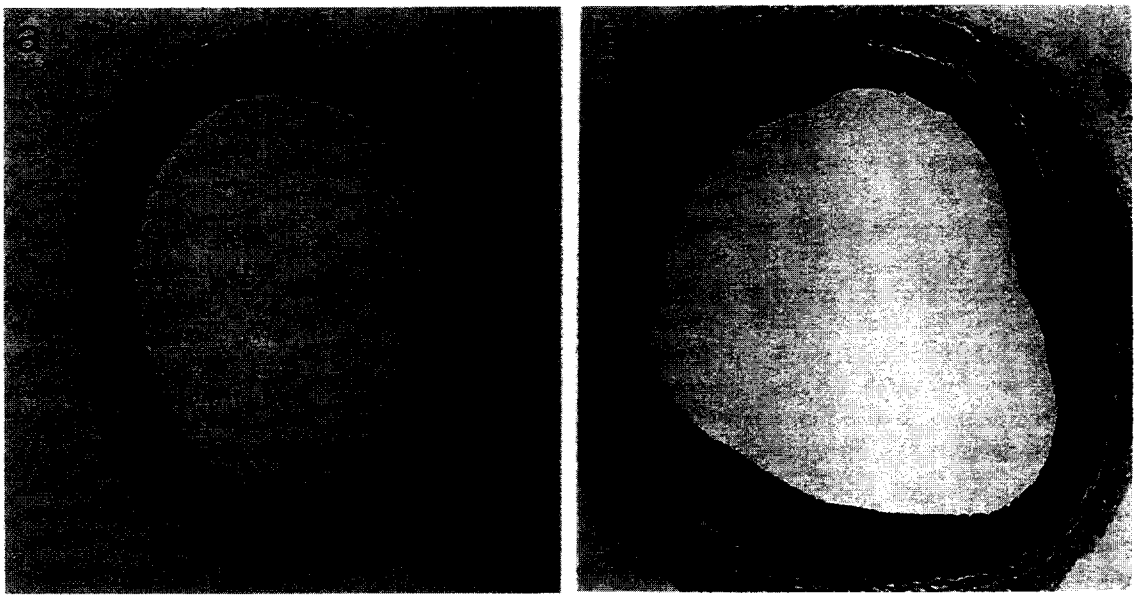

Fig. 2. Histological cross-sections of control (a) and hyperplastic (b) artery 6 weeks after ballooning procedures. Area of the intima in the hyperplastic artery was 38% of that of the media. Elastica-Van Gieson stain. Arrows point to the internal elastica lamina. Original magnification ×30.

plastic artery obtained when stimulated with 60 mM K^+ was also significantly higher than that of the control (314.3 ± 18.9 g/cm^2 vs. 232.2 ± 24.1 g/cm^2). These concentration-response curves were not affected by phentolamine (3 μM) or atropine (1 μM), when these drugs were added to the bath 10 min before stimulation (data not shown). The K^+ stimulation therefore may not involve α-adrenergic or muscarinic mechanisms, in either the hyperplastic or control arteries.

The fura-PE3-loaded hyperplastic and control arteries were stimulated with 60 mM K^+. Changes in $[Ca^{2+}]_i$ are shown in Fig. 4. $[Ca^{2+}]_i$ in both cases increased rapidly for up to 15 s to a maximal value, then slightly decreased and was sustained for up to 5 min. The $[Ca^{2+}]_i$ response to K^+ was the same in the two types of arteries. These results suggest that calcium influx through voltage-dependent calcium channels activated by K^+ stimulation may not be enhanced in the hyper-

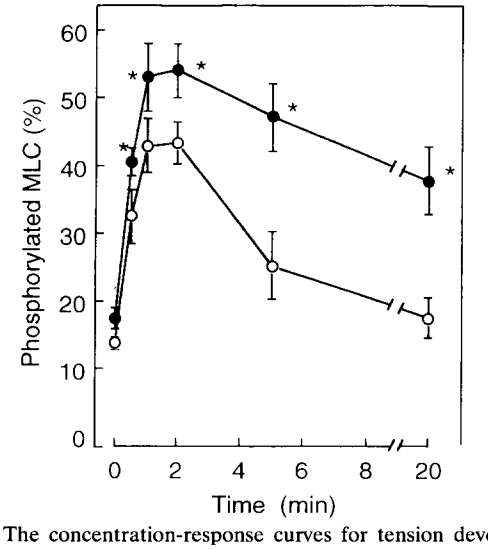

Fig. 3. The concentration-response curves for tension development elicited by K^+. Each curve was obtained by the cumulative addition of K^+ (10, 20, 30,40, 60 mM) to the control (○) and hyperplastic (●) arteries. Each point is the mean ± S.D. of four experiments. * Significantly different ($P < 0.05$) from values for the control artery.

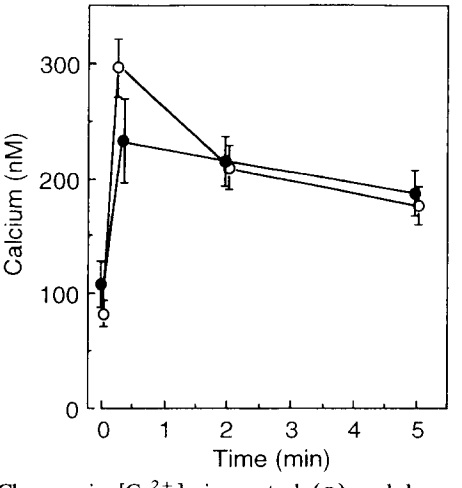

Fig. 4. Changes in $[Ca^{2+}]_i$ in control (○) and hyperplastic (●) arteries stimulated with 60 mM K^+. The fura-PE3 (5 μM)-loaded control and hyperplastic arteries were placed horizontally in a organ bath. Fluorescence induced by 340 nm and 380 nm excitation was monitored after stimulation with 60 mM K^+. Each data point is the mean ± S.D. of 12 experiments. Each point for hyperplasia is not significantly different ($P < 0.05$) from the corresponding control artery point.

plastic artery. If this is indeed the case, the increase in vascular responsiveness cannot be explained by an enhancement of voltage-dependent calcium channel functions in the vascular wall. An alteration of the intracellular contractile mechanisms of smooth muscle may be involved in the changes in the vascular response.

The time course of myosin light chain phosphorylation in both hyperplastic (A) and control (B) arteries stimulated with 60 mM K^+ is shown in Fig. 5. In the case of a hyperplastic artery stimulated with 60 mM K^+, the extent of myosin light chain phosphorylation (18.6 ± 2.0% before stimulation) increased for up to 2 min to a maximal value of 54.1 ± 4.4%, decreased gradually and was sustained for up to 20 min (39.5 ± 5.5%). In case of a control artery, the extent of myosin light chain phosphorylation (15.0 ± 3.0%, before stimulation) increased up to 2 min to a maximal value of 43.1 ± 3.9% and then rapidly decreased to the basal level (19.5 ± 3.9%, 20 min after stimulation). The extent of myosin light chain phosphorylation in the hyperplastic arteries was significantly greater than that of the control artery and the rate of dephosphorylation of myosin light chain phosphorylation in the hyperplastic artery was slower than that in the control artery. These results suggest that the myosin light chain phosphorylation system in the hyperplastic artery is enhanced, particularly in the sustained phase.

The inhibitory effects of HA1077 (protein kinase inhibitor) and nifedipine (voltage-dependent calcium channel inhibitor) on tension development, both in hyperplastic and control arteries, were investigated. HA1077 or nifedipine was added 10 min before the cumulative addition of K^+ (10, 20, 30, 40 and 60 mM). HA1077 dose dependently attenuated the tension development in both hyperplastic and control arteries. The IC_{50} value for tension development in the hyperplastic artery stimulated with 60 mM K^+ was 31.1 ± 9.1 μM and that in the control artery was 78.5 ± 18.1 μM (Table 1). The tension development in the presence of hyperplasia was more sensitive to HA1077 than that in the control artery. In contrast, nifedipine dose dependently attenuated tension development in both hyperplastic and control arteries, in a similar manner. The IC_{50} value for tension development in hyperplastic artery was 19.4 ± 5.9 nM and that in control artery was 18.5 ± 8.7 nM (Table 1). The results were similar for 40 or 30 mM K^+ stimulation.

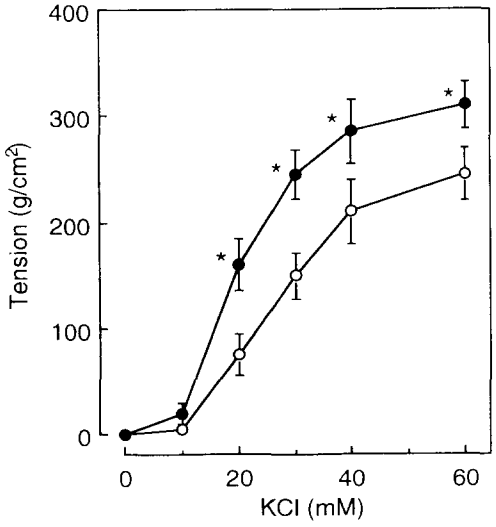

Fig. 5. Time course of myosin light chain phosphorylation in control (○) and hyperplastic (●) arteries stimulated with 60 mM K^+. Each point is the mean ± S.D. of four experiments. * Significantly different ($P < 0.05$) from values for the control artery.

Table 1
IC_{50} values of HA1077 and nifedipine for tension and myosin light chain (MLC) phosphorylation in control and hyperplastic arteries

	Tension		MLC phosphorylation	
	Control	Hyperplasia	Control	Hyperplasia
HA1077 (μM)	78.5 ± 18.1	31.1 ± 9.1 [a]	90.2 ± 5.1	30.0 ± 4.1 [a]
Nifedipine (nM)	18.5 ± 8.7	19.4 ± 5.9	18.5 ± 4.1	13.4 ± 5.2

Various concentrations of HA1077 (0–300 μM) or nifedipine (0–1000 nM) were added 10 min before stimulation with K^+. The IC_{50} values for maximal tension and MLC phosphorylation were calculated. Each value is the mean ± S.D. of five experiments. [a] Significantly different ($P < 0.05$) from values for the control artery.

The effect of HA1077 and nifedipine on myosin light chain phosphorylation in both hyperplastic and control arteries was also investigated. The level of myosin light chain phosphorylation was measured 2 min after the addition of 60 mM K^+ in the presence of various concentrations of HA1077 (0–300 μM) or nifedipine (0–1000 nM). At this time, myosin light chain phosphorylation was maximal. With an increase in HA1077 concentrations, the extent of myosin light chain phosphorylation in both hyperplastic and control arteries decreased. The myosin light chain phosphorylation induced by 60 mM K^+ in the hyperplastic artery was more sensitive than that in the control artery. The IC_{50} value for myosin light chain phosphorylation in the hyperplastic artery was 30.0 ± 4.1 μM and that in the control was 90.2 ± 5.1 μM (Table 1). Nifedipine also dose dependently attenuated the myosin light chain phosphorylation induced by 60 mM K^+ in both hyperplastic and control arteries in a similar manner. The IC_{50} for myosin light chain phosphorylation in the hyperplastic artery was 13.4 ± 5.2 nM and that in the control artery was 18.5 ± 4.1 nM (Table 1). These two values were not significantly different.

4. Discussion

Endothelial denudation and the resultant intimal hyperplasia may play a major role in the enhanced responsiveness of an artery. In the present work, we

focused on this mechanism operative in hyperplastic arteries.

The enhanced contractile response to K^+ in hyperplastic arteries was accompanied by an increase in myosin light chain phosphorylation. One reason for this enhancement may be a greater calcium influx in these arteries. However, our fluorometric study, done using fura-PE3, revealed no difference in the $[Ca^{2+}]_i$ response to elevated K^+ between control and hyperplastic arteries. These results suggest that the function of the calcium channel remained unchanged during the hyperplasia. Despite the same $[Ca^{2+}]_i$ level during K^+ stimulation in hyperplastic arteries, myosin light chain phosphorylation was significantly increased in hyperplastic arteries as compared with that in the control arteries. This clearly means that the increased phosphorylation at a given $[Ca^{2+}]_i$ was responsible for the enhanced contractile response to K^+. The resting $[Ca^{2+}]_i$ value and myosin light chain phosphorylation level, measured before the addition of K^+, was the same in the two arteries. Thus, K^+ stimulation may recruit the altered myosin light chain phosphorylation mechanism in the hyperplastic artery. Nifedipine, an L-type calcium channel blocker, inhibited the contraction and myosin light chain phosphorylation to a similar extent in control and hyperplastic arteries, since the IC_{50} values for both parameters were much the same. This means that the hyperplasia probably did not alter the process from membrane depolarization to elevation in $[Ca^{2+}]_i$, in particular the function and characteristics of the L-type voltage-dependent calcium channel.

In contrast to nifedipine, HA1077 inhibited tension development more strongly in the hyperplastic artery. We reported that HA1077 inhibited myosin light chain kinase in vitro with a K_i value of 36 μM (Sakurada et al., 1994) and that it also inhibited myosin light chain phosphorylation induced by various agonists in the rabbit aortic artery (Seto et al., 1991). These findings lead to the tentative assumption that the major part of the HA1077-induced inhibition of contraction in both control and hyperplastic arteries is a consequence of the inhibition of myosin light chain kinase. In particular, the enhanced phosphorylation of myosin light chain in the hyperplastic artery was more sensitive to HA1077. The HA1077-sensitive myosin light chain phosphorylation mechanism may be altered in hyperplastic arteries. We have reported that HA1077 is 10 times more potent against protein kinase C ($K_i = 3.3$ μM) than against myosin light chain kinase (Seto et al., 1991). Therefore, protein kinase C may be also linked to the alteration of myosin light chain phosphorylation in hyperplastic arteries.

The myosin light chain phosphorylation level is regulated by a balance between myosin light chain phosphorylation by myosin light chain kinase and dephosphorylation by myosin light chain phosphatases (Kamm and Stull, 1989). One possible interpretation for the enhanced and HA1077-sensitive myosin light chain phosphorylation is that myosin light chain kinase is modified (should be phosphorylated by HA1077-sensitive kinase(s)) and that the reactivity of myosin light chain kinase for Ca^{2+} is increased in hyperplastic arteries. Another interpretation is that the activity of the phosphatases is impaired in hyperplastic arteries. A number of protein kinases, including protein kinase C, phosphorylate myosin light chain kinase (Nishikawa et al., 1984; Somlyo et al., 1989). However, the phosphorylation of myosin light chain kinase does not increase the affinity of the enzyme for Ca^{2+}-calmodulin, rather it reduces it (Kamm and Stull, 1989). Thus, it is unlikely that myosin light chain kinase is modified in hyperplastic arteries. The slower rate of dephosphorylation of myosin light chain phosphorylation induced by K^+ in the hyperplastic artery suggests that an impairment of phosphatases is a more plausible cause for the increased myosin light chain phosphorylation. Similar results have been obtained with prostaglandin $F_{2\alpha}$- or norepinephrine-stimulated hyperplastic arteries (Seto et al., 1993).

The activity of myosin light chain phosphatases is thought to be regulated through activation and inactivation cycles of endogenous protein phosphatase inhibitors (Cohen, 1989; Somlyo et al., 1989). Somlyo and colleagues (Somlyo et al., 1989) have suggested that these inhibitors are activated through phosphorylation catalyzed by protein kinase C. HA1077 is a potent inhibitor of protein kinase C. Protein kinase C may be activated by as yet to be identified factors in hyperplastic arteries, and if so, this would contribute to the increased myosin light chain phosphorylation.

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