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Effect of nitro-L-arginine on electrical and mechanical responses to acetylcholine in the superior mesenteric artery from stroke-prone hypertensive rat

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1 High salt diet is known to aggravate the vascular pathology in spontaneously hypertensive stroke-prone rats (SHR-SP). The aim of the present study was to assess the involvement of endothelial dysfunction in this effect. Contractile tension and membrane potential were simultaneously recorded in superior mesenteric artery rings of untreated and NaCl-loaded (1% NaCl in the drinking water) SHR-SP and normotensive Wistar Kyoto rats (WKY).

2 In unstimulated artery, hyperpolarization evoked by acetylcholine was not different in WKY and in NaCl-loaded WKY; it was reduced in SHR-SP and further reduced in NaCl-loaded SHR-SP. Hyperpolarization was unaffected by N^{ω}-nitro-L-arginine (L-NA) but was abolished in high-KCl solution.

3 In noradrenaline-stimulated artery, ACh-evoked hyperpolarization and relaxation were not different in WKY and in SHR-SP. NaCl-treatment did not affect the responses to ACh in WKY but decreased maximum relaxation in SHR-SP from $93 \pm 2\%$ to $72 \pm 7\%$ of the contraction. In WKY, in NaCl-loaded WKY and in SHR-SP, L-NA similarly shifted the concentration-relaxation curve to ACh to the right and depressed its maximum but L-NA did not affect the hyperpolarization to ACh. In NaCl-loaded SHR-SP, L-NA blunted the effects of ACh on membrane potential and on contraction.

4 The NO donor SNAP abolished the depolarization and the contraction evoked by noradrenaline with the same potency in WKY and in untreated SHR-SP but was more potent in NaCl-loaded SHR-SP.

5 In KCl-contracted arteries the relaxations to ACh were not different in WKY and SHR-SP but NaCl-loaded SHR-SP were more sensitive to ACh.

6 The results showed that NaCl-rich diet markedly reduced the L-NA-resistant responses to ACh and increased the sensitivity to NO in SHR-SP.

Keywords: Endothelium; membrane potential; relaxation; acetylcholine; mesenteric artery; NO; EDHF; N^{ω}-nitro-L-arginine; hypertension; stroke

Abbreviations: ACh, acetylcholine; ANOVA, analyse of variance; EDHF, endothelium-derived hyperpolarizing factor; Em, membrane potential; L-NA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-L-arginine methylester; Nad, noradrenaline; NO, nitric oxide, NOS nitric oxide synthase; SHR-SP, spontaneously hypertensive stroke-prone rat; SHR-SP NaCl, NaCl-loaded spontaneously hypertensive stroke-prone rat; SNAP, S-nitroso-N-acetylpenicillamine, WKY, Wistar Kyoto rat; WKY NaCl, NaCl-loaded Wistar Kyoto rat

Introduction

The endothelium plays an important role in the regulation of vascular tone through the release of contracting and of relaxing factors. Endothelium-dependent relaxation has been reported to be mediated by several factors: NO, prostaglandins and an hyperpolarizing factor (EDHF) insensitive to blockers of NO synthases and prostaglandins (Vanhoutte *et al*., 1986). In many arteries, muscarinic stimulation evokes the release of EDHF in addition to the release of NO (Bolton *et al*., 1984; Chen *et al*., 1988; Feletou & Vanhoutte, 1988). The contribution of hyperpolarization due to EDHF to the endothelium-dependent vaso-relaxation is still debated. NO-evoked hyperpolarization has also been reported in some arteries (Tare *et al*., 1990; Krippeit-Drews *et al*., 1992; Cohen *et al*., 1997), but was not observed everywhere (Vanheel *et al*., 1994; Garland & McPherson, 1992). The existence of a cross-talk between NO and EDHF has also been suggested from the observation that EDHF release can be modulated by NO donors (Bauersachs *et al*., 1996).

Impairment of endothelium-dependent relaxation has been associated with several vacular pathologies like essential hypertension (see Vanhoutte, 1996, for a review) although contradictory results have been published (Angus, 1996). In spontaneously hypertensive stroke-prone rats (SHR-SP) and in stroke-prone F2 hybrids, a cosegregation of stroke and impaired endothelium function has been reported (Volpe *et al*., 1996). Alteration in the NO release or effect has been reported in basilar artery of SHR-SP (Salomone *et al*., 1997) while in contrast an increased activity of constitutive NOS has been found in aorta from SHR-SP compared to normotensive Wistar Kyoto rats (WKY, McIntyre *et al*., 1997). In SHR-SP, a NaCl-rich diet markedly increases the incidence of stroke without a pronounced change in blood pressure. NaCl-load also increases the hypertrophy of heart and vessels, increases the contractile reactivity of arteries and augments the cardiac expression of pre-proendothelin-1 (Feron *et al*., 1995; Salomone *et al*., 1996). Preliminary results have shown that

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NaCl-load impairs the endothelium-dependent relaxation in the rat superior mesenteric artery of SHR-SP (Morel *et al*., 1996). The purpose of the present investigations was to characterize the effect of NaCl-rich diet on the endothelial factors involved in the vasorelaxation evoked by acetylcholine (ACh) in the superior mesenteric artery isolated from WKY and from SHR-SP. Indomethacin was added to all solutions to eliminate the influence of prostaglandins. Simultaneous measurement of contractile responses and membrane potential was used to investigate the contribution of hyperpolarization to the relaxation. The respective role of NO and of the putative EDHF was assessed by measuring changes observed after blockade of NO synthase. Results showed that supplying the rats with 1% NaCl in their drinking water reduced EDHF-mediated responses to ACh in SHR-SP but not in WKY. Reduction of EDHF was accompanied by an increase in the sensitivity to NO.

Methods

Normotensive Wistar Kyoto (WKY) and spontaneously hypertensive stroke-prone (SHR-SP) male rats (Iffa Credo, L'arbresle, France) were used. The rats were divided in two groups: one was untreated, the other received 1% NaCl in their drinking water from week 8 to week 14. Blood pressure was measured by the tail-cuff method in conscious animals (Physiograph Narco, Houston, Texas, U.S.A.). All rats were killed by decapitation at 14 weeks. The superior mesenteric artery was rapidly removed and immersed in physiological solution (composition in mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, glucose 10, $MgCl_2$ 1.25 and $CaCl_2$ 1.25) gassed with a mixture of 95% O_2 – 5% CO_2. The mesenteric artery was carefully cleaned of all fat and connective tissue. The heart and a segment of the mesenteric artery were rapidly dried between filter papers and weighed.

Simultaneous measurement of contractile tension and membrane potential

A segment of the superior mesenteric artery, ± 2 mm in length, was everted and mounted in a myograph. Briefly, the vessel segment was threaded onto two 40 μm wires. One wire was attached to a stationary support driven by a micrometer, while the other was attached to an isometric force transducer (UC 2 Gould). Vessels were maintained under zero force for 60 min. A passive diameter-tension curve was constructed as described (Mulvany & Halpern, 1977). From this curve the effective transmural pressure was calculated. The vessel was set at a tension equivalent to that generated at $0.9\times$ the diameter of the vessel at 100 mmHg. The bath of the myograph was continuously perfused with physiological solution.

Measurement of the smooth muscle membrane potential was made with a glass microelectrode (Clark Electromedical Instruments, type GC 120F-15) filled with 1.5 M KCl and advanced through the luminal surface of the arterial segment with a micromanipulator (Leitz). After a first drop in voltage, the microelectrode was further advanced into the arterial wall to ensure that a smooth muscle cell was impaled.

The input resistance of the microelectrodes varied between 30 and 80 MΩ. Potential differences were measured with reference (reference electrode: Clark Electromedical Instruments, type E208) to the grounded bath by means of a Dagan amplifier (8100, Minneapolis, MN, U.S.A.). Electrical responses were monitored on an oscilloscope (Hitachi, oscilloscope V-252, 20 MHz). Membrane potential and tension were simultaneously recorded with a pen recorder (Kontron, 500 SP). Criteria for a successful impalement were an abrupt drop in voltage on entry of the microelectrode into the cell, a stable membrane potential for at least 2 min, and a sharp return to zero on withdrawal of the electrode.

After being mounted in the organ chamber, the rings were maintained in gassed physiological solution (see above) containing indomethacin 10 μM at 37°C for 30 min before the initiation of the experiment. Endothelium integrity was assessed at the beginning of each experiment by the application of 1 μM ACh on the plateau of the contraction evoked by noradrenaline (1 μM). The agonists tested were applied in the perfusion solution. When high KCl solutions were used, NaCl concentration was reduced on an equimolar basis. ACh concentration-response curves for the change in resting membrane potential were established by the successive application of different concentrations of the agonist with 30 min time interval between two concentrations in order to avoid the development of tachyphylaxis. Concentration-response curves to ACh in stimulated arteries were obtained by cumulative increase of the concentration of ACh in the superfusion solution. When contraction and depolarization were evoked by noradrenaline, concentration of noradrenaline was adapted to produce a contraction similar to that evoked by 100 mM KCl solution (0.3 – 1 μM). When required, endothelium removal was obtained by gently rubbing the endothelial surface with cotton wool.

Drugs

Acetylcholine chloride (ACh), noradrenaline bitartrate (Nad), N^{ω}-nitro-L-arginine (L-NA), N^{ω}-nitro-L-arginine methylester (L-NAME), L-indomethacin, S-nitroso-N-acetylpenicillamine (SNAP) were from Sigma. Stock solution of indomethacin was prepared in 2% Na_2CO_3.

Statistics

Results are given as mean $\pm$ s.e.mean. Comparisons were made using Student's *t*-test, or by analysis of variance (ANOVA) followed by a Bonferroni test when more than two groups were involved in the comparison. Significant differences were indicated by P values lower than 0.05. EC_{50} values (concentration producing half-maximal effect) were obtained by non-linear regression of the individual concentration-response curves (Multifit, Day Computing, Cambridge, U.K. and KaleidaGraph, Synergy Software, Reading, U.K.). Logarithmic values of EC_{50} (pD_2) were used for the statistical analysis.

Results

Biometric parameters of the rats

Table 1 summarizes the biometric parameters of the rats used in the present study. The systolic blood pressure of the SHR-SP rats was significantly higher than that of the WKY. Confirming previous results (Feron *et al*., 1995), 1% NaCl in the drinking water of the rats increased the blood pressure of SHR-SP and of WKY by 10 – 20 mm Hg, but this difference did not reach a statistically significant level in SHR-SP. The ratio of the heart wet weight on the body weight as well as the wet weight per length unit of the superior mesenteric artery were significantly higher in SHR-SP than in WKY. NaCl-load significantly increased the weight of the mesenteric artery and

of the heart in SHR-SP ($P<0.05$), but not in WKY. The internal diameter measured at 100 mm of Hg was significantly greater in WKY compared to SHR-SP but was not modified by NaCl-load.

Effect of acetylcholine on resting membrane potential of the superior mesenteric artery

The resting membrane potential of mesenteric artery smooth muscle cells was significantly lower in SHR-SP compared to WKY (Table 2). NaCl-load of the rats resulted in a significant depolarization of about 3 mV in WKY as in SHR-SP (Table 2). In the mesenteric artery of WKY, ACh evoked a rapid and sustained hyperpolarization. Concentration-effect curves were established by applying successive concentrations of ACh at random to avoid tachyphylaxis. The effect of ACh was concentration-dependent; the maximal amplitude of the hyperpolarization was obtained with 1 μM (Figure 1). The response often decreased with higher concentrations. The amplitude of the hyperpolarization to 1 μM ACh was significantly lower in arteries from SHR-SP than from WKY, but the pD_2 values of ACh were similar. NaCl-load did not significantly affect the hyperpolarization to ACh in WKY but significantly decreased its amplitude in SHR-SP (Figure 1 and Table 3).

Incubation of arteries with L-NA (100 μM) slightly depolarized the resting membrane potential in WKY

Table 2 Mean values of resting membrane potential of the smooth muscle cells of the superior mesenteric artery

		Membrane potential (mV) *Without L-NA*		*With L-NA*	
WKY	untreated	-45.7 ± 0.3	(51)	-44.4 ± 0.4^{c}	(45)
WKY	NaCl-loaded	-42.2 ± 0.6^{a}	(12)	-43.4 ± 0.8	(9)
SHR-SP	untreated	-40.8 ± 0.2^{b}	(52)	-40.5 ± 0.3^{b}	(48)
SHR-SP	NaCl-loaded	$-38.1\pm0.3^{a,b}$	(44)	$-38.3\pm0.2^{a,b}$	(40)

Data are means$\pm$s.e.mean from (n) cells from the superior mesenteric artery of normotensive Wistar Kyoto (WKY) and spontaneously hypertensive stroke-prone (SHR-SP) rats supplied without (untreated) or with 1% NaCl (NaCl-loaded) in their drinking water. N^{ω}-nitro-L-arginine (L-NA) was applied for 30 min at 100 μM. Data without L-NA and with L-NA are not from the same cells. $^{a}P<0.01$ NaCl-loaded rats versus untreated rat. $^{b}P<0.01$ SHR-SP versus WKY. $^{c}P<0.01$ with L-NA versus without L-NA (ANOVA).

Table 1 Biometric parameters of Wistar Kyoto normotensive rats (WKY) and spontaneously hypertensive stroke-prone rats (SHR-SP) supplied without (untreated) or with 1% NaCl (NaCl-loaded) in their drinking water

		n	*Blood pressure* (mm Hg)	*Heart weight/ body weight* (mg g^{-1})	*Mesenteric artery* *Diameter at 100 mm Hg* (mm)	*Weight* (mg mm^{-1})
WKY	untreated	34	145 ± 3	2.9 ± 0.04	1.06 ± 0.01	0.27 ± 0.014
WKY	NaCl-loaded	12	167 ± 4^{a}	2.9 ± 0.06	1.04 ± 0.03	0.27 ± 0.10
SHR-SP	untreated	36	242 ± 5^{a}	3.5 ± 0.04^{a}	0.91 ± 0.02^{a}	0.32 ± 0.012^{a}
SHR-SP	NaCl-loaded	35	253 ± 5^{c}	$4.1\pm0.05^{b,c}$	0.90 ± 0.02^{c}	$0.38\pm0.014^{b,c}$

Data represent means$\pm$s.e.mean from n rats. $^{a}P<0.05$ versus untreated WKY. $^{b}P<0.05$ versus untreated SHR-SP. $^{c}P<0.05$ versus NaCl-loaded WKY.

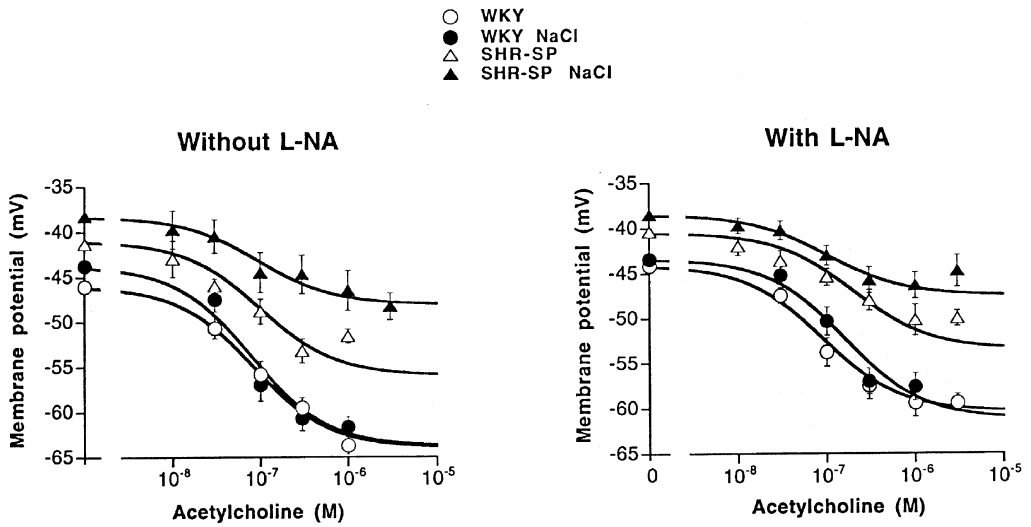

Figure 1 Concentration-response curves for the hyperpolarization to acetylcholine in the superior mesenteric artery from normotensive Wistar Kyoto rats (WKY) and from spontaneously hypertensive stroke-prone rats (SHR-SP) supplied with 1% NaCl drinking water (NaCl-loaded) or with normal water (untreated). Left panel: membrane potential changes evoked by acetylcholine in the absence of N^{ω}-nitro-L-arginine (L-NA). Right panel: membrane potential changes evoked by acetylcholine in the presence of N^{ω}-nitro-L-arginine (L-NA, 100 μM). Points are mean values$\pm$s.e.mean obtained from ten and six determinations in WKY, four and four determinations in NaCl-loaded WKY, six and five determinations in untreated SHR-SP and four and four determinations in NaCl-loaded SHR-SP, in the absence and in the presence of L-NA, respectively. Data without and with L-NA were not obtained from the same arteries.

(difference in resting membrane potential with L-NA and without L-NA: 1.3 ± 0.5 mV; $P<0.05$) but not in the other groups (Table 2). In the presence of L-NA, the hyperpolarization evoked by ACh (1 μM) was not significantly different from that recorded in its absence (Figure 1 and Table 3). No difference was noted when L-NAME was used instead of L-NA or in addition to L-NA. Hyperpolarization was markedly blunted in 40 mM KCl solution: in mesenteric artery from WKY, hyperpolarization to ACh was -2.5 ± 0.6 mV ($n=4$) in 40 mM KCl, compared to -17.8 ± 0.6 mV ($n=23$) in 5.9 mM KCl ($P<0.01$).

In endothelium-denuded arteries the resting membrane potential was slightly depolarized compared to intact arteries and ACh (1 μM) failed to produce any hyperpolarization. Instead, a small depolarization of about 2 mV was observed. It was associated with a small increase in tension (Figure 2). No difference was noted between the different groups of rats in the responses to ACh in endothelium-denuded artery rings.

Responses to acetylcholine in superior mesenteric artery exposed to noradrenaline

Noradrenaline (1 μM) depolarized WKY mesenteric artery smooth muscle cells by 15.2 ± 1.2 mV and simultaneously increased tone by 7.5 ± 1.3 mN ($n=5$). In some arteries, the depolarization was associated with a rhythmic electrical activity of about 10 mV amplitude. ACh added on the plateau of the responses evoked by noradrenaline induced a rapid but transient repolarization and a parallel relaxation (Figure 3a). At concentrations higher than 0.1 μM ACh hyperpolarized the cell membrane. The maximum change in membrane potential was observed in the presence of ACh 1 μM and was equal to -25.2 ± 4.1 mV ($n=4$) leading to a potential value 11 ± 2 mV more negative than the resting membrane potential. Simultaneously, ACh relaxed the contraction induced by noradrenaline by $96 \pm 2\%$ (Figure 3a,b). pD_2 values of ACh, for the relaxation and the change in membrane potential were not significantly different (Table 4).

In the mesenteric artery from SHR-SP, contracted and depolarized by noradrenaline (1 μM; mean value of depolarization: 16.2 ± 1.3 mV, mean value of contraction: 10.4 ± 0.7 mN, $n=14$), the responses to ACh were similar to those recorded in WKY (Figure 4a, Table 4). ACh pD_2 values for the relaxation and the change in membrane potential were not significantly different from the values measured in WKY (Table 4). The maximal change of membrane potential evoked by ACh was slightly but not significantly smaller than that recorded in WKY; nevertheless, it produced a smaller hyperpolarization than that recorded in WKY (membrane potential in the presence of ACh 1 μM was 5.8 ± 1.6 mV more negative than the resting membrane potential, $n=6$, $P<0.05$ versus WKY).

In NaCl-loaded SHR-SP the mean value of noradrenaline-evoked depolarization was of 15.0 ± 0.7 mV and was associated with a contraction of 9.3 ± 1.0 mN ($n=10$). The effects of ACh on membrane potential and on contractile

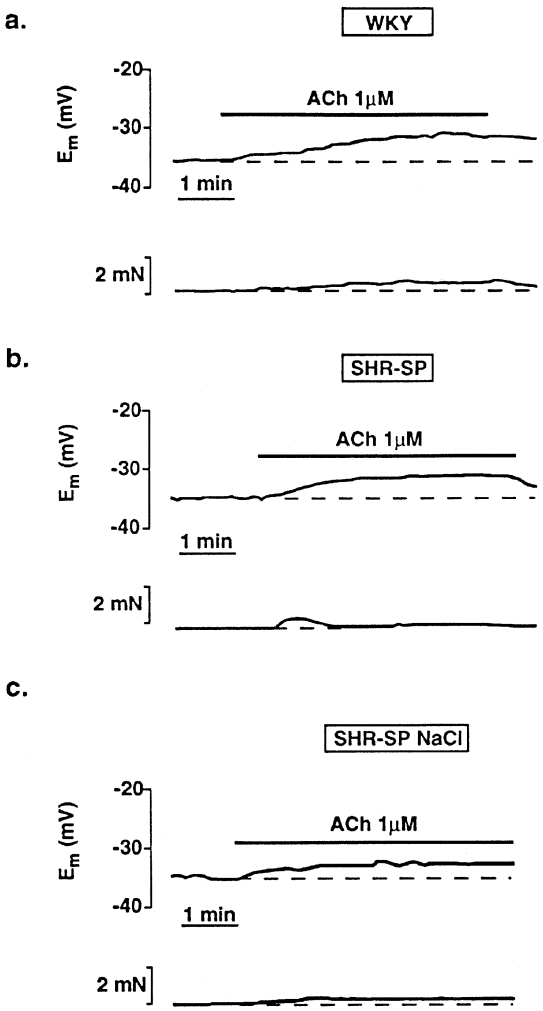

Figure 2 Representative records of the effect of acetylcholine on membrane potential (E_m, upper traces) and tension (lower traces) obtained simultaneously in endothelium-denuded superior mesenteric artery rings from Wistar Kyoto normotensive rats (WKY, a), untreated spontaneously hypertensive rats (SHR-SP, b), and SHR-SP supplied with 1% NaCl drinking water (NaCl-loaded SHR-SP, c). Acetylcholine (ACh, 1 μM) was applied as indicated.

Table 3 Effect of acetylcholine on the resting membrane potential of smooth muscle cells of the superior mesenteric artery of untreated and NaCl-loaded normotensive (WKY) and spontaneously hypertensive stroke-prone (SHR-SP) rats

		without L-NA			*with L-NA* (100 μM)		
		ΔE_m (mV)		*pD2*	ΔE_m (mV)		*pD2*
WKY	untreated	-17.8 ± 0.6	(23)	7.07 ± 0.22 (10)	-17.7 ± 1.2	(13)	7.19 ± 0.12 (5)
	NaCl-loaded	-16.5 ± 0.7	(7)	6.97 ± 0.18 (4)	-15.9 ± 1.7	(4)	6.75 ± 0.12 (3)
SHR-SP	untreated	-13.7 ± 1.2	(19)[a]	7.21 ± 0.11 (5)	-13.1 ± 1.6	(13)[a]	7.21 ± 0.11 (4)
	NaCl-loaded	-10.5 ± 0.6	(11)[a,b,c]	7.41 ± 0.05 (4)	-8.9 ± 1.3	(10)[a,b,c]	6.84 ± 0.16 (4)

NaCl-loaded rats were given 1% NaCl in their drinking water. ΔE_m represents the hyperpolarization evoked by 1 μM acetylcholine. pD_2 values are the negative log of the concentrations of acetylcholine producing an effect equal to 50% of the maximum. They were determined by fitting the data of the acetylcholine concentration-effect curves to the equation $E_m = E_{m(rest)} - \frac{\Delta E_{m(max)} * [ACh]}{[ACh] + EC_{50}}$ with $E_{m(rest)}$ being the resting membrane potential, $\Delta E_{m(max)}$, the maximum amplitude of the hyperpolarization produced by acetylcholine and [ACh], the concentration of acetylcholine. Data are means $\pm$ s.e.mean from (n) determinations. [a]Significantly different from untreated WKY. [b]Significantly different from untreated SHR-SP. [c]Significantly different from NaCl-loaded WKY.

tension were depressed compared to untreated SHR-SP, although only the decrease in relaxation reached a statistically significant level (Figure 5b, Table 4).

In normotensive WKY, NaCl-load did not significantly affect the concentration-relaxation curve to ACh in noradrenaline-contracted mesenteric artery rings (Table 4).

Effect of L-NA on responses to acetylcholine in superior mesenteric artery exposed to noradrenaline

To investigate the involvement of NO in the responses to ACh, WKY arteries were incubated in the presence of NO synthases (NOS) inhibitors (L-NA, L-NAME, or both). The inhibition of endothelial NOS activity increased the contraction to noradrenaline (noradrenaline pD_2 values were equal to 6.56 ± 0.16 and 7.26 ± 0.13, in the absence and in the presence of L-NA, respectively, $n=6$, $P<0.01$). Therefore, in order to obtain a contractile tone of similar amplitude as in the absence of NOS inhibitor, the concentration of noradrenaline was lowered to 0.5 μM. In this condition, noradrenaline produced a depolarization of 17.0 ± 1.7 mV and a contraction of 8.4 ± 1 mN ($n=4$, Figure 3a).

L-NA produced a slight, not significant shift of the concentration-response curve of the ACh-evoked change in membrane potential, with an unchanged maximum. The relaxation to ACh was more affected: the maximum was significantly decreased ($P<0.01$ versus in the absence of NOS inhibitor) and the concentration-relaxation curve was significantly displaced to the right (Figure 3b, Table 4). No difference was noted when L-NAME (100 μM) or L-NA plus L-NAME were used instead of L-NA.

In arteries isolated from SHR-SP incubated in the presence of L-NA, noradrenaline (0.5 μM) produced a depolarization of 14.5 ± 1.8 mV ($n=8$), and contracted the artery ring by 10.5 ± 0.8 mN ($n=12$). As observed in WKY, L-NA only slightly affected the electrical responses to ACh. Simultaneously, the relaxation curve was significantly shifted to higher concentrations and its maximum was decreased ($P<0.05$ compared to the responses measured in the absence of L-NA; Figure 4b, Table 4). However, compared to WKY, the

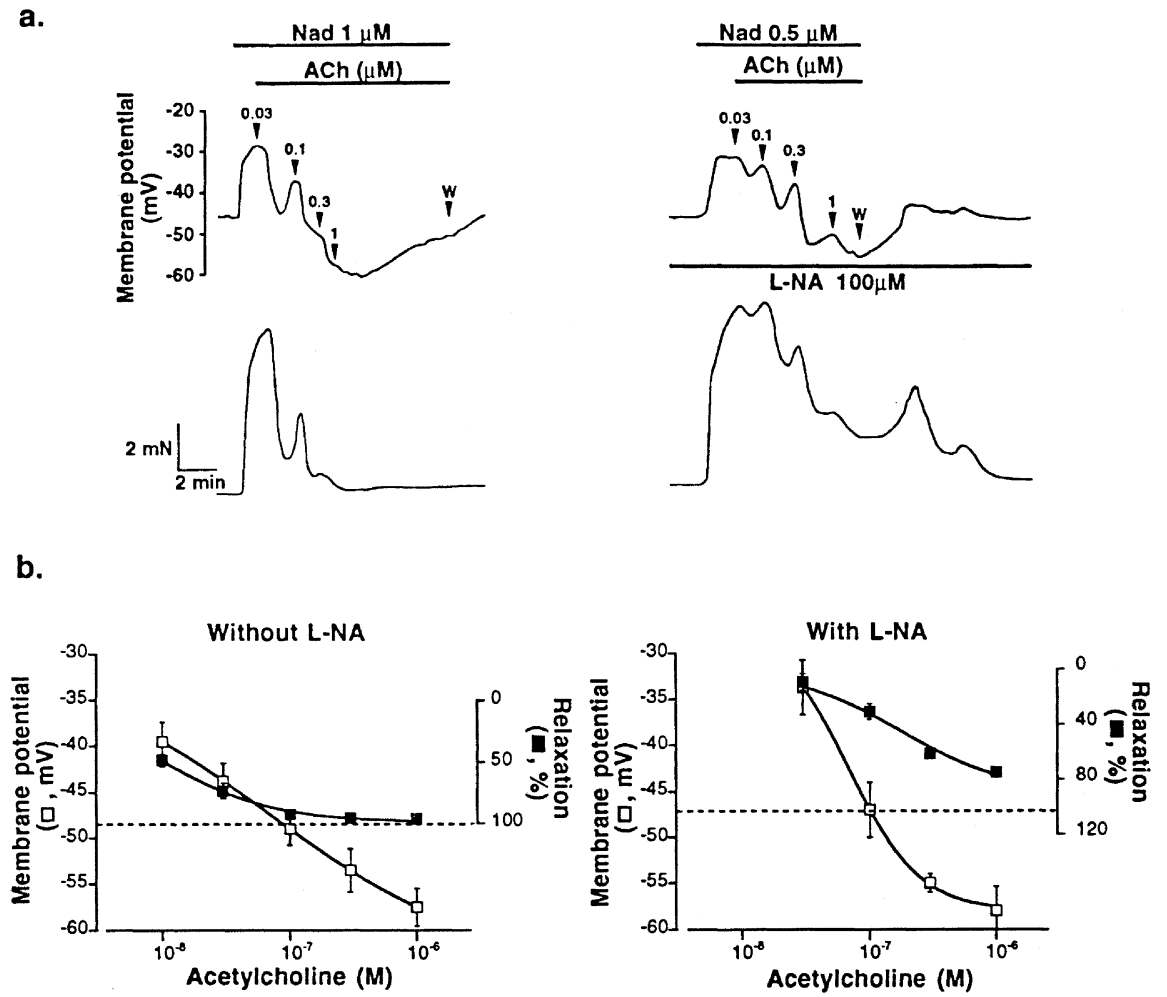

Figure 3 Effects of acetylcholine on the membrane potential and the contractile tension simultaneously recorded in Wistar Kyoto (WKY) mesenteric artery rings contracted and depolarized by noradrenaline. (a) Representative traces of the membrane potential (upper panel) and the contractile tension (lower panel) recorded simultaneously. Mesenteric artery rings were stimulated with noradrenaline in the absence of N^{ω}-nitro-L-arginine (L-NA) (left panel), or in the presence of N^{ω}-nitro-L-arginine (L-NA 100 μM) (right panel). L-NA was added to the perfusion solution 30 min before noradrenaline. Acetylcholine (ACh) and noradrenaline (Nad) were applied as indicated. (b) Mean concentration-response curves for the hyperpolarization and for the relaxation to acetylcholine in the absence (left panel) and in the presence (right panel) of L-NA. Ordinate scales are adjusted so that 0 and 100% relaxation correspond to the level of membrane potential recorded in the presence of noradrenaline and to the resting potential, respectively. Level of resting membrane potential is indicated by the dotted line. Relaxation is expressed as percentage of the contraction evoked by noradrenaline. Points are means $\pm$ s.e.mean from four determinations.

Table 4 Influence of N^{ω}-nitro-L-arginine (L-NA) on the effects of acetylcholine on membrane potential and contractile tension in noradrenaline-stimulated mesenteric artery

A. pD_2 values of acetylcholine

		Change in membrane potential				*Relaxation*			
		without L-NA		*with L-NA*		*without L-NA*		*with L-NA*	
WKY	untreated	7.32±0.12	(4)	7.08±0.08	(3)	7.67±0.19	(4)	6.71±0.15	(3)[a]
WKY	NaCl-loaded	N.D.		N.D.		7.29±0.10	(5)	6.78±0.08	(6)[a]
SHR-SP	untreated	7.12±0.13	(4)	6.77±0.34	(4)	7.49±0.07	(8)	7.14±0.05	(6)[a,b]
SHR-SP	NaCl-loaded	6.96±0.08	(4)	<6		6.81±0.11	(4)[b,c]	<6	

B. Responses to acetylcholine 1 μM

		Change in membrane potential (mV)				*Relaxation* (%)			
		without L-NA		*with L-NA*		*without L-NA*		*with L-NA*	
WKY	untreated	−25.2±4.1	(4)	−29.8±2.5	(4)	96±2	(4)	75±2	(4)[a]
WKY	NaCl-loaded	N.D.		N.D.		96±1	(5)	77±5	(6)[a]
SHR-SP	untreated	−22.5±2.8	(6)	−16.0±1.0	(6)[b]	93±2	(12)	79±3	(11)[a]
SHR-SP	NaCl-loaded	−20.5±1.5	(4)	−6.2±3.5	(6)[a,b,c]	72±7	(7)[b,c]	14±6	(6)[a,b,c]

Data are means±s.e.mean from (*n*) arteries from Wistar Kyoto normotensive rats (WKY) or from spontaneously hypertensive stroke-prone rats (SHR-SP). NaCl-loaded rats were supplied with 1% NaCl in their drinking water. Relaxation is expressed as a percentage of the contraction evoked by noradrenaline. Change in membrane potential is the difference between the membrane potential recorded in the presence of acetylcholine and the value recorded in the presence of noradrenaline immediately before the addition of acetylcholine. Contractions were evoked by noradrenaline 1 μM (without L-NA) or 0.5 μM (with L-NA). L-NA was applied at 100 μM. N.D. not determined. [a]$P<0.05$ versus in the absence of L-NA. [b]$P<0.05$ versus WKY. [c]$P<0.05$ versus SHR-SP.

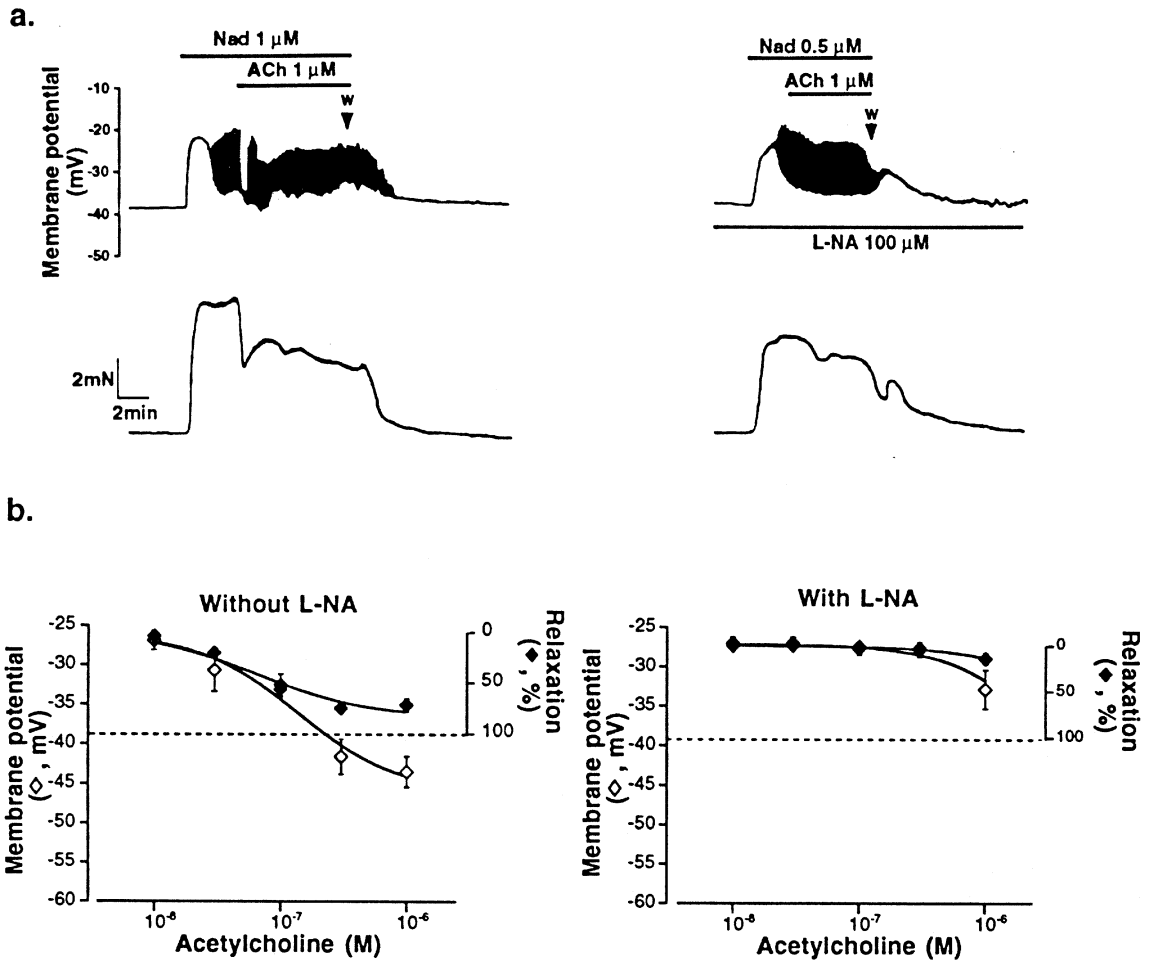

Figure 4 Effect of acetylcholine on the membrane potential and the contractile tension simultaneously recorded in noradrenaline-stimulated mesenteric artery rings from untreated spontaneously hypertensive stroke-prone rat (SHR-SP). (a) Representative recording of the membrane potential (upper trace) and the contractile tension (lower trace) in the absence of N^{ω}-nitro-L-arginine (left panel) and in the presence of N^{ω}-nitro-L-arginine (L-NA – 100 μM) (right panel). L-NA was added to the perfusion solution 30 min before noradrenaline. Acetylcholine (ACh) and noradrenaline (Nad) were applied as indicated. Traces without and with L-NA were from different arteries. (b) Mean concentration-response curves for the hyperpolarization and for the relaxation to acetylcholine in the absence (left panel) and in the presence (right panel) of L-NA. Ordinate scales are adjusted so that 0 and 100% relaxation correspond to the level of membrane potential recorded in the presence of noradrenaline and to the resting potential, respectively. Level of resting membrane potential is indicated by the dotted line. Relaxation is expressed as percentage of the contraction evoked by noradrenaline. Points are means±s.e.mean from four–eight determinations.

maximum change of membrane potential evoked by ACh in the presence of L-NA was significantly smaller in SHR-SP ($n=6$; $P<0.05$), but there was no significant difference in the relaxation to ACh between SHR-SP and WKY (Table 4).

In NaCl-loaded SHR-SP, in the presence of L-NA (100 μM), noradrenaline (0.5 μM) produced a depolarization of 11.6 ± 1.8 mV and simultaneously contracted the artery by 8.1 ± 1.0 mN ($n=7$). L-NA dramatically reduced the effects of ACh on both tension and membrane potential (Figure 5a): ACh failed to reverse the depolarization evoked by noradrenaline and, simultaneously, the relaxation was markedly depressed ($P<0.05$ versus in the absence of L-NA) (Table 4).

In NaCl-loaded WKY rats, the responses to ACh exhibited the same sensitivity to L-NA as that observed in unloaded rats (Table 4).

Effect of the NO donor SNAP on membrane potential and tension in noradrenaline-contracted mesenteric artery

The observation that the electrical response to ACh in mesenteric artery isolated from NaCl-loaded SHR-SP was sensitive to L-NA indicated that NO could affect membrane potential in noradrenaline-depolarized arteries. The following experiments were performed in order to determine whether an electrical effect of NO could be found in arteries from WKY and from untreated SHR-SP despite the fact that the hyperpolarization evoked by 1 μM ACh was unaffected by NO synthase blockade. With this aim, we investigated the effects of the NO donor SNAP in mesenteric artery rings perfused with L-NA-containing physiological solution. As in

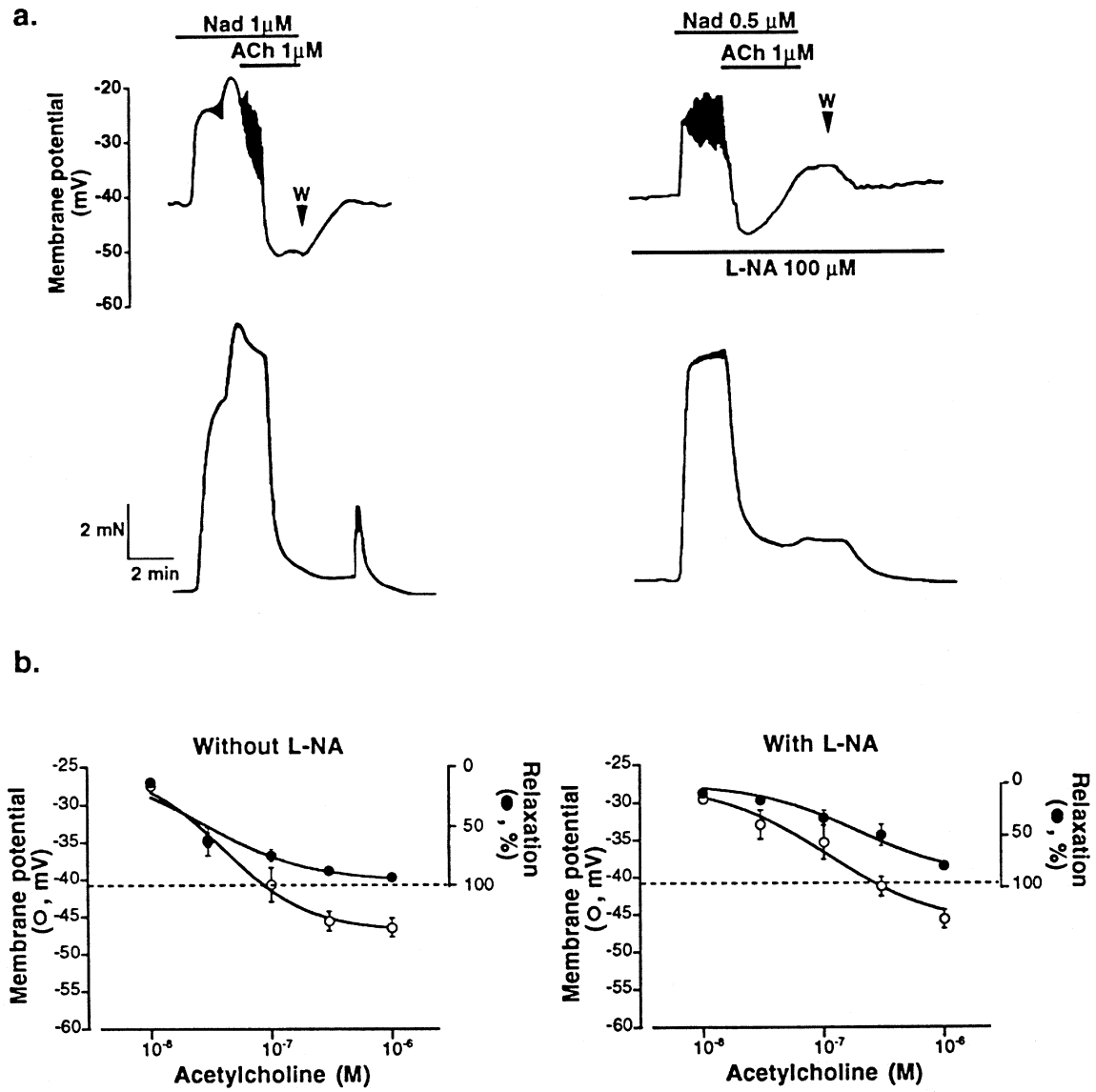

Figure 5 Effect of acetylcholine on the membrane potential and the contractile tension in noradrenaline-stimulated mesenteric artery rings from spontaneously hypertensive stroke-prone rats given 1% NaCl in their drinking water (NaCl-loaded SHR-SP). (a) Representative simultaneous recording of the membrane potential (upper trace) and the contractile tension (lower trace) in the absence of N^{ω}-nitro-L-arginine (left panel) and in the presence of N^{ω}-nitro-L-arginine (L-NA 100 μM) (right panel). L-NA was added to the perfusion solution 30 min before noradrenaline. Acetylcholine (ACh) and noradrenaline (Nad) were applied as indicated. Traces without and with L-NA were from different arteries. (b) Mean concentration-response curves for the hyperpolarization and for the relaxation to acetylcholine in the absence (left panel) and in the presence (right panel) of L-NA. Ordinate scales are adjusted so that 0 and 100% relaxation correspond to the level of membrane potential recorded in the presence of noradrenaline and to the resting potential, respectively. Level of resting membrane potential is indicated by the dotted line. Relaxation is expressed as percentage of the contraction evoked by noradrenaline. Points are means$\pm$s.e.mean from four determinations.

the experiments performed with ACh, the concentration of noradrenaline was adapted to produce similar depolarizations. The depolarization evoked by noradrenaline was of 12.2 ± 0.9 mV ($n=11$), 12.9 ± 0.6 mV ($n=5$) and 13.4 ± 0.9 mV ($n=8$) in WKY, SHR-SP and NaCl-loaded SHR-SP. In normotensive as in hypertensive rats, the addition of SNAP on the plateau of the responses evoked by noradrenaline produced a concentration-dependent, rapid and complete reversal of the depolarization and of the contraction evoked by noradrenaline (Figure 6). Close values of pD_2 were found for the change in membrane potential and the relaxation. NaCl-loaded SHR-SP were significantly more sensitive to the actions of SNAP on membrane potential and on tension compared to WKY and unloaded SHR-SP: pD_2 values in WKY ($n=5$), SHR-SP ($n=4$) and NaCl-loaded SHR-SP ($n=4$) were respectively 6.58 ± 0.13, 6.72 ± 0.10 and 7.22 ± 0.07 ($P<0.05$ versus WKY and versus SHR-SP) for the change in membrane potential and 6.72 ± 0.06, 6.85 ± 0.3 and 7.25 ± 0.07 ($P<0.05$ versus WKY and versus SHR-SP) for the relaxation.

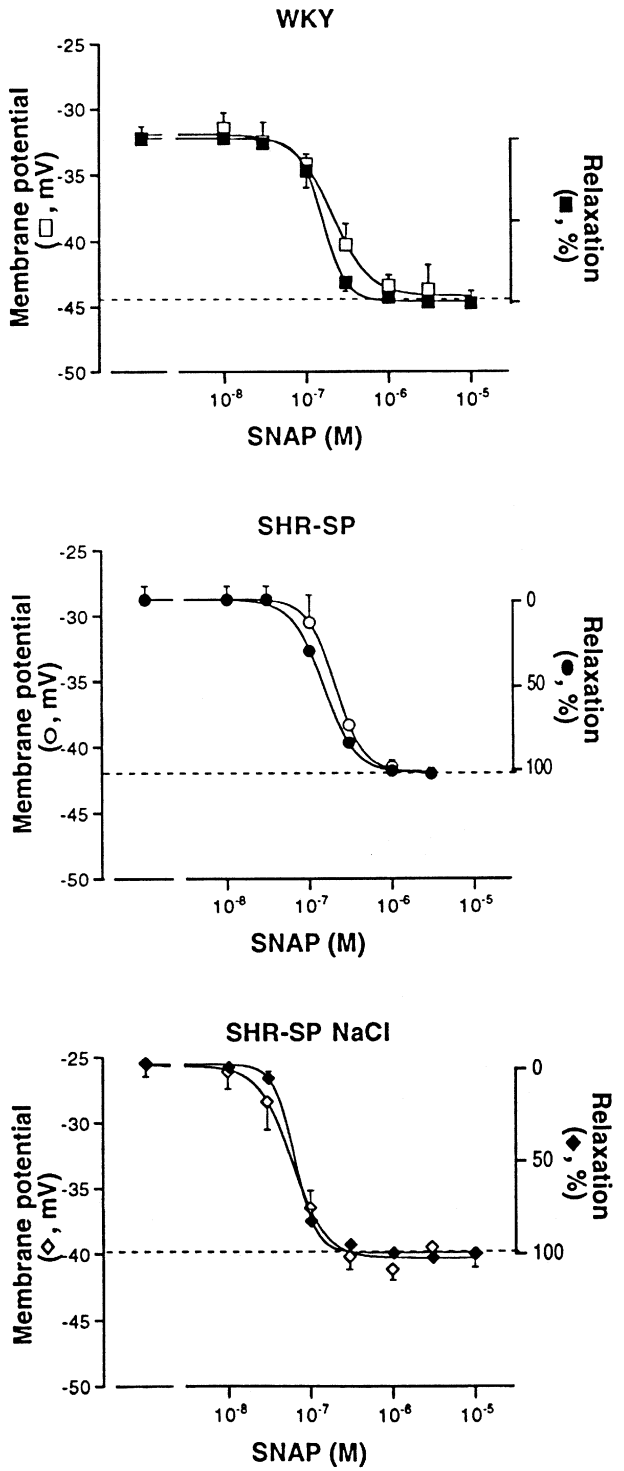

Figure 6 Mean concentration-response curves for the change in membrane potential and the relaxation to SNAP in noradrenaline-contracted mesenteric artery rings isolated from normotensive Wistar Kyoto rats (WKY), from spontaneously hypertensive stroke-prone rats (SHR-SP) and from SHR-SP given 1% NaCl in the drinking water (NaCl-loaded SHR-SP). Ordinate scales are adjusted so that 0 and 100% relaxation correspond to the level of membrane potential recorded in the presence of noradrenaline and to the resting potential, respectively. Level of resting membrane potential is indicated by the dotted line. Relaxation is expressed as a percentage of the contraction evoked by noradrenaline. Points are means $\pm$s.e.mean from five–seven determinations.

Relaxation to acetylcholine in KCl-contracted mesenteric artery rings

As mentioned above, ACh-evoked hyperpolarization was nearly abolished by a high concentration of KCl. Relaxation was also markedly depressed, as compared with noradrenaline-contracted artery: maximum relaxation of the contraction evoked by 100 mM KCl solution was $45 \pm 6\%$ ($n=5$), $42 \pm 5\%$ ($n=5$) and $41 \pm 4\%$ ($n=4$) of the contraction in WKY, SHR-SP and NaCl-loaded SHR-SP, respectively (Figure 7). However, NaCl-loaded SHR-SP were significantly more sensitive to ACh than were unloaded SHR-SP: pD_2 values were 7.18 ± 0.11 ($n=5$), 7.17 ± 0.22 ($n=5$) and 7.71 ± 0.06 ($n=4$) in WKY, SHR-SP and NaCl-loaded SHR-SP, respectively ($P<0.05$ NaCl-loaded SHR-SP versus WKY and versus untreated SHR-SP). The responses to ACh in KCl-activated arteries were completely blocked by L-NA.

Discussion

Release of both NO and EDHF after stimulation of endothelium with ACh has been reported in many arteries (for a review see Garland *et al.*, 1995). The respective role of

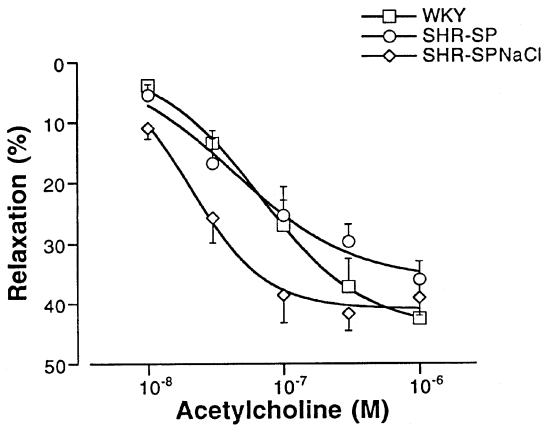

Figure 7 Mean concentration-response curves for the relaxation to acetylcholine in KCl-contracted mesenteric artery rings isolated from normotensive Wistar Kyoto rats (WKY), from spontaneously hypertensive stroke-prone rats (SHR-SP) and from SHR-SP given 1% NaCl in the drinking water (NaCl-loaded SHR-SP). Relaxation is expressed as a percentage of the contraction evoked by 100 mM-KCl solution. Points are means $\pm$s.e.mean from five–seven determinations.

these two factors varies with the artery, EDHF being more involved in the endothelium-dependent relaxation in resistance arteries than in conduit arteries (Garland *et al*., 1995; Shimokawa *et al*., 1996). The present results show that the relative contribution of NO and EDHF is modified in a pathological situation.

In the unstimulated superior mesenteric artery of WKY, the endothelium-dependent changes in membrane potential evoked by ACh in the presence of indomethacin were not significantly affected by NOS inhibitors. This observation is in agreement with the activation by ACh of an endothelium-derived hyperpolarizing factor (EDHF) distinct of NO (Bolton *et al*., 1984; Adeagbo & Triggle, 1993; McCullogh *et al*., 1997). This factor has been reported to activate K^+-channels and/or Na^+, K^+-ATPase in vascular smooth muscle cells (Chen & Suzuki, 1989; Edwards *et al*., 1998). In the rat superior mesenteric artery, the L-NA-resistant hyperpolarization induced by ACh was reduced by increasing the extracellular concentration of KCl to 40 mM, in agreement with a contribution of K^+-channels to this effect.

The observation that, in the superior mesenteric artery, basal permanently released NO, as well as NO released from endothelial cells stimulated by ACh, had little effect on the resting membrane potential is at variance with reports by us and others in several vascular preparations (Krippeit-Drews *et al*., 1992; Garland & McPherson, 1992; Parkington *et al*., 1993), but is in agreement with other reports (Vanheel *et al*., 1994; Plane & Garland, 1993), indicating that important differences in the action of NO could exist, depending on the vascular bed.

In noradrenaline-contracted artery, ACh hyperpolarized the smooth muscle cell membrane and relaxed the contraction with a similar potency. L-NA produced a shift in the concentration-relaxation curve to ACh and a depression of the maximum response, but did not abolish the relaxation, in agreement with the involvement of both NO and EDHF in the relaxation (Waldron & Garland, 1994). In KCl-contracted artery any effect on the membrane potential was abolished and relaxation to ACh was dependent on NO only, as indicated by its abolition by L-NA. The relaxing effect of NO could then be related to the effect of cyclic GMP on the contractile machinery of vascular smooth muscle cells (Salomone *et al*., 1995). The change in membrane potential evoked by ACh in noradrenaline-contracted artery was less affected by L-NA than was the relaxation: L-NA did not significantly change either the maximum effect of ACh or its EC_{50} value. An effect of NO on membrane potential in noradrenaline-stimulated artery was suggested by the experiments using the NO donor SNAP, which simultaneously relaxed the contraction and repolarized the membrane potential in the noradrenaline-contracted artery. It is worthwhile noting that SNAP did not hyperpolarize the cell membrane but only reversed the depolarization evoked by noradrenaline. Similar repolarization of membrane potential evoked by NO has been reported in uterine artery contracted with phenylephrine (Tare *et al*., 1990). The effect of NO on membrane potential was not observed when the artery was depolarized with KCl or in an unstimulated artery (Ghisdal & Morel, 1998); it can then be considered as an antagonistic effect on noradrenaline-evoked responses resulting from the interaction of NO with the excitation-contraction coupling process activated by noradrenaline, at a level upstream of the activation of the depolarization (Ghisdal & Morel, 1998). The observation that in WKY artery the NO-dependent effect of ACh on the depolarization evoked by noradrenaline was hardly detected by using L-NA can be explained by the simultaneous release by ACh of both NO and EDHF, the effect of NO on the membrane potential being masked by the hyperpolarizing effect of EDHF.

It is generally accepted that endothelium-dependent relaxation is decreased in hypertension (Vanhoutte, 1996), although contradictory results have been reported. Unaltered endothelium-dependent relaxation has been reported in young and adult SHR mesenteric artery (Wirth *et al*., 1996), while in old SHR and in 6–8 month-old SHR-SP (Fujii *et al*., 1992) relaxation to ACh is impaired. Not only has age an important influence on the endothelial function but also the vascular preparation, since hypertension in combination with ageing has been shown to induce an endothelial dysfunction in conduit arteries but not in resistance vessels (Husken *et al*., 1994). In the present study, the relaxation evoked by ACh was well preserved in noradrenaline- or KCl-contracted mesenteric artery from SHR-SP compared to WKY. NaCl-rich diet is known to markedly aggravate the vascular pathology and to increase the mortality in those rats (Yamori *et al*., 1984). The low concentration of NaCl used in the present study (1% in the drinking water) increased the blood pressure but did not cause cardio-vascular hypertrophy in WKY, in contrast with the marked cardiac and renal hypertrophy produced by 8% NaCl in both WKY and SHR (Yu *et al*., 1998). The present results showed that NaCl decreased the relaxation to ACh in noradrenaline-contracted artery from 93% of the contraction in untreated SHR-SP to 72% of the contraction in NaCl-loaded SHR-SP, but that NaCl did not affect relaxation in WKY. Moreover, NaCl-load selectively impaired the L-NA-resistant component of the relaxation, which was reduced to 14% of the contraction (with 1 μM ACh) compared to 79% in unloaded rats. An alteration in EDHF response in NaCl-loaded SHR-SP was more directly indicated by the decrease in the L-NA-resistant hyperpolarization to ACh in resting arteries and in noradrenaline-activated arteries. Impaired EDHF-mediated responses to ACh in NaCl-loaded SHR-SP could result from alterations occurring at the level of endothelial or muscular cells. Functional changes of the mesenteric artery in NaCl-loaded SHR-SP are illustrated by their increased reactivity to the Ca^{2+} channel agonist Bay K 8644 (Godfraind *et al*., 1997), which could be related to the depolarization of the smooth muscle cells. The depolarization evoked by the NaCl-rich diet was added to a depolarization associated with hypertension which has already been reported in several arteries and in different models of hypertension (Cheung & MacKay, 1986; Tomobe *et al*., 1991; Fujii *et al*., 1992; Van de Voorde *et al*., 1992; Morel & Godfraind, 1994) although not everywhere (Chai & Webb, 1992; Kuriyama & Suzuki, 1978; Silva *et al*., 1994). The hypertension-associated depolarization of vascular smooth muscle cells has not yet been explained. Several hypotheses have been proposed: a change in ionic distribution (Jones, 1973), a change in Na^+, K^+-ATPase activity (Hermsmeyer, 1986) or in K^+-channel activity (Rusch *et al*., 1992; Liu *et al*., 1994). The effect of NaCl-load on membrane potential was not specific to hypertensive rats as it was also observed in WKY. It has been reported that high sodium diet increases the amount of a circulating digitalis-like factor (Castaneda Hernandes & Godfraind, 1984), which could be the cause of the depolarization observed in both WKY and SHR-SP. Whether the decrease in EDHF could be related to the depolarization of the smooth muscle cells remains to be determined. It is possible that the combination of the depolarization caused by hypertension and NaCl-load eventually leads to alteration in synthesis or action of EDHF. A membrane potential change in smooth muscle cells has been

reported to electrotonically spread into endothelial cells (Bény, 1997). The resulting depolarization of endothelial cells could affect EDHF production. EDHF has been proposed to pass through gap junctions (Taylor *et al*., 1998); the hypothesis that cell–cell communications could be disturbed in the hypertrophied arteries from NaCl-loaded SHR-SP is attractive. Alternatively, a change in smooth muscle cells K^+-channel activity cannot be excluded. Further experiments will be designed in order to investigate these hypotheses.

Although EDHF was impaired in NaCl-loaded SHR-SP, the relaxation to ACh 1 μM was maintained at about 70% in the absence of NOS blocker. This relaxation was mainly dependent on the release of NO, as indicated by its nearly complete abolition by L-NA, suggesting that the NO-dependent component of the relaxation was not altered. Also the relaxation of the KCl contraction, which only was caused by endothelium-derived NO since EDHF was inhibited in high-KCl solution, was not depressed. Moreover, arteries from NaCl-loaded rats were more sensitive to NO than arteries from unloaded SHR-SP or from WKY: this was observed in KCl-depolarized arteries, which were more sensitive to ACh, and in noradrenaline-contracted arteries, when SNAP was used as source of NO. The present results, showing an increase in NO-mediated relaxation associated with an attenuation in EDHF-mediated effects, could agree with the existence of a cross-talk between NO and EDHF. Increase in the basal production of NO has been suggested to cause a reduction in the production of EDHF (Bauersachs *et al*., 1996). However, in NaCl-loaded SHR-SP, the increased effect of NO appeared to result from an increased sensitivity since it was observed with a NO donor, more than from an increased production of NO. Nevertheless, the production of NO was not measured directly and an increased production therefore cannot be excluded as increased activity of NO synthases has been reported in SHR and SHR-SP arteries (McIntyre *et al*., 1997; Bennai *et al*., 1999). An increased sensitivity to NO has also been reported in the mesenteric artery bed (Qiu *et al*., 1998), and in aortic rings from SHR where higher levels of messenger RNA for the beta 1 subunit of soluble guanylate cyclase were revealed (Papapetropoulos *et al*., 1994).

An important observation reported in the present paper was that the effect of ACh on membrane potential was not sensitive to L-NA in WKY artery but was markedly depressed by the NOS inhibitor in NaCl-loaded SHR-SP, suggesting that in the latter group of rats, NO was the principal mediator of the change in membrane potential evoked by ACh. In view of the effects of the NO donor SNAP on the membrane potential in noradrenaline-contracted artery, which was observed in all the groups of rats, it may be hypothesized that in NaCl-loaded SHR-SP, impairment of EDHF-mediated hyperpolarization was such that it revealed the effect of NO on the membrane potential.

In conclusion, the NaCl-rich diet of SHR-SP markedly impaired the EDHF-mediated effects of ACh. Simultaneously, the sensitivity to NO was increased. The reduction in EDHF-evoked response revealed a change in membrane potential mediated by NO in noradrenaline-stimulated artery. Despite the decrease in EDHF, endothelium-dependent relaxation to ACh was only slightly affected.

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