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Preservation of vascular contraction during ageing: dual effect on calcium handling and sensitization

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1 The present study was aimed to characterize the effects of ageing on vascular contraction by noradrenaline in rat isolated arteries. The existence of vascular bed heterogeneity was investigated in endothelium-denuded conductance (aorta) and resistance (small mesenteric artery, SMA) arteries, with respect to Ca^{2+} handling, Ca^{2+} sensitization or Ca^{2+}-independent mechanisms.

2 In both arteries, contractions to noradrenaline were not different between adult and aged rats.

3 In Ca^{2+}- free medium, noradrenaline elicited a transient increase in tension that was reduced by the Ca^{2+} mobilizing agents, ryanodine and thapsigargin, in arteries from adult rats. A loss of the thapsigargin- but not the ryanodine-sensitive component of noradrenaline-induced contraction was observed in the two arteries from aged rats.

4 After depletion of Ca^{2+} stores with noradrenaline, addition of exogenous $CaCl_2$ produced a sustained contraction that was decreased to the same extent by the protein kinase C inhibitor, GF 109203X and the tyrosine kinase inhibitor, tyrphostin A-23, in arteries from adult and aged rats. The Rho-associated protein kinase inhibitor, Y-27632, caused identical relaxation of noradrenaline pre-contracted arteries from both age groups.

5 Basal intracellular calcium ($[Ca^{2+}]_i$) was higher in SMA from aged than from adult rats. In addition, the noradrenaline $[Ca^{2+}]_i$-force relationship was significantly shifted to the right in the SMA from aged rats.

6 Altogether, these data indicate that responsiveness to noradrenaline is preserved both in conductance and resistance arteries with ageing. The latter results from the association of increased basal $[Ca^{2+}]_i$, changes in Ca^{2+} handling at the level of thapsigargin-sensitive sarcoplasmic reticulum Ca^{2+}-ATPases and decreased myofilament sensitivity to Ca^{2+}.

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CICR, calcium-induced calcium release; HR, heart rate; MAP, mean arterial pressure; NO, nitric oxide; PKC, protein kinase C; PSS, physiological salt solution; ROK, Rho-associated kinases; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SMA, small mesenteric artery, branch II or III of superior mesenteric artery; TK, tyrosine kinase

Introduction

Vascular ageing is associated with both structural and functional changes that can take place at the level of the endothelium, smooth muscle cells and the extracellular matrix of blood vessels. Endothelial dysfunction occurs in large as well as in resistance arteries with ageing (Matz *et al*., 2000). Recently, reduced vasodilatation in response to agonists was reported both in the aorta and in small mesenteric arteries (SMA) of the rat in the course of ageing (Matz *et al*., 2000). With regard to smooth muscle contraction, controversial data have been reported in the literature consisting of either decreased or unchanged responsiveness to vasoconstrictor agonists (Tuttle, 1966; Duckles *et al*., 1985; Docherty, 1990). Possible reasons for these different results could be firstly, the studies of changes related to maturation but not to senescence, secondly the influence of the hormonal status by the use of female rats that might mask the changes related to ageing *per se*, or thirdly the use of an hypertensive strain in which it is difficult to discriminate between the consequences of ageing, the increase in blood pressure, or both.

It is commonly agreed that an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is a determinant for contraction in response to Ca^{2+} mobilizing agonists such as noradrenaline (Somlyo & Somlyo, 1994). In addition, most of these agonists are able to modulate contraction by altering myofilament Ca^{2+} sensitivity or through Ca^{2+}-independent pathways (Khalil & van Breemen, 1988; Horowitz *et al*., 1996). Phosphorylation mechanisms involving protein kinase C (PKC), tyrosine kinase (TK) and Rho-associated protein kinases (ROK) may account for both increase in $[Ca^{2+}]_i$ and Ca^{2+} sensitization. However, little information is available on changes in Ca^{2+} handling and regulation of contractility during ageing.

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Therefore, the present study was aimed to characterize the effects of ageing on contraction induced by the vasoconstrictor agonist, noradrenaline, in rat isolated arteries. In order to investigate the effect of ageing *per se*, senescent male Wistar rats that did not develop hypertension in the course of ageing have been used in the present study. Recently, we have reported vascular bed heterogeneity in age-related endothelial dysfunction with respect to nitric oxide (NO) and eicosanoids (Matz *et al*., 2000). The existence of such anatomical heterogeneity in the course of ageing was investigated with respect to Ca^{2+} handling, Ca^{2+} sensitization or Ca^{2+}-independent mechanisms in endothelium-denuded aorta and SMA, the latter playing a role in the regulation of local blood flow.

Methods

Animals

Male Wistar rats of 12–14 weeks old (adult) or 70–100 weeks old (aged) were bred in our institute from progenitors provided by Iffa-Credo (Lyon, France). This investigation conforms to the authorization (number 01918) for the use of laboratory animals given by the French government (Department of Agriculture). Mean arterial blood pressure was determined in pentobarbitone anaesthetized rats (60 mg kg^{-1}) by direct measurement with a carotid catheter and a Statham 23 DB transducer.

Arterial preparation and mounting

Thoracic aorta and branch II or III of superior mesenteric artery (SMA) (normalized internal diameter (μm) : 172 ± 8 and 198 ± 12 for adult and aged rats respectively) were carefully removed. Segments of arteries (2–3 mm or 1.6–2.0 mm in length for aorta and for SMA, respectively) were mounted on myographs filled with physiological salt solution (PSS) under normalized tension as previously described (Matz *et al*., 2000). In all vessels, the endothelial layer was removed immediately after dissection either by gently rubbing the intimal surface with curved forceps for the aorta or by intraluminal perfusion with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1 propane sulphonate (CHAPS) in PSS for 25 s followed by repeated washing with PSS for the SMA. The absence of functional endothelium was confirmed by the absence of a relaxation response to acetylcholine (1 μM) in arteries pre-contracted with noradrenaline (1 and 10 μM in the aorta and the SMA, respectively).

Contraction experiments

Vessels were contracted by cumulative addition of increasing concentrations of noradrenaline in the organ bath. In another set of experiments, the vessels were left for 15 min in Ca^{2+}-free PSS and ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, 0.5 mM) was added 5 min before the start of the experiment. The vessels were then challenged with noradrenaline at a maximally active concentration (1 and 10 μM, for the aorta and for the SMA, respectively). When the tension had returned to the baseline, $CaCl_2$ (1 mM) was added to the bath in the continuous presence of the agonist. The same experimental protocol was used in the presence of ryanodine (10 μM), thapsigargin (1 μM), GF 109203X (5 μM), or tyrphostin A-23 (100 μM). All the inhibitors were incubated with the vessels for 15 min. In another set of experiments the vessels were pre-contracted with noradrenaline in normal PSS and when the tension reached a steady state, Y-27632 was added cumulatively (0.1–30 μM).

Measurements of $[Ca^{2+}]_i$

Contraction and $[Ca^{2+}]_i$ were measured simultaneously in the SMA; contraction was measured as described above, and changes in $[Ca^{2+}]_i$ were determined by measuring the fluorescence of trapped fura-2 with a dual-excitation wavelength fluorometer (Fluorolog II, SPEX, Edison, NJ, U.S.A.) as previously described (Martinez *et al*., 2000) except that the incubation period with fura-2 AM was 90 min. Concentration-response curves to noradrenaline were constructed by cumulative addition of increasing concentrations of noradrenaline. The change in $[Ca^{2+}]_i$ was calculated by use of the equation described by Grynkiewicz *et al*. (1985) and was expressed in nanomoles per liter (nM).

Expression of results and statistical analysis

The increase of tension was measured at the peak of contraction induced by noradrenaline. Contractions were expressed in g mg^{-1} and mN mm^{-1} for the aorta and the SMA respectively, except for the Ca^{2+}-force relationship where it was expressed in per cent of the maximal contractile response. All results are expressed as means $\pm$ s.e.mean of n experiments, n representing the number of rats. Sensitivities to noradrenaline are expressed as pD_2 values, where $pD_2 = -\log EC_{50}$, EC_{50} being the molar concentration of the agonist that produces 50% of the maximal effect (E_{max}), and were calculated by log-logit regression. Unpaired and paired Student's t-test were used for statistical analysis, for the aorta and the SMA respectively. $P < 0.05$ was considered significant.

Drugs

Ryanodine and tyrphostin A-23 were from Calbiochem (Meudon, France), GF 109203X was from Interchim (Montluçon, France), Fura 2-AM was from Molecular Probes (Interchim, Montluçon, France). The other chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Results

Body weight, blood pressure and heart rate

Body weight significantly increased with age (g) (405 ± 17.1 ($n = 14$), 805 ± 26.1 ($n = 15$), $P < 0.001$, adult and aged rats respectively). Mean arterial blood pressure (MAP) and heart rate (HR) were not significantly different between the two groups of rats (MAP (mmHg): 114 ± 9.2 *versus* 126 ± 6.3; HR (beats min^{-1}): 373 ± 10 *versus* 367 ± 11; adult and aged rats respectively).

Effect of noradrenaline on contraction and $[Ca^{2+}]_i$

Noradrenaline produced contraction in a concentration-dependent manner in aortic rings and SMA from both adult and aged rats. The pD_2 and E_{max} values were not significantly different in the aorta between the two groups of rats being respectively 7.82 ± 0.24 and 8.09 ± 0.40 for the pD_2 and 3.20 ± 0.45 and 3.16 ± 0.30 for the E_{max} ($g\ mg^{-1}$, $n = 10-12$). Also, the response to noradrenaline was not altered with ageing in SMA, the pD_2 values being 5.88 ± 0.07 and 5.99 ± 0.05 and the E_{max} ($mN\ mm^{-1}$) values being 3.59 ± 0.41 and 3.89 ± 0.13, in adult and aged rats respectively ($n = 8-10$).

Mechanisms involved in noradrenaline-induced contraction

In Ca^{2+}-free medium, noradrenaline produced a fast transient increase in tension in the aorta and the SMA from adult and aged rats (Figure 1A). Addition of $CaCl_2$ after tension had returned to the baseline induced a large and sustained rise in contraction in both types of arteries from adult and aged rats. Contractions obtained in Ca^{2+}-free medium and that obtained after addition of $CaCl_2$ were not significantly different in arteries from adult and aged rats, both in the aorta and the SMA (Figure 1B,C).

Effects of agents interfering with phosphorylation-dependent pathways In Ca^{2+}-free medium, the PKC inhibitor, GF 109203X, did not modify the increase in tension induced by noradrenaline in the aorta from adult and aged rats (Figure 2A). However, this inhibitor significantly decreased the noradrenaline-induced contraction in Ca^{2+}-free medium in the SMA from both adult and aged rats (Figure 2B). In addition, GF 109203X significantly reduced the $CaCl_2$-induced response in the noradrenaline-exposed aorta and SMA from both adult and aged rats (Figure 2C,D).

The TK inhibitor, tyrphostin A-23, did not significantly affect the responses to noradrenaline in Ca^{2+}-free medium but it greatly reduced contractions obtained after subsequent addition of $CaCl_2$ (Tables 1 and 2). The ROK inhibitor, Y-27632, produced a concentration-dependent relaxation of aorta and SMA pre-contracted by noradrenaline that was not significantly different between adult and aged rats (Figure 3A,B).

Intracellular Ca^{2+} measurement and Ca^{2+}-force relationship in the SMA In the resting state, basal $[Ca^{2+}]_i$ was significantly higher in the SMA from aged rats as compared to adult rats (Figure 4A). The $[Ca^{2+}]_i$-force relationship obtained by plotting $[Ca^{2+}]_i$ *versus* the corresponding values of contractions for each concentration of noradrenaline (from 0.1 to 30 μM) was greatly shifted to the right in aged rats (Figure 4B).

Effects of agents interfering with Ca^{2+} storage mechanisms The inhibitor of calcium-induced calcium release mechanism (CICR), ryanodine, reduced the increase in tension induced by noradrenaline in Ca^{2+}-free medium, but it had no effect on the response to the agonist after addition of $CaCl_2$ both in the aorta and the SMA from adult and aged rats (Tables 1 and 2).

The inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), thapsigargin, significantly reduced the increase in tension induced by noradrenaline in both types of arteries from adult rats but it had no effect in those from aged rats (Figure 5A,B). Furthermore, thapsigargin did not modify the noradrenaline-induced contractions after addition of $CaCl_2$ in both types of arteries from adult and aged rats

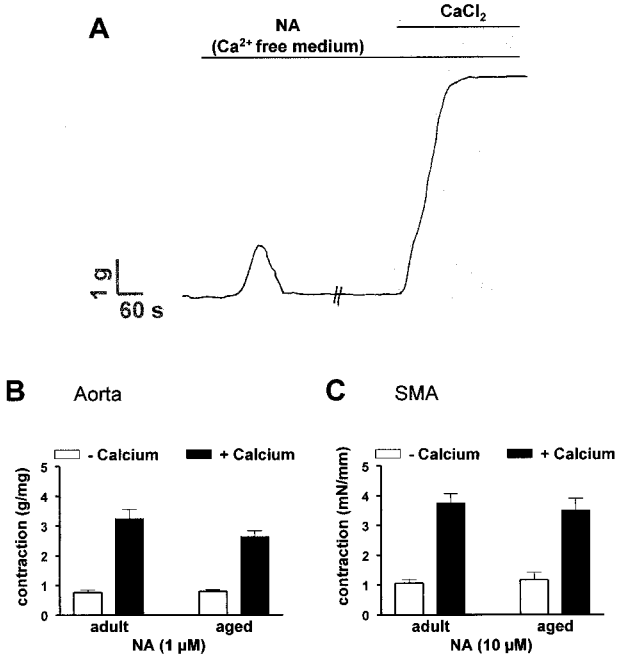

Figure 1 Representative trace showing the effect of noradrenaline (NA) on small mesenteric arteries (SMA) in calcium-free medium and after addition of $CaCl_2$ (1 mM) in the organ bath (A) and contraction values obtained in the same conditions in aorta (B) and SMA (C). Values are mean$\pm$s.e.mean ($n = 12-15$ for the aorta, $n = 23-24$ for the SMA).

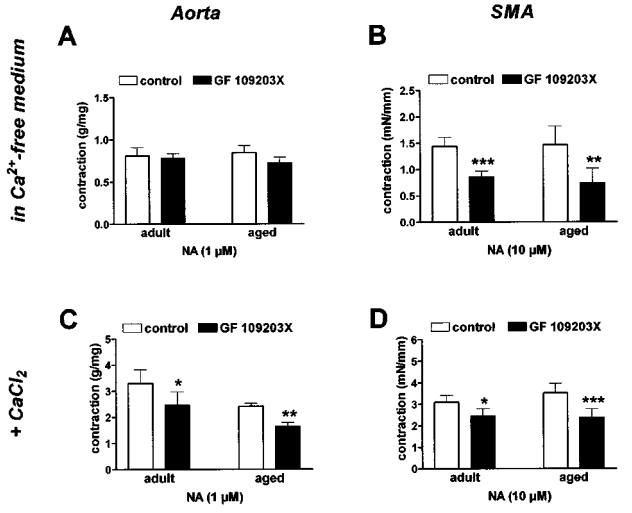

Figure 2 Effects of GF 109203X (5 μM) on noradrenaline (NA)-induced contractions in aorta and small mesenteric arteries (SMA) from adult and aged rats, in calcium-free medium (A and B) and after addition of $CaCl_2$ (1 mM) (C and D) in the organ bath. Values are mean$\pm$s.e.mean ($n = 8-12$). $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$: *versus* in the absence of GF 109203X.

Table 1 Noradrenaline-induced contractile values obtained in the aorta (g mg^{-1}) and small mesenterc arteries (SMA, mN mm^{-1}) from adult and aged rats in a calcium free medium in the absence (control) or presence (+) of inhibitors in the organ bath

Aorta				*SMA*			
Adult		*Aged*		*Adult*		*Aged*	
Control	*+ Ryanodine*	*Control*	*+ Ryanodine*	*Control*	*+ Ryanodine*	*Control*	*+ Ryanodine*
0.81±0.10	0.12±0.04***	0.84±0.09	0.17±0.04***	1.16±0.22	0.56±0.10*	1.41±0.26	0.41±0.08**
Control	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*
0.70±0.13	0.46±0.17	0.61±0.16	0.37±0.17	1.05±0.18	1.21±0.27	0.83±0.20	0.65±0.13

Values are mean±s.e.mean ($n=8-12$ and 6–8 with ryanodine and tyrphostin A-23 respectively). ***$P<0.001$, **$P<0.01$, *$P<0.05$: *versus* in the absence of inhibitor.

Table 2 Noradrenaline-induced contractile values obtained in the aorta (g mg^{-1}) and small mesenterc arteries (SMA, mN mm^{-1}) from adult and aged rats after addition of $CaCl_2$ in the organ bath in the absence (control) or presence (+) of inhibitors

Aorta				*SMA*			
Adult		*Aged*		*Adult*		*Aged*	
Control	*+ Ryanodine*	*Control*	*+ Ryanodine*	*Control*	*+ Ryanodine*	*Control*	*+ Ryanodine*
3.30±0.52	2.45±0.31	2.41±0.19	2.14±0.18	3.20±0.29	3.32±0.79	3.60±0.15	2.80±0.49
Control	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*	*Control*	*+ Tyrphostin A-23*
3.16±0.26	1.33±0.66*	3.20±0.34	1.06±0.59*	3.65±0.59	1.56±0.31*	4.28±0.54	2.55±0.41**

Values are mean±s.e.mean ($n=8-12$ and 6–8 with ryanodine and tyrphostin A-23 respectively). **$P<0.001$, *$P<0.05$: *versus* in the absence of inhibitor.

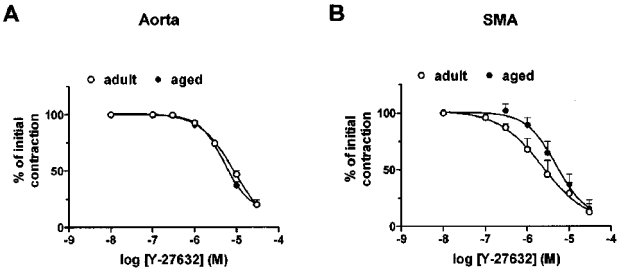

Figure 3 Cumulative concentration-response curves to Y-27632 in the aorta (A) and the small mesenteric artery (B, SMA) from adult and aged rats precontracted with noradrenaline (1 or 10 μM, for the aorta and the SMA respectively). Values are mean±s.e.mean ($n=6-8$).

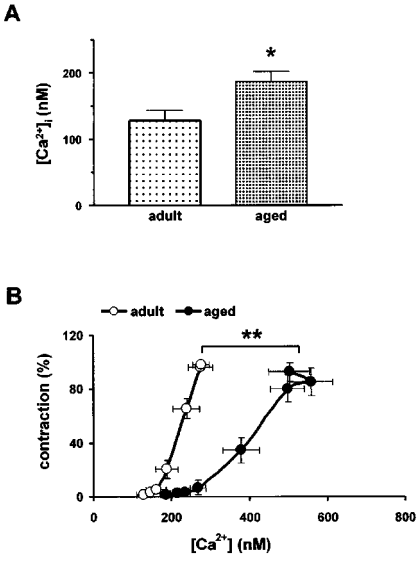

Figure 4 Intracellular basal calcium concentration ($[Ca^{2+}]_i$) (A) and calcium-contraction curves (B) obtained in response to noradrenaline (0.1–30 μM) in small mesenteric arteries from adult and aged rats. Contractions are expressed as percentage of the maximal contractile responses. Values are means±s.e.mean ($n=5$). **$P<0.01$, *$P<0.05$: adult *versus* aged.

(Figure 5C,D). Neither ryanodine nor thapsigargin had any detectable effect on contraction during the preincubation prior to the addition of noradrenaline.

Discussion

In a model that allows the determination of the effect of ageing *per se*, the regulation of Ca^{2+} handling was markedly altered despite the preservation of contractility in responses to noradrenaline both in conductance (aorta) and resistance (SMA) arteries of the rat. Indeed, increased basal $[Ca^{2+}]_i$ and changes in Ca^{2+} handling at the level of thapsigargin-sensitive sarcoplasmic reticulum Ca^{2+} ATPases were observed in the course of ageing. In addition, vessels from aged rats displayed decreased sensitivity of contractile proteins to Ca^{2+}, at least in the SMA. Taken together, these data suggest that adjustments of Ca^{2+} handling and sensitization occur with ageing allowing the maintenance of responsiveness to vasoconstrictor agonists within physiological limits.

In vessels from adult rats, it is reported that the pathways leading to the contractile response to noradrenaline are composed of Ca^{2+} release from ryanodine-sensitive stores

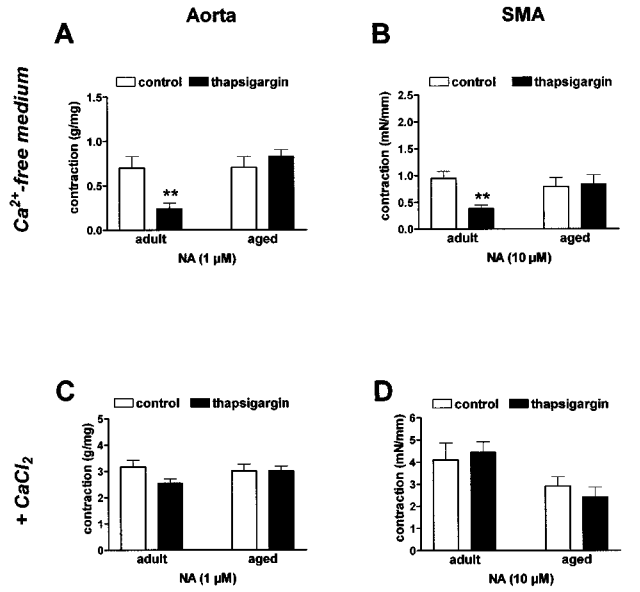

Figure 5 Effects of thapsigargin (1 μM) on noradrenaline (NA)-induced contractions in aorta and small mesenteric arteries (SMA) from adult and aged rats, in calcium-free medium (A and B) and after addition of $CaCl_2$ (1 mM) (C and D) in the organ bath. Values are mean$\pm$s.e.mean ($n=8-12$). $^{**}P<0.01$: *versus* in the absence of thapsigargin.

and from thapsigargin-sensitive stores, Ca^{2+} entry and Ca^{2+} sensitization pathways both in the aorta and the SMA.

The ryanodine-sensitive Ca^{2+} stores are known to be involved in the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism in vascular smooth muscle (Blatter & Wier, 1992; Berridge, 1993). The thapsigargin-sensitive Ca^{2+} stores are known to be involved, at least partly, in the IP_3-induced Ca^{2+} release mechanism (Bian *et al*., 1991). The latter is a common pathway signalling downstream of G protein-coupled receptors (Bolton *et al*., 1984; Shima & Blaustein, 1992; Nilsson *et al*., 1994; Lagaud *et al*., 1999). In accordance with our previous work, Ca^{2+} entry in response to noradrenaline may occur via voltage-operated Ca^{2+} channels, receptor-operated Ca^{2+} channels or via Na^+-Ca^{2+} exchanger (Lagaud *et al*., 1999).

The use of agents interfering with phosphorylation-dependent pathways showed that both TK and PKC contributed to the contraction induced by the addition of $CaCl_2$ both in the aorta and the SMA exposed to noradrenaline. This component of noradrenaline-induced contraction is known to result from both Ca^{2+} entry and Ca^{2+} sensitization (Khalil & van Breemen, 1988; Hughes, 1995; Jinsi *et al*., 1996; Buus *et al*., 1998; Lagaud *et al*., 1999; Martinez *et al*., 2000).

Anatomical heterogeneity between the two vascular preparations is reported here with regard to the participation of PKC-inhibitor sensitive pathway in the component of the contraction linked to Ca^{2+} release. Thus, a GF 109203 X-sensitive component of the response to noradrenaline obtained in Ca^{2+} free medium has been observed in the SMA but not in the aorta. The participation of PKC in the Ca^{2+}- release mechanisms has also been described in the human small omental arteries (Martinez *et al*., 2000). Further studies are needed to sort out the underlying mechanisms.

One of the findings of the present study is the involvement of Rho-kinase (ROK) sensitive to Y-27632 in the contractile response to noradrenaline both in the conductance and resistance arteries of the rat. Y-27632 is known to selectively inhibit smooth muscle contraction by inhibiting Ca^{2+} sensitization through small GTP-binding proteins (Uehata *et al*., 1997). The relaxation of noradrenaline-precontracted vessels by Y-27632 strongly suggests the implication of such pathway in the two arteries studied.

Turning now to the effects of ageing, the results point out the preservation of vascular contractility in response to noradrenaline in the two arteries studied despite the reported changes in these vessels (Folkow & Svanborg, 1993).

The use of agents interfering with the Ca^{2+} release mechanisms suggest a decrease in the control of Ca^{2+} at the level of the sarcoplasmic reticulum. A change in CICR mechanism is unlikely since, in aged rats, ryanodine was still effective in inhibiting the component of contraction linked to the release of Ca^{2+} from intracellular stores. However, the effectiveness of thapsigargin, a selective inhibitor of SERCAs, in reducing noradrenaline response in Ca^{2+} free medium in aorta and SMA from adult rats, was lost in the two types of arteries from aged rats. These data suggest an alteration of the contribution of thapsigargin-sensitive Ca^{2+} stores in arteries from aged rats. The decrease in the control of Ca^{2+} signaling at the level of SERCA in the course of ageing has never been described in vascular smooth muscle. Several hypothesis can be advanced to explain the ineffectiveness of thapsigargin under the experimental conditions used, such as a change in expression, a decrease in activity of SERCA isoforms or both. A decreased expression of SERCA 2 mRNA has been reported in the heart from aged rats (Lompre *et al*., 1991). Also, an age-dependent loss of SERCA2a activity and Ca^{2+} uptake rate in sarcoplasmic reticulum isolated from Fisher-344 rat skeletal muscle were demonstrated (Viner *et al*., 1999). SERCA 2 isoforms (SERCA 2a and SERCA2b) have been described both in the aorta and the SMA from the rat (Lompre, 1999). In the present study, the loss of thapsigargin-sensitive component of noradrenaline-induced contraction suggest an age-related decrease in Ca^{2+} buffering function of sarcoplasmic reticulum. This assumption is corroborated by the increase in basal $[Ca^{2+}]_i$ observed in the SMA of aged rats. Such a decrease in Ca^{2+} buffering function of the sarcoplasmic reticulum with ageing has been described by Tsai *et al*. (1998) in different sympathetic nerves of the tail artery from Fischer-344 rat. Thus, this alteration might be a generalized feature of ageing.

The present study also provides evidence that in vessels from aged rats the contractile response to noradrenaline was not modified despite the increase in basal $[Ca^{2+}]_i$, suggesting a dissociation between $[Ca^{2+}]_i$ and contraction in the course of ageing. Since the $[Ca^{2+}]_i$-force relationship to noradrenaline was shifted to higher calcium concentrations in vessels from aged animals when compared to those from adult rats, it is most likely that ageing is associated with a decrease in sensitivity of contractile myofilaments to Ca^{2+}. The use of agents interfering with phosphorylation mechanisms showed that the inhibitory effect of these compounds were not modified in aorta and SMA both from adult and aged rat. These data suggest that the decreased sensitivity of contractile myofilaments to Ca^{2+} during ageing may not involve changes in PKC, TK and ROK pathways. Changes of either Ca^{2+}-

independent light chain kinase mechanism or cytoskeleton-dependent pathways involving calponin or caldesmon cannot however be ruled out. Whether the increases in $[Ca^{2+}]_i$ may decrease the sensitivity of contractile apparatus to $[Ca^{2+}]_i$ as in other smooth muscle (Himpens *et al*., 1989) is not known. However, evidence is provided that ageing is associated with an increase in basal $[Ca^{2+}]_i$ and decreased sensitivity of contractile apparatus to $[Ca^{2+}]_i$ within the arterial wall, which together maintain unchanged agonist-induced contractility.

In summary, the present study points out the preservation of the contractile responsiveness to noradrenaline within physiological limits in both conductance and resistance arteries with ageing. However, the mechanisms involved in calcium handling and sensitization are markedly altered in the course of ageing. The preservation of the contractile response to noradrenaline results from multiple adjustments at the level of calcium signalling allowing a novel intracellular calcium homeostasis in vessels from aged rats. The setting of this phenomenon within the vascular wall may play a role in prevention of age-associated cardiovascular diseases.

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