

Effects of Aging on Contraction and Ca^{2+} Mobilization in Smooth Muscle Cells of the Rat Coronary Artery

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The present study was undertaken to investigate agonist-induced contraction and Ca^{2+} mobilization in the smooth muscles of coronary arteries isolated from young (16 weeks) and aged (more than 72 weeks) rats. Ring segments of the rat coronary anterior descending arteries without endothelium were loaded with $40 \mu\text{mol/L}$ fura-2 acetoxymethyl ester and mounted in an organ bath. Isometric tension was recorded using a force displacement transducer, and the percentage of phosphorylated myosin light chain (MLC_{20}) was measured with Western blotting. Administration of vasoactive substances such as 5-hydroxytryptamine (5-HT), prostagrandin $\text{F}_{2\alpha}$, histamine, endothelin-1 and angiotensin II (A-II) caused dose-dependent contraction in the isolated coronary arteries, and the active tension (mN force/mg tissue weight) evoked by these agonists was significantly greater in the young rats than in the aged ones. On the other hand, the increase in cytoplasmic calcium ($[\text{Ca}^{2+}]_i$) induced by the maximum concentration of the agonists was not significantly different between the 2 groups. The percentage of phosphorylated MLC_{20} induced by a maximum concentration of 5-HT or A-II was significantly greater in the young rats than in the aged ones. The number of nuclei per medial area was fewer in the aged rats than in the young ones. These results suggest that the age-dependent decrease in the agonist-induced contraction of rat coronary arteries is attributable to a reduction of smooth muscle density in the media and/or an impairment in the signal transduction pathway of the smooth muscle cells. One possible site for the impaired pathway is either downstream from the $[\text{Ca}^{2+}]_i$ increase or in some Ca^{2+} independent secondary mechanisms.

Key words: aging; calcium; coronary artery; myosin light chain; vascular smooth muscle

It is generally accepted that the rise and fall of cytoplasmic free calcium ($[\text{Ca}^{2+}]_i$) initiate contraction and relaxation, respectively, in smooth muscles. The calcium ion binds to calmodulin, and the calcium-calmodulin complex activates myosin light chain kinase (MLCK), which in turn leads to the phosphorylation of serine at position 19 on the myosin light chain (MLC_{20}) and subsequent contraction of the muscles (Johnson, 1987; Sweeney et al., 1994). It is also known, however, that $[\text{Ca}^{2+}]_i$ is not necessarily proportional to the levels of MLC_{20} phosphorylation

and developed tension. Thus, secondary mechanisms that can modify Ca^{2+} sensitivity by changing the activities of phosphorylating and dephosphorylating enzymes of the MLC_{20} , have been reported (Somlyo and Somlyo, 1994). A number of neurotransmitters, hormones, and autacoids increase $[\text{Ca}^{2+}]_i$ and cause a contraction of vascular smooth muscles. Such agonists can increase force in permeabilized muscles in which $[\text{Ca}^{2+}]_i$ is maintained at a constant level, indicating an increase in Ca^{2+} sensitivity (Kitazawa et al., 1989; Nishimura et al., 1990).

Abbreviations: A-II, angiotensin II; AM, acetoxymethyl ester; DTT, dithiothreitol; ET-1, endothelin-1; His, histamine; 5-HT, 5-hydroxytryptamine; MLC, myosin light chain; MLCK, MLC kinase; PAGE, polyacrylamide gel electrophoresis; $\text{PGF}_{2\alpha}$, prostagrandin $\text{F}_{2\alpha}$; PSS, physiological salt solution; TCA, trichloroacetic acid

Numerous studies concerning age-dependent changes in the contraction (Tuttle et al., 1966; Tschudi et al., 1995) and relaxation (Fleisch et al., 1970; Hayashi and Toda, 1978; Kawai and Ohhashi, 1989) of vascular smooth muscles have been reported. These changes may lead to ischemic heart disease, which occurs more often in older patients than in the young, if coronary arteries are involved. Little is known in the literature, however, about age-dependent changes in Ca^{2+} mobilization and MLC_{20} phosphorylation in the smooth muscles of the coronary artery. In the present study, agonist-induced changes in $[\text{Ca}^{2+}]_i$, MLC_{20} phosphorylation, and developed tension were examined in the isolated coronary arteries excised from young and aged rats. We found that the level of MLC_{20} phosphorylation in the smooth muscles of coronary arteries stimulated with vasoactive substances was lower in aged rats than in young ones with no difference in $[\text{Ca}^{2+}]_i$.

Materials and Methods

Wistar male rats aged 16 weeks (young, $n = 21$) and 72 weeks (aged, $n = 21$) were killed with 25% urethane (0.02 mL/g, intraperitoneally), and the left anterior descending coronary artery was excised from the heart and placed in normal physiological salt solution (PSS). All procedures were reviewed and approved in accordance with the Guidelines for Animal Experimentation at Tottori University Faculty of Medicine, Yonago, Japan, and conformed to "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" published by the Physiological Society of Japan.

Tissue preparation

The isolated arterial segment was trimmed of fat, connective tissue and myocardia, and was cut into a ring segment of 4 mm long. The intimal surface of the segment was rubbed gently with a single human hair to remove the endothelium. Removal of the endothelium was confirmed by the absence of relaxant response to acetylcholine.

Measurement of isometric tension

After each ring was loaded with fura-2 acetoxy-methyl ester (AM), it was mounted horizontally between 2 hooks in an organ bath filled with 5 mL PSS (pH 7.4) on the stage of an inverted fluorescence microscope (TMD-300, Nikon, Tokyo, Japan). The PSS was maintained at $37 \pm 0.5^\circ\text{C}$ and bubbled with 100% O_2 . A hook holding one end of the ring was fixed to the wall of the bath, and another hook was connected to the lever of a force displacement transducer (UL-10GR, Minebea, Nagano, Japan). The isometric tension detected by the transducer was amplified and recorded in a data analyzer (MacLab Mk III, AD Instrument, Castle Hill, Australia). The specimen was allowed to equilibrate for 60 min at a resting tension of 1.8 mN. The optimal range of resting tension for obtaining maximum contraction was 1.5 to 1.9 mN in young rats and 1.6 to 2.2 mN in aged rats. After the equilibration period, the rings were contracted by 80 mmol/L KCl solution; then the dose-response curves for 5-hydroxytryptamine (5-HT), prostagrandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$), histamine (His), endothelin-1 (ET-1) and angiotensin II (A-II) were obtained by adding each agonist into the PSS for 15 to 20 min for ET-1 and 5 to 10 min for the rest of agonists. At the end of each experiment, the ring was removed from the organ bath, and the wet weight of the preparation was measured. The active tension produced by the agonists was normalized as force (mN) per wet tissue weight (mg).

Measurement of $[\text{Ca}^{2+}]_i$

The arterial ring was loaded for 3 h at 37°C with fluorescence dye 40 $\mu\text{mol/L}$ fura-2 AM. Chremophor EL (0.02%) was added to improve the dye loading. After loading, the tissue was washed out 2 or 3 times with the PSS. The measurement of fluorescence was performed using an inverted fluorescence microscope equipped with a fluorometric system (Quanti Cell 700, Applied Imaging, Newcastle, United Kingdom). Fura-2 was excited with the light of a 100 W xenon lamp at wavelengths of 340 nm and 380

nm. The fluorescence emission (F340 and F380) was recorded at 510 nm and monitored with an intensified computer-controlled display camera. After the noise signal and autofluorescence had been subtracted, the ratio (F340/F380) of fluorescence was calculated as an indicator of $[Ca^{2+}]_i$.

Measurement of MLC₂₀ phosphorylation

The extent of MLC₂₀ phosphorylation in the arterial ring was measured by separation of non-, mono- and diphosphorylated forms by glycerol-polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer of the proteins to a poly vinylidene di-fluoride membrane (Persechini et al., 1986). The relative amount of each form was quantified by an immunoblot procedure as described elsewhere (Hathaway and Haeberle, 1983). After incubation with each agonist, arterial rings were frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol (DTT) cooled with dry ice. The frozen tissues were washed twice with acetone containing 10 mmol/L DTT to remove the TCA and then dried. The dried tissues were cut into small pieces and homogenized for 2 min at 4°C, using a homogenizer in 35 µL of glycerol-PAGE sample buffer which contained 20 mmol/L Tris base-22 mmol/L glycine (pH 8.6), 10 mmol/L DTT, 8 mol/L urea and 0.1% bromphenol blue. The urea-solubilized samples (12 µL) were subjected to glycerol-PAGE / immunoblot analysis, using the specific MLC₂₀ antibody (Seto et al., 1990). The region containing MLC₂₀ was visualized as bands, using an enhanced chemiluminescence Western blotting detection re-

agent. Densitometry of immunoblot and quantitation of absorbance peaks were performed with a National Institutes of Health image analysis (MD 20892) equipped with a recording integrator. The extent of MLC₂₀ phosphorylation is expressed as the percentage of MLC₂₀ in either the mono- or diphosphorylated form.

Morphometric analysis

The isolated left anterior descending coronary artery was gently flushed with 10% formaldehyde and fixed in the solution for at least 24 h. After routine processing, the tissues were embedded in paraffin. They were cut and stained with hematoxylin and eosin or elastica van Gieson stains. The thickness of the muscular media was measured under a light microscope at 4 different sites, and finally the average of the 4 sites was determined. The area of the media was planimetrically quantified using the National Institutes of Health image analysis, and the number of nuclei in the media was counted.

Solutions

The composition of the standard PSS was as follows (mmol/L): 130.0 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 5.5 glucose and 10.0 HEPES. Substituting KCl for equimolar NaCl in the standard PSS made a high K⁺ solution (80 mmol/L).

Drugs and chemicals

The following compounds were used: fura-2 AM (Dojin, Kumamoto, Japan), A-II, cremophor EL, His, 5-HT, PGF_{2α}, (Sigma, St. Louis, MO) and ET-1 (Peptide, Osaka, Japan). The concentration of agonists was expressed as the final organ bath concentration.

Statistics

The data were expressed as mean ± SEM (*n*), where *n* refers to the number of animals. Student's *t* test was used to determine the statistical difference; *P* < 0.05 was considered significant.

Table 1. Body weight, heart weight and coronary ring weight

	Body weight (g)	Heart weight (g)	Coronary ring weight (mg)
Young [16]	388.3 ± 8.3	1.3 ± 0.4	0.34 ± 0.01
Aged [16]	578.8 ± 10.8**	1.7 ± 0.5**	0.96 ± 0.03**

The data are given as mean ± SEM.

[], number of animals.

Significantly different from young rats: ** *P* < 0.01.

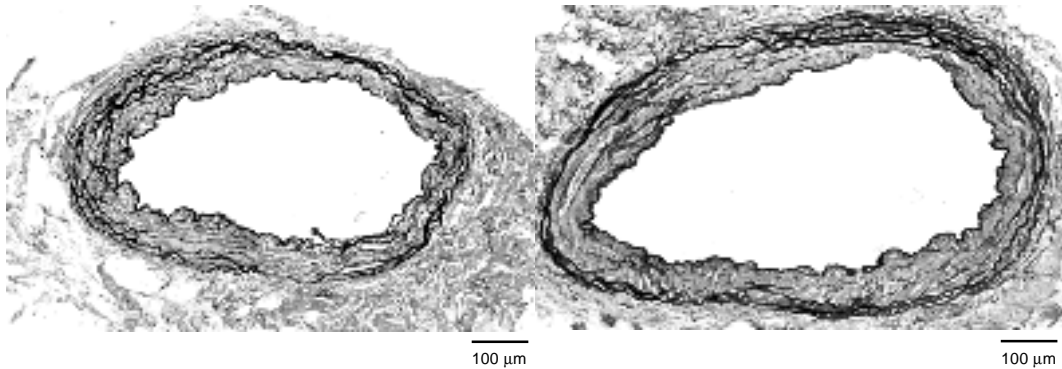


Fig. 1. Left anterior descending coronary arteries isolated from a young rat (**left**) and an aged rat (**right**). The media is thick and the vessel wall is dilated in the aged. Elastica van Gieson stain, original magnification $\times 65$.

Results

Body weight, heart weight and coronary ring weight were significantly heavier in aged rats than in young rats (Table 1). Figure 1 shows cross-sections of the left anterior descending coronary artery of young (left) and aged rats (right). The medial thickness of the arteries was $53.0 \pm 1.5 \mu\text{m}$ in young rats and $75.3 \pm 2.4 \mu\text{m}$ in aged rats, being significantly ($P < 0.01$) different between the 2 groups (Table 2). The number of nuclei per medial area was significantly less in aged rats than in young ones (Table 2).

Active tension (mN force/mg tissue weight) induced by 80 mmol/L KCl was smaller in aged

Table 2. Morphometric parameters of the coronary arteries

	Medial thickness (μm)	Medial area ($10^4 \mu\text{m}^2$)	Density of nuclei ($/10^3 \mu\text{m}^2$ medial area)
Young [16]	53.0 ± 1.5	3.3 ± 0.03	4.4 ± 0.4
Aged [16]	$75.3 \pm 2.4^{**}$	$10.3 \pm 0.6^{**}$	$1.3 \pm 0.1^{**}$

The data are given as mean \pm SEM.

[], number of animals.

Significantly different from young rats: $^{**} P < 0.01$.

rats (0.72 ± 0.06) than in young rats (2.01 ± 0.11). Figure 2 shows the dose-response curves of contraction in isolated rings of the coronary artery for 5-HT (left), $\text{PGF}_{2\alpha}$ (middle) and His

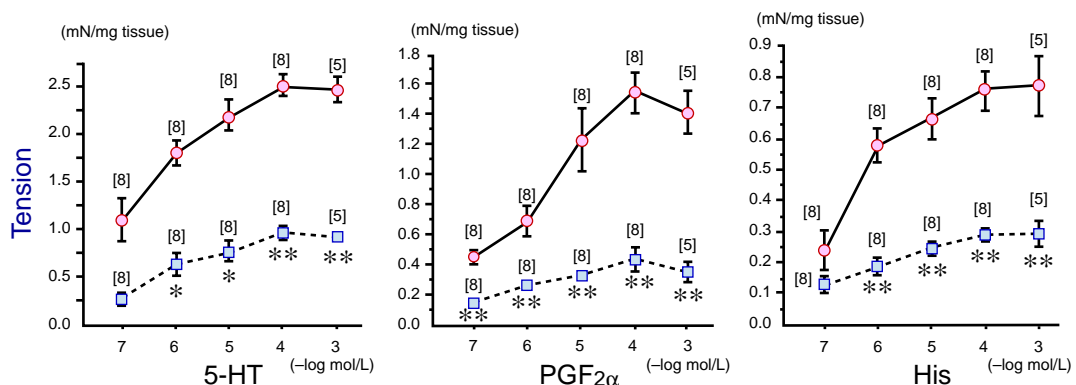


Fig. 2. Dose-response curves of 5-hydroxytryptamine (5-HT, 10^{-7} – 10^{-3} mol/L), prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$, 10^{-7} – 10^{-3} mol/L) and histamine (His, 10^{-7} – 10^{-3} mol/L) in isolated rings of the coronary arteries of young (○) and aged (□) rats. The contractile responses are expressed as active tension in mN/mg tissue and shown as mean \pm SEM. Significantly different between groups ($^*P < 0.05$, $^{**}P < 0.01$). [n], number of animals.

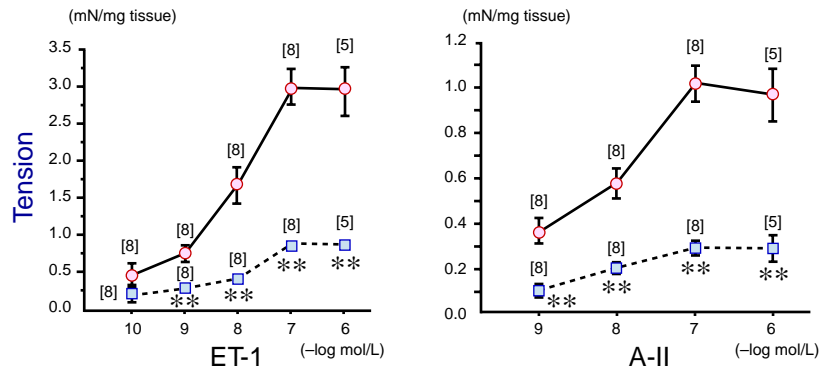


Fig. 3. Dose-response curves of endothelin-1 (ET-1, 10^{-10} – 10^{-6} mol/L) and angiotensin II (A-II, 10^{-9} – 10^{-6} mol/L) in isolated rings of the left anterior coronary arteries of young (○) and aged (□) rats. The contractile responses are expressed as active tension in mN/mg tissue and shown as mean \pm SEM. Significantly different between groups (* $P < 0.05$, ** $P < 0.01$). [n], number of animals.

(right). Active tension evoked by 10^{-6} mol/L to 10^{-3} mol/L of these agonists was significantly greater in young rats (solid line) than in aged rats (dotted line). ET-1 and A-II also caused concentration-related contractions (Fig. 3) in the coronary rings from young (solid line) and aged rats (dotted line). The active tension induced by 10^{-9} to 10^{-6} mol/L of these peptides was greater in young rats than in aged rats. In Table 3, the maximum contraction of the arteries induced by the agonists is compared between young and aged animals. It was 3 times greater in the young group than in the aged group when it was expressed as mN force per mg tissue weight. On the other hand, when it was normalized as percentage of 80 mmol/L

KCl-induced contraction, there was no significant difference between the 2 groups. The increase in $[Ca^{2+}]_i$ induced by 80 mmol/L KCl and maximum concentration of agonists was comparable in the 2 groups (Fig. 4).

Table 4 shows the percentage of phosphorylated MLC₂₀ (mono- and diphosphorylated) in rat coronary arteries. The percentage of phosphorylated MLC₂₀ during rest (control) was significantly lower in the young group than in the aged group. Addition of high K solution, 10^{-4} mol/L 5-HT, and 10^{-7} mol/L A-II increased the percentage in both groups. The phosphorylation level during contraction was significantly higher in the young rats than in the aged rats.

Table 3. Summary of agonist-induced contraction

	Maximum active tension		Percentage of KCl contraction	
	Young (mN/mg)	Aged (mN/mg)	Young (%)	Aged (%)
5-HT (10^{-4} mol/L)	2.46 ± 0.11	$0.96 \pm 0.06^*$	122.4 ± 5.4	133.4 ± 7.8^{NS}
PGF _{2α} (10^{-4} mol/L)	1.56 ± 0.13	$0.45 \pm 0.09^*$	77.5 ± 6.3	62.2 ± 12.2^{NS}
His (10^{-4} mol/L)	0.76 ± 0.06	$0.29 \pm 0.02^*$	37.6 ± 3.1	40.1 ± 2.8^{NS}
ET-1 (10^{-7} mol/L)	2.96 ± 0.23	$0.88 \pm 0.04^*$	147.6 ± 11.7	122.4 ± 6.0^{NS}
A-II (10^{-7} mol/L)	1.04 ± 0.07	$0.36 \pm 0.03^*$	51.7 ± 3.7	49.5 ± 3.9^{NS}

The data are given as mean \pm SEM.

A-II, angiotensin II; ET, endothelin; His, histamine; 5-HT, 5-hydroxytryptamine; NS, not significant; PGF_{2 α} , prostaglandin F_{2 α} .

Significantly different from young rats: * $P < 0.05$.

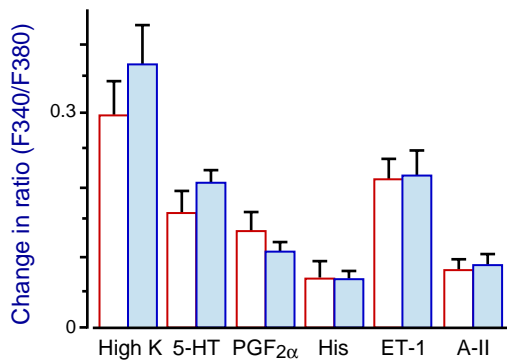


Fig. 4. Increases in ratio (F340/F380) induced by maximal concentration of agonists in isolated rings of the coronary arteries of young (□, $n = 8$) and aged (■, $n = 8$) rats. The data are expressed as mean \pm SEM. Maximal concentration; 10^{-4} mol/L for 5-hydroxytryptamine (5-HT), prostagrandin F_{2α} (PGF_{2α}), and histamine (His) or 10^{-7} mol/L for endothelin-1 (ET-1) and angiotensin II (A-II).

Discussion

The present results showed that the medial thickness and cross-sectional area of rat coronary arteries increased with age, and the number of nuclei per medial area decreased with age. On the other hand, the active tension (mN force/mg of tissue weight) induced by agonists decreased with age. The decrease in active tension was associated with a reduction in the agonist-induced elevation of the phosphorylated MLC₂₀ level in the tissues from aged animals, although there was no significant difference in $[Ca^{2+}]_i$.

A number of studies have demonstrated age-dependent changes in contractile and relaxant responses of vascular smooth muscles to vasoactive agents. The sensitivity to norepinephrine of rat aortic strips was greatly reduced with age when the contractile response was estimated as milligram force per milligram wet weight (Tuttle, 1966; Cohen and Berkowitz, 1976), which was the same method we used in the present study. The decreased sensitivity was associated with an increased thickness of the media and a coarsening of the smooth muscle fibers (Tuttle, 1966). The number of nuclei per medial area decreased in the coronary arteries

from aged rats compared with those from younger rats (Table 2), which is consistent with previous histological observation (Hüsken et al., 1997). Thus, the smooth muscle fibers seem to be dispersed in the media of coronary arteries from aged rats, and this dispersion is probably one of the reasons for the decrease in agonist-induced active tension. On the other hand, contractile responses of rat coronary arteries to 5-HT (Nyborg and Mikkelsen, 1988; Tschudi and Lüscher, 1995), ET-1 (Tschudi and Lüscher, 1995) and KCl (Tschudi and Lüscher, 1995) have been shown to increase with age. In these studies, however, the amount of response was estimated as the developed force divided by the length of ring segment. Thus, the increase in wall thickness of the vessel isolated from aged animals was not taken into account.

Not only the dispersed arrangement of smooth muscle fibers but also a reduction in contractile response of the individual muscles to agonists seems to take part in the age-dependent decrease in active tension, because the level of MLC₂₀ phosphorylation in the coronary arteries stimulated with the agonists was lower in the aged rats than in the young ones (Table 4). Thus, it is important to know which part of the signal transduction for agonist-induced contraction is modified with age. The function of certain receptors, such as the beta adrenoceptor, decreases during the aging process (Fleisch et al., 1970; Hayashi and Toda, 1978). In the present experiment, however, the decrease in active tension was observed with all

Table 4. Percentage of phosphorylated MLC₂₀ in the smooth muscle cells of the coronary arteries

	Young	Aged
Control	[5] 19.9 \pm 1.9	28.0 \pm 2.6**
High K (80 mmol/L)	[5] 63.5 \pm 1.1	56.8 \pm 2.2**
5-HT (10 ⁻⁴ mol/L)	[5] 71.3 \pm 1.5	66.3 \pm 2.2**
A-II (10 ⁻⁷ mol/L)	[5] 76.2 \pm 4.1	58.2 \pm 6.5**

The data are given as mean \pm SEM.

[], number of animals.

A-II, angiotensin II; K, KCl solution; 5-HT, 5-hydroxytryptamine; MLC, myosin light chain.

Significantly different from young rats: ** $P < 0.01$.

agonists including 5-HT, PGF_{2α}, His, ET-1 and A-II. Furthermore, the active tension of coronary arteries induced by 80 mmol/L KCl solution was also smaller in the aged rats than in the young ones. Hence, the age-dependent change in rat coronary smooth muscles is likely to occur at a common pathway of the signal transduction distal to the receptor. This is also supported by the result that the agonist-induced increase in [Ca²⁺]_i was not significantly different between young and aged ones (Fig. 4). Taken together, the age-dependent change in the signal transduction pathway may occur either downstream from the [Ca²⁺]_i increase, including the binding of Ca²⁺ to calmodulin and the activation of MLCK by Ca-calmodulin complex, or in the secondary mechanism(s) which is (are) independent of [Ca²⁺]_i.

The sensitivity of coronary arteries to Ca²⁺ has been investigated in pigs (Nyborg and Mikkelsen, 1988; Noda et al., 1996) but not in rats. The present results show that 10⁻⁴ mol/L 5-HT and 10⁻⁷ mol/L ET-1 increased [Ca²⁺]_i to almost the same level as 80 mmol/L KCl did (Fig. 4), and caused a 120–150% contraction of the KCl contracture (Table 3) in the rat coronary arteries. This is consistent with previous results in porcine coronary arteries where agonists such as carbachol, 5-HT, PGF_{2α}, endoperoxide analogue U-46619 and ET-1 produced a greater force for a given [Ca²⁺]_i compared to that seen during KCl contracture (Bradley and Morgan, 1987; Kodama et al., 1994). These results suggest that a secondary mechanism(s) that can modify, independently of [Ca²⁺]_i, the phosphorylation of MLC₂₀ is (are) playing a certain role in the regulation of smooth muscle contraction in the coronary arteries.

Studies using permeabilized smooth muscles revealed that a GTP-binding protein inhibits MLC₂₀ phosphatase and increases Ca²⁺ sensitivity (Somlyo and Somlyo, 1994) and that the rho p21 family is involved in the GTPγS-enhanced Ca²⁺ sensitivity of smooth muscle contraction (Ichi-Hirata et al., 1992; Gong et al., 1996; Noda et al., 1996). A recent study further demonstrated that rho inhibits MLC phosphatase through activation of rho-kinase (Kimura et al., 1996). On the other hand, Eto and coworkers

(Eto et al., 1995; Li et al., 1998) suggested that CPI-17 protein accounts largely for the pathway between protein kinase C and MLC phosphatase. Effects of aging on the function of rho, rho-kinase and CPI-17 have not yet been investigated in smooth muscle cells. Further studies will be needed to clarify whether they are involved in the age-dependent change in the contractile response of coronary arteries to vasoactive substances.

In conclusion, the active tension (mN of force/mg of tissue weight) of isolated rat coronary arteries induced by agonists decreased with age, which was associated with the increase in the medial thickness and the reduction of the agonist-induced elevation of phosphorylated MLC₂₀. The age-dependent change is attributable to a reduction of smooth muscle density in the media and/or an impairment in the signal transduction pathway in the smooth muscle cells. A possible site for the impaired pathway is either downstream from the [Ca²⁺]_i increase or in some Ca²⁺-independent secondary mechanism(s).

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