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Evidence for reduced β-adrenoceptor coupling to adenylate cyclase in femoral arteries from spontaneously hypertensive rats

[1]Masahisa Asano, Kaoru Masuzawa & Tomohiro Matsuda

Department of Pharmacology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

1 Arterial relaxant responses via β-adrenoceptors have been demonstrated to be decreased in spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto rats (WKY). To determine which process of the β-adrenoceptor · adenylate cyclase (AC) system is involved in the decreased responsiveness to β-adrenoceptor stimulation, relaxant responses to forskolin and dibutyryl cyclic AMP (db cyclic AMP) were compared between strips of femoral and mesenteric arteries isolated from 13 week-old SHR and age-matched WKY.

2 The relaxant response to either forskolin, an activator of AC, or db cyclic AMP was not significantly different between the SHR and WKY, when the strips of both arteries from both strains were contracted with K^+ to an equivalent magnitude (85% of the maximum).

3 Under the same conditions, however, the relaxant response to noradrenaline (NA) via β-adrenoceptors was significantly decreased in the SHR arteries.

4 When the strips of femoral arteries were contracted with the same concentration of K^+, there was a precontraction of greater magnitude in response to the K^+ and a decreased relaxation in response to forskolin, db cyclic AMP or NA in the SHR. On the other hand, when the strips of mesenteric arteries were contracted with the same concentration of K^+, the precontraction was smaller in magnitude and there was an increased relaxation in the SHR.

5 The relationship between the relaxant responses and the K^+-induced precontractions clearly showed that the ability of forskolin and NA to relax the K^+-contracted strips depends on the magnitude of precontraction. Therefore, a difference in magnitude of precontraction between the two groups may produce a meaningless difference.

6 The relaxant responses to forskolin and NA were significantly potentiated by the addition of isobutyl methylxanthine (IBMX), an inhibitor of cyclic AMP phosphodiesterase. Even in the presence of IBMX, relaxant responses to forskolin were the same for the two strains. The difference in the pD_2 value for NA-induced relaxation between the two strains was the same in the presence and absence of IBMX.

7 The relaxant effect of either nitroprusside or nifedipine, agents which are independent of this system, was not significantly different between the strips from SHR and WKY. These relaxations were not potentiated by IBMX.

8 From these results, it is concluded that the reduced β-adrenoceptor coupling to AC is mainly involved in the decreased responsiveness to β-adrenoceptor stimulation. Furthermore, for an accurate comparison to be made, it is necessary to minimize the influence of variations in the magnitude of precontraction on the relaxant responses.

Introduction

The relaxation of vascular smooth muscle induced by β-adrenoceptor stimulation has been demonstrated to be decreased in a variety of hypertensive animals including spontaneously hypertensive rats (SHR) (Amer, 1973; Amer *et al.*, 1974; Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Asano *et al.*, 1982; Silver *et al.*, 1985). β-Adrenoceptor-mediated relaxation has been proposed to involve increased cellular

[1] Author for correspondence.

cyclic AMP through the activation of adenylate cyclase (AC), and subsequent activation of cyclic AMP-dependent protein kinase, in a variety of smooth muscles including vascular smooth muscles (for reviews, see Anderson & Wilsson, 1977; Hardman, 1981; Kukovetz *et al.*, 1981; Namm, 1982; Krall *et al.*, 1983). Since cyclic AMP serves as a second messenger and translates hormonal and neuronal effects into biochemical events in vascular smooth muscle cells, it is not surprising that abnormalities in cyclic AMP metabolism have been postulated to be involved in the pathogenesis of essential hypertension. In fact, in strips of femoral arteries from SHR, the decrease in β-adrenoceptor responsiveness was accompanied by an enhanced vasoconstriction induced by noradrenaline (NA) (Asano *et al.*, 1982). Thus, the decreased β-adrenoceptor component in the femoral artery is pathophysiologically important in regulating arterial contraction in SHR. The decreased β-adrenoceptor responsiveness may lead to the elevation of total peripheral resistance which is present in essential hypertension. On the other hand, β-adrenoceptors of the mesenteric artery and aorta seem to be physiologically inactive or less active, because contractile responses of these two arteries to NA are not potentiated by the presence of a β-adrenoceptor antagonist (Asano *et al.*, 1982). To this end, the SHR femoral artery is one of the most suitable preparations for investigating the role of the decreased β-adrenoceptor responsiveness in the pathogenesis of essential hypertension. The decreased responsiveness might be related to aberrations at different cellular processes of the β-adrenoceptor · adenylate cyclase (AC) system, including β-adrenoceptors. β-adrenoceptor coupling to AC, AC activity, phosphodiesterase activity or cyclic AMP-dependent protein kinase activity. Several investigations are presented in the literature which either implicate or eliminate the involvement of these components in the decreased β-adrenoceptor responsiveness (Amer, 1973; Ramanathan & Shibata, 1974; Amer *et al.*, 1974; Klenerova *et al.*, 1975; Donnelly, 1978). In view of the difficulties in demonstrating the effects of agents which interact with different processes of this system, by use of biochemical methods, in small resistance vessels, these investigations were limited to the aorta. No studies have been done in which the precise mechanism responsible for these changes in the SHR femoral artery has been examined.

Forskolin, a diterpene isolated from the roots of the plant *Coleus forskohlii*, is a potent, reversible activator of soluble and particulate AC in various tissues. The activation is not blocked by a variety of receptor antagonists, including β-adrenoceptor antagonists and appears to be mediated by direct activation of the catalytic subunit of AC (Seamon & Daly, 1981; Daly, 1984; Bender *et al.*, 1984).

The present study was designed to define which process of the β-adrenoceptor · AC system is involved in the decreased responsiveness to β-adrenoceptor stimulation in the SHR femoral artery. Vascular relaxant responses to a β-adrenoceptor agonist, forskolin or dibutyryl cyclic AMP (db cyclic AMP) were compared between strips of femoral arteries isolated from SHR and Wistar-Kyoto rats (WKY). Evidence is presented that reduced β-adrenoceptor coupling to AC is involved in the decreased responsiveness to β-adrenoceptor stimulation.

Methods

Preparation of arterial strips for recording of mechanical activity

Male SHR, 13 weeks of age, and age-matched male WKY were inbred in our laboratory. Systolic blood pressures, measured by tail-cuff plethysmography, were 119 ± 6 mmHg (WKY, $n = 20$) and 194 ± 12 mmHg (SHR, $n = 20$, significantly different from WKY, $P < 0.001$), respectively. Body weights at this age were not significantly different between the WKY (260 ± 6 g, $n = 20$) and SHR (268 ± 5 g, $n = 20$).

The rats were stunned and exsanguinated. The femoral artery (0.7–0.9 mm outside diameter) and distal portion of the superior mesenteric artery (0.7–0.9 mm o.d.) were quickly dissected. A 100 μm o.d. stainless steel wire was passed through the lumen of the artery segment and the wire was fixed to a paraffin base in a Petri dish containing Krebs-bicarbonate solution of the following composition (in mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. Surgical scissors and forceps were used to remove any loosely adhering adventitia and remaining fat under a stereoscopic microscope. The segment was then carefully cut into a helical strip while it was allowed to rotate on the wire. To avoid the possible influence of the endothelium-derived relaxing factor, the endothelium of the strip was removed intentionally by gently rubbing the endothelial surface with cotton pellets. Successful removal of the endothelium was confirmed later by the inability of acetylcholine (10^{-6} M) to induce relaxation (Furchgott & Zawadzki, 1980).

Strips (0.8 mm in width and 7 mm in length) of femoral and mesenteric arteries were mounted vertically between hooks in water-jacketed (37 ± 0.5°C) muscle baths containing 20 ml of the Krebs-bicarbonate solution. Muscle bath solutions were maintained at 37 ± 0.5°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The hook

anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan). The strips were stretched passively to optimal length by imposing the optimal resting tension, which resulted in the development of maximum isometric tension after stimulation with 60 mM KCl (K^+). To determine the optimal resting tension, a length-passive tension study was performed for the two kinds of strips from both strains. The resting tensions determined were as follows: SHR femoral, 0.6 g; WKY femoral, 0.6 g; SHR mesenteric, 0.5 g; WKY mesenteric, 0.5 g. These resting tensions were maintained throughout the experiments. After application of the resting tension, the strips were equilibrated for 90 min in oxygenated Krebs-bicarbonate solution, and during this period the solutions were replaced every 20 min.

After the 90 min equilibration period, a submaximally effective concentration of K^+ (30 mM) was administered two or three times at 40 min intervals until the responses were reproducible. At the final response, 60 mM K^+ was cumulatively added to obtain the maximum contraction of the strip. Isometric contractions were recorded on an ink-writing oscillograph.

Relaxation of arterial strips.

To compare agonists that relax blood vessels, strips of femoral and mesenteric arteries were contracted with K^+ to equivalent magnitude of tension (85% of the maximum contraction developed by 60 mM K^+) before challenge with the agonists. Because it is known that differences in induced tension could be a variable in the examination of relaxant drugs (Cohen & Berkowitz, 1976) and that the sensitivity of the contractile response of femoral arterial strips to exogenously added K^+ is increased in the SHR when compared with the WKY (Aoki & Asano, 1986; Asano *et al.*, 1986), it is necessary to minimize the influence of variations in precontraction upon relaxant responses. This may be accomplished by contracting femoral arterial strips to an equivalent magnitude of tension. Preliminary experiments indicated that for the WKY femoral artery 17 or 18 mM K^+ and for the SHR femoral artery 12 or 13 mM K^+ produced a contraction which was approximately 85% of the maximum contraction developed by 60 mM K^+. With regard to the strips of mesenteric arteries, preliminary experiments indicated that for the WKY 18 or 19 mM K^+ and for the SHR 20 or 21 mM K^+ were required to obtain the equivalent 85% contraction. These contractions induced by ED_{85} K^+ were well sustained for at least 3 h.

Cumulative dose-response curves for the arterial relaxant effects of agonists were determined by producing a stepwise increase in the concentration of the agonist as soon as a stable response to the preceding dose had been obtained. The relaxant response to dibutyryl (db) cyclic AMP was a more gradual process so that it took approximately 3 h to complete the full dose-response curve. At the end of experiments, 10^{-4} M papaverine was added to obtain the maximum relaxation of the strip. Relaxant responses are expressed as % of the maximum relaxation induced by papaverine (Asano & Hidaka, 1985).

All the experiments were conducted in phenoxybenzamine (Pbz)-treated strips to eliminate possible α-adrenoceptor responses. In these experiments, strips were treated with 2×10^{-6} M Pbz during the first 60 min of the 90 min equilibration period. Using these Pbz-treated strips, the relaxant response to NA via β-adrenoceptors could be consistently demonstrated (Asano *et al.*, 1982).

Statistical analysis

When assessing the ED_{50} value, responses to agonists were calculated as % of the maximum response obtained with each agonist. The ED_{50} value was obtained visually from a plot of % response vs. log concentration of the agonist and expressed as a negative log (pD_2 value).

Unless specified, results shown in the text, tables and figures are expressed as the mean value ± s.e. (n = number of preparations). Statistical analysis of the data was done by Student's t test for paired or unpaired data, or by completely randomized design, one-way analysis of variance followed by Newman-Keuls test for a significant F ratio ($P < 0.05$), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

Drugs and chemicals

The following drugs were used: (−)-noradrenaline bitartrate (NA; Sigma Chemical Co., St Louis, MO), phenoxybenzamine hydrochloride (Pbz; Nakarai Chemicals, Kyoto, Japan), timolol maleate (Banyu Pharmaceutical Co., Tokyo, Japan), forskolin (Nippon Kayaku Co., Tokyo, Japan), dibutyryl cyclic AMP sodium salt (db cyclic AMP; Sigma), sodium nitroprusside dihydrate (Wako Pure Chemical Industries, Osaka, Japan), nifedipine (Bayer Yakuhin Ltd, Osaka, Japan), papaverine hydrochloride (Wako), 3-isobutyl-1-methylxanthine (IBMX; Sigma) and acetylcholine chloride (Sigma).

Stock solutions of Pbz (10^{-3} M), forskolin (10^{-3} M) and IBMX (10^{-2} M) were prepared using 50% ethanol with further dilution in distilled water. Nifedipine was dissolved in 99.5% ethanol to make a

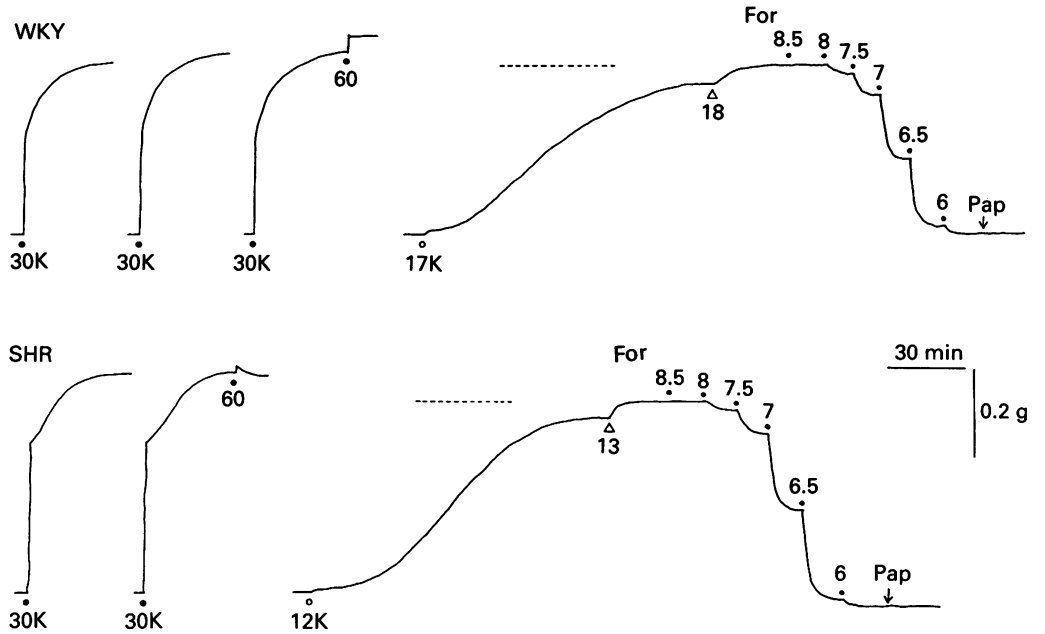

Figure 1 Typical recordings of the relaxant response to forskolin (For) in phenoxybenzamine (Pbz)-treated, ED_{85} K^+-contracted strips of femoral arteries isolated from 13 week-old WKY (top panel) and age-matched SHR (bottom panel). The strips were treated with 2×10^{-6} M Pbz for 60 min, and after its removal by repeated washing, the maximum contraction developed by 60 mM K^+ was determined. An 85% contraction was obtained by producing a stepwise increase in the concentration of K^+. Broken lines in each trace indicate the magnitude of an 85% contraction. Forskolin in concentrations ranging from 3×10^{-9} to 1×10^{-6} M (expressed as negative log of the molar concentration) was cumulatively added after the ED_{85} K^+-induced contraction had reached a plateau. At the end of each experiment, 10^{-4} M papaverine (Pap) was added to obtain the maximum relaxation of the strip.

stock solution of 10^{-3} M. NA was prepared daily in Krebs-bicarbonate solution and kept on ice during the course of the experiment. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations in the muscle bath.

Results

Relaxation of arterial strips induced by forskolin, NA and db cyclic AMP

Relaxant effects of forskolin were first compared between the Pbz-treated, K^+-contracted strips of femoral arteries isolated from WKY and those from SHR. The addition of 18 mM K^+ to a strip of the WKY femoral artery caused a sustained contraction which was approximately 85% of the maximum contraction developed by 60 mM K^+ (Figure 1). On the other hand, for an equivalent contraction to be observed in a SHR strip, 13 mM K^+ was required (Figure 1). The addition of forskolin in concentrations ranging from 3×10^{-9} to 1×10^{-6} M produced a dose-dependent relaxation in Pbz-treated, K^+-contracted strips of femoral arteries from both strains (Figure 1). Dose-response curves for forskolin were not significantly different between the WKY and SHR (Figure 2a). Under these conditions, the magnitude of the ED_{85} K^+-induced contraction was not significantly different between the two strains (Table 1). However, when the strips of femoral arteries from both strains were contracted with the same concentration of K^+, i.e., 18.1 mM K^+, the relaxant response to forskolin in the SHR was significantly weaker than that in the WKY (Figure 2b). Under these conditions, the magnitude of the K^+-induced contraction in the SHR was significantly greater than that in the WKY (Table 1), suggesting that the greater magnitude of precontraction in the SHR leads to a decreased relaxant response to forskolin.

Similar experiments were performed on the relaxant responses to the β-adrenoceptor agonist, nor-

Table 1 Arterial relaxant responses to forskolin, noradrenaline (NA) and dibutyryl (db) cyclic AMP in phenoxybenzamine-treated, K^+-contracted strips of femoral arteries isolated from 13 week-old WKY and age-matched SHR

		K^+-contraction[a]		*Agonist-relaxation*[b]		
Agonist	*Rat*	mM	% *of* K^+ max	pD_2	% max	n
Forskolin	WKY	18.1 ± 0.5	85.4 ± 1.9	7.08 ± 0.04	99.3 ± 0.3	15
	SHR	12.9 ± 0.7[d]	84.4 ± 1.7	7.06 ± 0.04	99.5 ± 0.4	15
	SHR	18.1	94.0 ± 1.0[e,f]	6.75 ± 0.03[e,g]	99.5 ± 0.3	11
NA	WKY	17.5 ± 0.6	86.2 ± 2.0	6.87 ± 0.06	99.2 ± 0.2	15
	SHR	12.7 ± 0.8[d]	85.2 ± 2.1	6.22 ± 0.06[e]	86.3 ± 2.8[e]	15
	SHR	17.5	93.9 ± 1.4[e,f]	6.03 ± 0.04[e,f]	66.5 ± 3.9[e,g]	11
db cyclic AMP	WKY	17.8 ± 0.3	85.4 ± 2.1	4.35 ± 0.06	99.6 ± 0.3	10
	SHR	12.6 ± 0.9[d]	86.5 ± 2.2	4.32 ± 0.04	99.4 ± 0.4	10
	SHR	17.8	94.6 ± 0.9[e,f]	3.86 ± 0.04[e,g]	98.9 ± 0.6	8

[a] Contractile tensions induced by each concentration of K^+ are expressed as % of the maximum tension to 60 mM K^+.

[b] Dose-response curves for the relaxant effect of forskolin. NA or db cyclic AMP were determined. pD_2 values (negative log of the molar concentration of ED_{50}) and the maximum relaxations induced by 1×10^{-6} M forskolin, 1×10^{-4} M NA and 1×10^{-3} M db cyclic AMP are listed. The maximum relaxation induced by each agonist is expressed as % of the maximum relaxation induced by 10^{-4} M papaverine. For details, see Methods. *n* indicates the number of preparations used. Data are expressed as mean ± s.e.

Significantly different from WKY: [e] $P < 0.05$; [d] $P < 0.01$; [e] $P < 0.001$.

Significantly different from the ED_{85} K^+ SHR: [f] $P < 0.05$; [g] < 0.001.

adrenaline (NA). The addition of NA in concentrations ranging from 1×10^{-9} to 1×10^{-4} M produced a dose-dependent relaxation in the Pbz-treated, K^+-contracted strips of femoral arteries from both strains. Under the experimental conditions in which the ED_{85} K^+ concentrations were used for the precontraction, the relaxant response to NA was significantly weaker in the SHR than in the WKY (Figure 2c). The maximum relaxation induced by NA in the SHR was significantly smaller than that in the WKY (Table 1). The pD_2 value for NA in the SHR was significantly smaller than that in the WKY (Table 1). When the strips from both strains were contracted with the same concentration of K^+, the difference in the relaxant response to NA between the two strains became more apparent when compared with experiments using the ED_{85} K^+-contraction (Figure 2d, Table 1); again suggesting that the variation in the magnitude of precontraction affects the relaxant response to NA.

Dose-response curves for forskolin were not significantly altered by 5×10^{-7} M timolol, whereas the relaxant effect of NA was drastically antagonized by this concentration of timolol (data not shown). These results suggest that forskolin produces the arterial relaxation by acting on a site other than the β-adrenoceptors at which NA acts.

Relaxant effects of both forskolin and NA were also compared between the Pbz-treated, K^+-contracted strips of mesenteric arteries from WKY and SHR (Figure 3). In the strips of mesenteric arteries, the ED_{85} K^+ in the SHR was significantly higher than that in the WKY (Table 2). Under the conditions in which the strips were precontracted with the ED_{85} K^+, dose-response curves for forskolin were not significantly different between the two strains (Figure 3). When the strips of SHR mesenteric artery were precontracted with 17.9 mM K^+, the same concentration as the WKY, the magnitude of precontraction was significantly smaller than the ED_{85} K^+-induced contraction in the SHR (Table 2). The pD_2 value for forskolin against the 17.9 mM K^+-induced contraction in the SHR was significantly greater than the pD_2 value in the same strain determined with the ED_{85} K^+-contraction (Table 2). However, no significant difference was observed in the pD_2 value for forskolin against the 17.9 mM K^+-contracted strips between the WKY and SHR (Table 2). The relaxant response to NA in the SHR was significantly weaker than that in the WKY, when ED_{85} K^+ concentrations were used for the precontraction (Figure 3, Table 2). When the strips were precontracted with the same concentration of K^+, i.e., 18.2 mM, the difference in the relaxant response to NA between the two strains became less apparent when compared with the difference determined on the ED_{85} K^+-contraction (Table 2).

The relaxant effect of dibutyryl cyclic AMP was not significantly different between the two strains, when ED_{85} K^+ concentrations were used for the precontraction (Figure 4). When the strips of femoral arteries were contracted with the same concentration

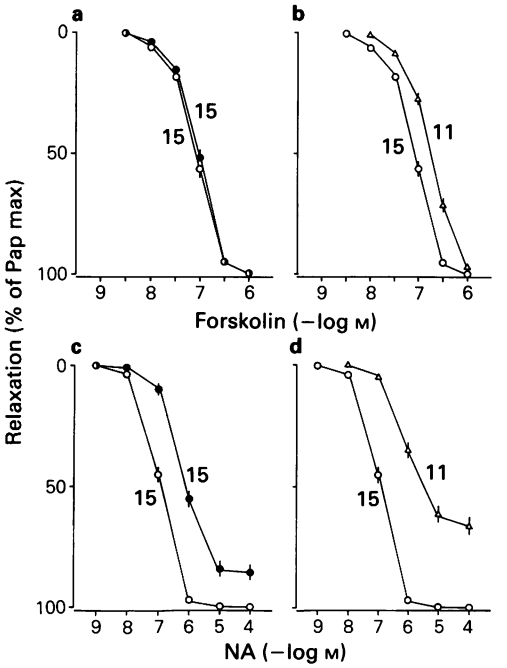

Figure 2 Dose-response curves for the relaxant effect of forskolin (a, b) and noradrenaline (NA; c, d) determined after precontraction with either ED_{85} K^+ (a, c) or the same concentration of K^+ (b, d) in phenoxybenzamine-treated strips of femoral arteries isolated from 13 week-old WKY (○) and age-matched SHR (●, △). Experimental conditions in (a) and (c) were the same as in Figure 1. ED_{85} K^+ = 18.1 mM and 12.9 mM in (a), and 17.5 mM and 12.7 mM in (c) for WKY and SHR, respectively. K^+ concentrations = 18.1 mM in (b) and 17.5 mM in (d) for WKY and SHR. Mean values of the contractile tensions developed by ED_{85} K^+ in (a) were 375 ± 15 mg (WKY, $n = 15$) and 430 ± 18 mg (SHR, $n = 15$, significantly different from WKY, $P < 0.01$), respectively. Mean values of K^+ concentrations used and resulting contractions are listed in Table 1. In (a–d), relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

of K^+, the relaxant response to db cyclic AMP in the SHR was significantly weaker than that in the WKY (Figure 4).

The addition of nitroprusside, an agent which is independent of the β-adrenoceptor · AC system, also produced a dose-dependent relaxation in Pbz-treated, ED_{85} K^+-contracted strips of femoral arteries from both strains. pD_2 values for nitroprusside were 8.90 ± 0.09 (WKY, $n = 7$) and 8.83 ± 0.06 (SHR, $n = 7$, not significantly different from WKY), respectively. Relaxant effects of

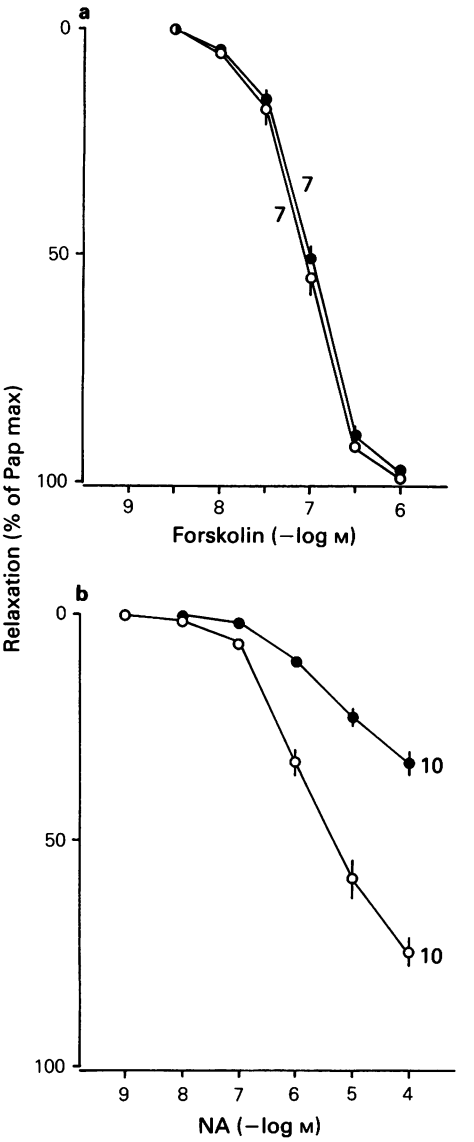

Figure 3 Dose-response curves for the relaxant effects of forskolin (a) and noradrenaline (NA, b) in phenoxybenzamine-treated, ED_{85} K^+-contracted strips of mesenteric arteries from 13 week-old WKY (○) and age-matched SHR (●). ED_{85} K^+ = 17.9 mM and 20.0 mM in (a), and 18.2 mM and 20.3 mM in (b) for WKY and SHR, respectively. Mean values of ED_{85} K^+ used and resulting contractions are listed in Table 2. Mean values of the contractile tensions developed by ED_{85} K^+ in (b) were 256 ± 14 mg (WKY, $n = 10$) and 332 ± 25 mg (SHR, $n = 10$, significantly different from WKY, $P < 0.05$), respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

Table 2 Arterial relaxant responses to forskolin, noradrenaline (NA) and dibutyryl (db) cyclic AMP in phenoxybenzamine-treated, K^+-contracted strips of mesenteric arteries isolated from 13 week-old WKY and age-matched SHR

		K^+-contraction[a]		*Agonist-relaxation*[b]		
Agonist	*Rat*	mM	% *of* K^+ max	pD_2	% max	n
Forskolin	WKY	17.9 ± 0.4	85.6 ± 1.9	7.06 ± 0.05	98.8 ± 0.5	7
	SHR	20.0 ± 0.4[d]	86.4 ± 2.1	7.01 ± 0.04	97.7 ± 0.9	7
	SHR	17.9	77.9 ± 2.2[c,f]	7.11 ± 0.04[f]	99.0 ± 0.4	5
NA	WKY	18.2 ± 0.3	85.6 ± 2.0	NC	74.8 ± 2.6	10
	SHR	20.3 ± 0.5[d]	84.9 ± 2.8	NC	32.8 ± 2.7[e]	10
	SHR	18.2	76.3 ± 2.2[c,f]	NC	48.2 ± 3.2[e,g]	6
db cyclic AMP	WKY	18.7 ± 0.4	85.8 ± 2.1	4.32 ± 0.05	99.7 ± 0.2	10
	SHR	20.8 ± 0.5[d]	84.3 ± 2.0	4.32 ± 0.05	99.6 ± 0.3	10

[a] Contractile tensions induced by each concentration of K^+ are expressed as % of the maximum tension to 60 mM K^+.

[b] Dose-response curves for the relaxant effect for forskolin, NA or db cyclic AMP were determined. pD_2 values (negative log of the molar concentration of ED_{50}) and the maximum relaxations induced by 1×10^{-6} M forskolin, 1×10^{-4} M NA and 1×10^{-3} M db cyclic AMP are listed. The maximum relaxation induced by each agonist is expressed as % of the maximum relaxation induced by 10^{-4} M papaverine. For details, see Methods. *n* indicates the number of preparations used. Data are expressed as mean ± s.e. NC: not calculated.

Significantly different from WKY: [c] $P < 0.05$; [d] $P < 0.01$; [e] $P < 0.001$.

Significantly different from the ED_{85} K^+ SHR: [f] $P < 0.05$; [g] $P < 0.01$.

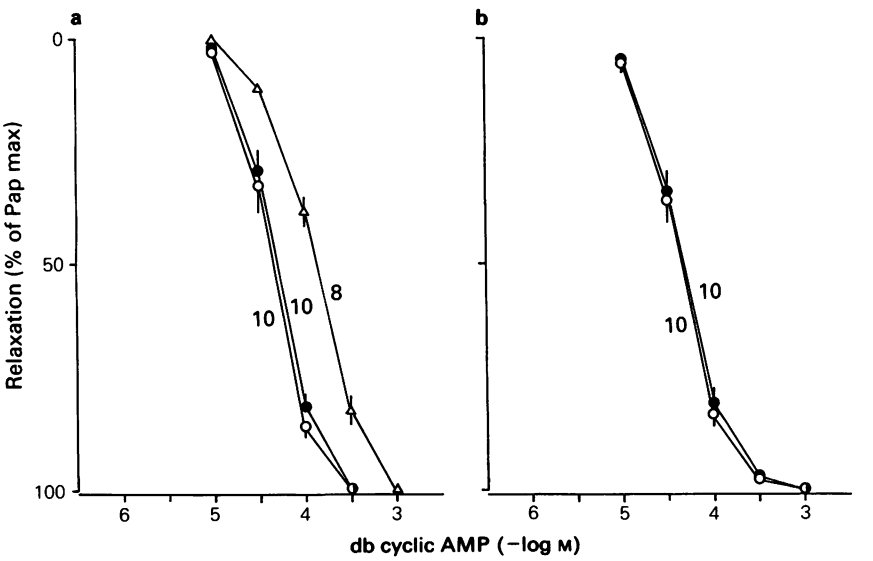

Figure 4 Dose-response curves for the relaxant effect of dibutyryl (db) cyclic AMP in phenoxybenzamine-treated, ED_{85} K^+-contracted strips of femoral (a) and mesenteric (b) arteries from 13 week-old WKY (○) and age-matched SHR (●, △). The dose-response curve of the SHR femoral artery determined after the precontraction with the same concentration of K^+ (17.8 mM) as the WKY is also included in (a) (△). K^+ concentrations = 17.8 mM and 12.6 mM (●) in (a), and 18.7 mM and 20.8 mM in (b) for WKY and SHR, respectively. Mean values of K^+ concentrations used and resulting contractions are listed in Table 1 (femoral artery) and Table 2 (mesenteric artery), respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

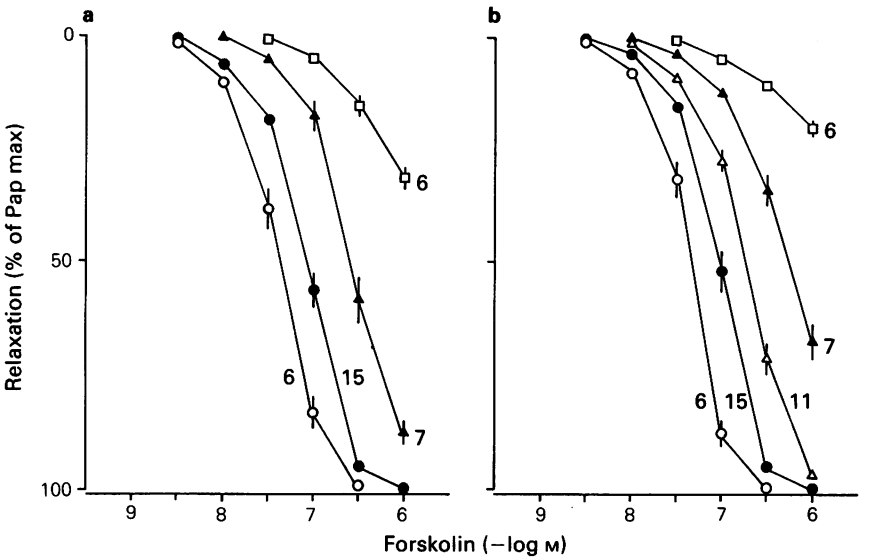

Figure 5 Dose-response curves for the relaxant effect of forskolin determined after a different magnitude of precontraction had been developed by the addition of several concentrations of K^+ in phenoxybenzamine-treated strips of femoral arteries isolated from 13 week-old WKY (a) and age-matched SHR (b). In (a) (WKY), the magnitude of precontraction developed by (○) 14.0 ± 0.4, (●) 18.1 ± 0.5, (▲) 30 and (□) 60 mM K^+ were 69.2 ± 2.3 (○), 85.4 ± 1.9 (●), 95.6 ± 0.5 (▲) and 100 (□) %, respectively. In (b) (SHR), the magnitude of precontraction developed by (○) 9.5 ± 0.5, (●) 12.9 ± 0.7, (△) 18.1, (▲) 30 and (□) 60 mM K^+ were 70.5 ± 2.0 (○), 84.4 ± 1.7 (●), 94.0 ± 1.0 (△), 97.5 ± 0.4 (▲) and 100 (□) %, respectively. In (a and b), relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

nifedipine, a Ca^{2+} channel antagonist, were also compared between the strips of femoral arteries from both strains. pD_2 values determined in the Pbz-treated, ED_{85} K^+-contracted strips of femoral arteries were 8.83 ± 0.04 (WKY, $n = 5$) and 8.72 ± 0.06 (SHR, $n = 5$, not significantly different from WKY), respectively. Thus, of the five relaxant agents used, only the β-adrenoceptor agonist exhibited a decreased responsiveness in the SHR.

Influence of variations in precontraction on relaxant responses

Figure 5 shows the dose-response curves for forskolin determined after a different magnitude of precontraction had been developed by several concentrations of K^+ in Pbz-treated strips of femoral arteries from the WKY and SHR. On increasing the concentration of K^+, that is increasing the magnitude of precontraction, the relaxant effect of forskolin was decreased in both strains. When the strips from both strains were contracted with the ED_{70} K^+, i.e., 14.0 ± 0.4 mM (WKY) and 9.5 ± 0.5 mM (SHR), respectively, dose-response curves for forskolin were not significantly different between the two strains. However, there was a significant difference in the dose-response curves for forskolin between the strips precontracted with 30 mM K^+ in the WKY and those in the SHR. Under these conditions, 30 mM K^+-induced contractions were 95.6 ± 0.5% (WKY, $n = 7$) and 97.5 ± 0.4% (SHR, $n = 7$), respectively (Figure 5). This is a small but significant ($P < 0.05$) difference in the magnitude of precontraction induced by 30 mM K^+. When the strips from both strains were maximally contracted with 60 mM K^+, the forskolin-induced relaxations were extremely weak (Figure 5). From these experiments, a relationship between the relaxant response to forskolin and the K^+-induced contraction was determined (Figure 6). When the pD_2 values for forskolin in both strains were plotted as a function of the magnitude of K^+-induced contraction (% of maximum), there was no significant difference in the pD_2 value for forskolin between the WKY and SHR (Figure 6a). Thus, the pD_2 value for forskolin in the SHR was the same as in the WKY under the conditions in which the strips from both strains were contracted to an equivalent magnitude (% of maximum). However, when the pD_2 values for forskolin were plotted as a

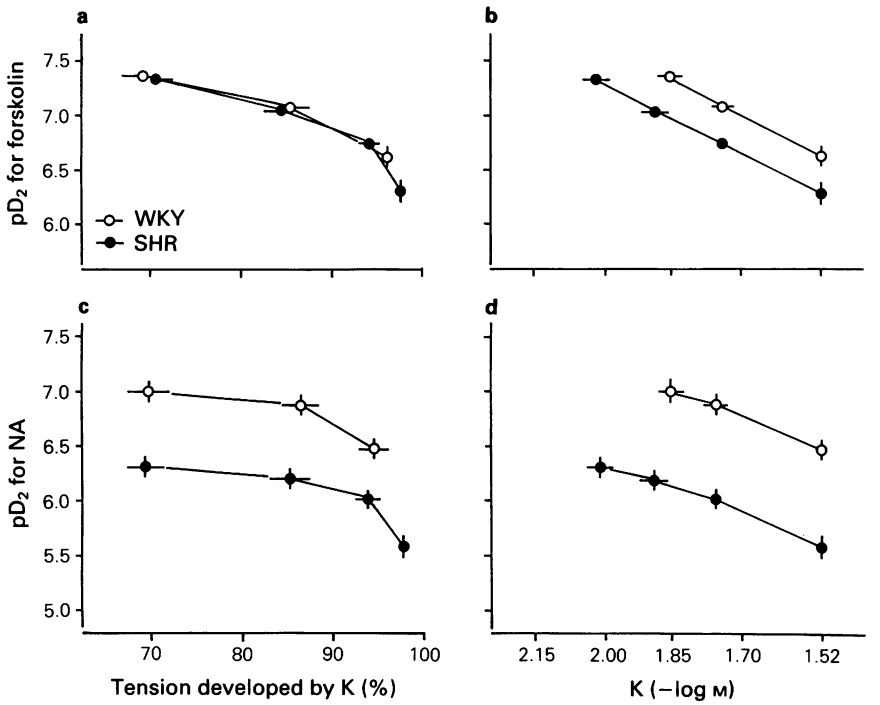

Figure 6 The effect of variations in precontraction on relaxant responses to forskolin (a, b) and noradrenaline (NA; c, d) determined in phenoxybenzamine-treated, K^+-contracted strips of femoral arteries isolated from 13 week-old WKY (○) and age-matched SHR (●). (a and c) Correlation between the pD_2 value for the relaxant effect of either forskolin (a) or NA (c) as a function of the magnitude of precontraction induced by K^+ (% of the maximum developed by 60 mM K^+). (b and d) Correlation between the pD_2 value for the relaxant effect of either forskolin (b) or NA (d) as a function of the concentration of K^+ used for the precontraction. All values are expressed as mean ± s.e.; values for (a) and (b) were obtained from Figure 5, and values for (c) and (d) were obtained from another series of experiments.

function of the concentration of K^+ used for the precontraction, there was a significant difference in the pD_2 value for forskolin between the WKY and SHR (Figure 6b). The difference in the pD_2 value between the two strains was almost constant (approximately 0.35 log scale) with increasing concentrations of K^+.

Similar experiments were conducted to determine the relationship between the relaxant response to NA and the K^+-induced contraction. When the pD_2 values for NA were plotted as a function of the magnitude of the K^+-induced contraction, there was a significant difference in the pD_2 value for NA between the two strains (Figure 6c). When the pD_2 values were plotted as a function of the concentration of K^+, the difference in the pD_2 value for NA between the two strains became more apparent (Figure 6d).

These results strongly suggest that the ability of forskolin and NA to relax the K^+-induced contraction depends on the magnitude of the precontraction. Therefore, a difference in the magnitude of the precontraction between the WKY and SHR affects the effects of relaxant agonists resulting in a meaningless difference between the two strains.

Relaxant responses to forskolin and NA in the presence of IBMX

To examine the possible influence of the activity of cyclic AMP phosphodiesterase, two kinds of additional experiment were undertaken in strips of femoral arteries. Direct relaxant effects of IBMX were first determined in the Pbz-treated, ED_{85} K^+-contracted strips of femoral arteries from both strains. The relaxant response to IBMX was not significantly different between the WKY and SHR, suggesting that the basal activity of cyclic AMP phosphodiesterase is the same between the two strains (Figure 7).

Then, the relaxant responses to forskolin and NA were determined in strips of femoral arteries in the

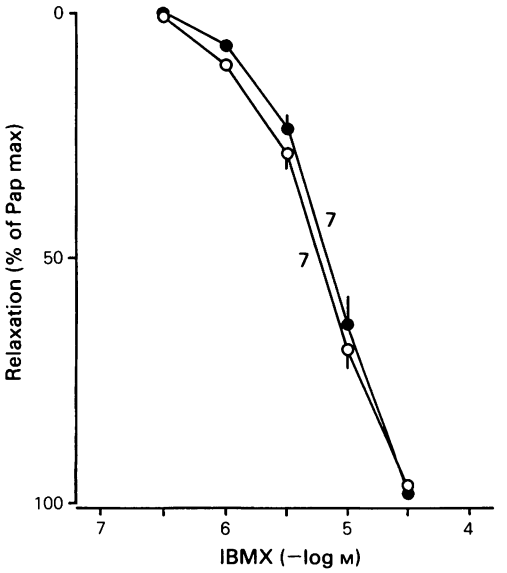

Figure 7 Dose-response curves for the relaxant effect of isobutyl methylxanthine (IBMX) in phenoxybenzamine-treated, ED_{85} K^+(18.1 mM for WKY and 12.3 mM for SHR)-contracted strips of femoral arteries from 13 week-old WKY (○) and age-matched SHR (●). Experimental conditions were the same as in Figure 2a. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

presence of 3×10^{-6} M IBMX. The magnitude of the ED_{85} K^+-induced contraction was not significantly altered by this concentration of IBMX, but the onset of contraction became slow, therefore, it took a longer time to reach a steady state. The relaxant responses to both forskolin and NA were significantly potentiated by the addition of IBMX (Figure 8, compared with Figure 2a and c). Even in the presence of IBMX, dose-response curves for forskolin were not significantly different between the two strains. On the other hand, the difference in the pD_2 value for NA between the WKY and SHR observed in the absence of IBMX (0.65 log scale; Table 1) was also observed to the same extent in the presence of IBMX (0.63 log scale; Figure 8), demonstrating that even in the presence of an inhibitor of cyclic AMP phosphodiesterase, the relaxant response to β-adrenoceptor stimulation is still impaired in strips of the SHR femoral artery. The relaxant responses of Pbz-treated, ED_{85} K^+-contracted strips of the SHR femoral artery to either nitroprusside or nifedipine were not potentiated by the addition of 3×10^{-6} M IBMX (data not shown). From these results, it may be concluded that both the forskolin- and the NA-induced stimulation of cyclic AMP phosphodiesterase activity are the same in the WKY and SHR.

Discussion

Forskolin, db cyclic AMP and NA, agents which interact with different processes of the β-adrenoceptor · AC system, were potent relaxants in arterial strips in the present study. The relaxant response of K^+-contracted strips of femoral and mesenteric arteries to forskolin was rapid in onset and dose-dependent in a concentration range comparable with that seen in other tissues (Seamon & Daly, 1981; Daly, 1984). The relaxant response to forskolin was not significantly different between the WKY and SHR. On the other hand, the relaxant response to NA via β-adrenoceptors was significantly decreased in the SHR arteries. The results of the experiments with forskolin and NA, when taken together, allow us to conclude that a reduced β-adrenoceptor coupling to AC is involved in the decreased β-adrenoceptor responsiveness present in the SHR arteries. This conclusion is also supported by the observation that the relaxant effect of db cyclic AMP was similar in the two strains. Therefore, the components of this system distal to and including AC are probably not responsible for the decreased responsiveness in the SHR.

β-Adrenoceptor-mediated relaxation has been proposed to involve an increased cellular cyclic AMP concentration through the activation of AC and subsequent activation of cyclic AMP-dependent protein kinase in a variety of smooth muscles, including vascular smooth muscles (Anderson & Wilsson, 1977; Hardman, 1981; Kukovetz *et al.*, 1981; Namm, 1982; Krall *et al.*, 1983). According to current models (Gilman, 1986), the activation by β-adrenoceptor agonists of AC-catalyzed cyclic AMP formation involves the interaction of at least three membrane-bound components: β-adrenoceptor, stimulatory GTP-binding protein (Gs) and catalytic subunit (C). The interaction of agonists with β-adrenoceptors results in the binding of GTP to Gs, which then activates C, resulting in the conversion of ATP to cyclic AMP. Cleavage by GTPase of the terminal phosphate group from the bound GTP turns off this process. This system can be modified by agents which interact directly with C, e.g., forskolin, with Gs, e.g., cholera toxin, or with β-adrenoceptors, e.g., β-adrenoceptor-agonists and antagonists.

Forskolin has been demonstrated to relax a variety of smooth muscles including vascular smooth muscles (Vegesna & Diamond, 1984; Silver *et al.*, 1985; Tsujimoto *et al.*, 1986). The mechanism by which forskolin produces an elevation of cyclic AMP is assumed to be through activation of AC by direct

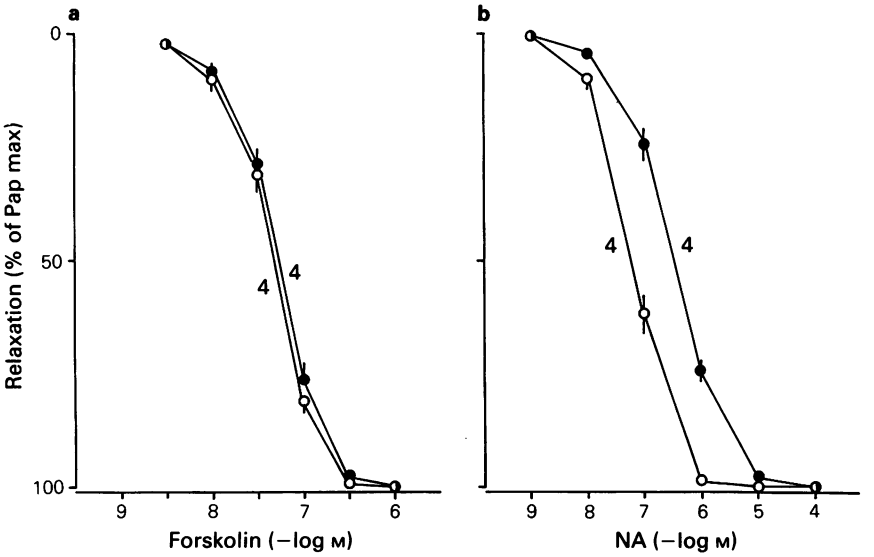

Figure 8 Dose-response curves for the relaxant effects of forskolin (a) and noradrenaline (NA; b) determined in the presence of 3×10^{-6} M isobutyl methylxanthine in phenoxybenzamine-treated, ED_{85} K^+-contracted strips of femoral arteries from 13 week-old WKY (○) and age-matched SHR (●). ED_{85} K^+ = 18.1 mM, 12.9 mM in (a) and 17.5 mM, 12.7 mM in (b) for WKY and SHR, respectively. Experimental conditions were the same as in Figure 2a and c. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

interaction with C (Seamon & Daly, 1981; Daly, 1984; Bender *et al.*, 1984). Therefore, it seems logical to assume that the forskolin-induced arterial relaxation may be a consequence of the elevation of cyclic AMP in arterial smooth muscle cells by forskolin. In the present study of femoral and mesenteric arteries, the relaxant responses to both forskolin and NA, but not to nitroprusside or nifedipine, were potentiated by IBMX, a cyclic AMP phosphodiesterase inhibitor. Although cyclic AMP levels were not actually measured, it is concluded that the arterial relaxations induced by forskolin and NA are mediated through the elevation of cyclic AMP.

The extent of the potentiation induced by IBMX of the relaxant response to either forskolin or NA was the same between the WKY and SHR, therefore suggesting that the decreased β-adrenoceptor responsiveness seen in the SHR arteries is not due to altered phosphodiesterase activity. This assumption is in good agreement with the biochemical analysis of Triner *et al.* (1975) and of Donnelly (1978), where it was demonstrated that the level of cyclic AMP phosphodiesterase was unchanged in the SHR aorta as compared with WKY.

For an accurate comparison of the relaxant effects between the two groups, it is necessary to minimize the influence of variations in the magnitude of precontraction on the relaxant responses. In the femoral and mesenteric arteries, the relaxant response to forskolin was the same between the WKY and SHR when the strips were precontracted to an equivalent magnitude. At any magnitude of precontraction ranging from approximately 70 to 96%, there was no difference in the forskolin-relaxation between the two strains (Figure 6). On the other hand, when the same concentration of K^+ was used for the precontraction in femoral arteries from both strains, there was a decreased response to forskolin in the SHR. Moreover, when the SHR mesenteric arteries were contracted with the same concentration of K^+ as the WKY, there was an increased response to forskolin compared with when the ED_{85} K^+ was used to induce a contraction in the SHR. These decreased or increased relaxations in the SHR are due to variations in the precontraction amplitude, since the same concentration of K^+ produced a greater contraction in the femoral artery and a smaller contraction in the mesenteric artery in the SHR. Inasmuch as the response of vascular smooth muscle to K^+ is known to depend on influx of extracellular Ca^{2+} (for review, see Bolton, 1979), the increase or decrease in K^+ sensitivity may reflect the increased or decreased permeability of plasma membrane to Ca^{2+} in the SHR. Femoral arteries from SHR are also more sensitive to Bay K 8644 (a dihydropyridine receptor agonist known to increase Ca^{2+} influx through the

voltage-dependent Ca^{2+} channels) than those from WKY, suggesting that the voltage-dependent Ca^{2+} channels in the SHR femoral artery are in different states of activation from those in the WKY (Aoki & Asano, 1986; Asano *et al.*, 1986). The state of activation might be related to the degree of membrane depolarization. Maximum contractile tensions developed by 60 mM K^+ (expressed as mg) were significantly greater in the SHR femoral and mesenteric arteries than the respective WKY arteries (see Figures 2 and 3). Therefore, it is important to contract the strips to equivalent magnitude, i.e., equivalent % of the maximum contraction. When the strips were contracted with the same concentration of K^+, there was a difference in the magnitude of precontraction followed by a meaningless difference between the two strains. Furthermore, appropriate comparisons cannot be made under conditions in which the strips from both strains were contracted with K^+ to the same mg of tension. Since the tension development (expressed as mg) depends on the size of the strip used, the term 'same mg of tension' does not mean the equivalent % of the maximum contraction. These methods were also applicable to the determination of the relaxant response to NA as demonstrated in the present study (Figure 6). Since the increased responsiveness to exogenously added K^+ was observed in the SHR femoral artery as compared with the WKY (Aoki & Asano, 1986; Asano *et al.*, 1986), lower concentrations of K^+ were sufficient to produce the equivalent % magnitude of contraction in the SHR compared to the WKY.

Silver *et al.* (1985) have demonstrated that the relaxant responses to both forskolin and isoprenaline are decreased in aortic strips isolated from 20 to 24 week-old SHR, when the strips were contracted to the same mg of tension (approximately 2.0 g). For the same mg of tension to be observed in aortic strips, 100 mM K^+ plus 1 mM Ca^{2+} and 100 mM K^+ plus 3 mM Ca^{2+} were used for the WKY and SHR, respectively. They have further shown that the rate and extent of activation by forskolin of cyclic AMP-dependent protein kinase were similar between the WKY and SHR, even though the rate and extent of relaxation induced by forskolin was markedly less in the SHR. Therefore, they proposed that the decreased relaxant response of SHR arteries to cyclic AMP-increasing vasodilators is probably not related to events proximal to and including activation of cyclic AMP-dependent protein kinase. With regard to the forskolin-induced relaxation, our present findings are not consistent with the findings by Silver *et al.* (1985). Possible reasons for this discrepancy include the different methods used for determining the forskolin-relaxation in the K^+-contracted strips (equivalent % magnitude vs same mg of tension), different vascular tissues (femoral and mesenteric arteries vs thoracic aorta) and different ages of the rats (13 weeks vs 20–24 weeks).

The present study failed to demonstrate a regional difference in the relaxant response to forskolin between the femoral and mesenteric arteries from both strains. Under an equivalent (85% of the maximum) contraction, the relaxant response to forskolin was the same in the femoral and mesenteric arteries (Tables 1 and 2). However, NA showed a typical regional difference in the relaxant effect. pD_2 values for the relaxant effect of NA in the WKY arteries were 6.87 (femoral) and >5.8 (mesenteric), and those in the SHR arteries were 6.22 (femoral) and >5.4 (mesenteric), respectively. These results suggest that the stimulation by forskolin of AC activity is the same in the femoral and mesenteric arteries, whereas the NA-induced stimulation of AC activity is less in the mesenteric artery. The result that higher concentrations of NA were required to produce the same magnitude of relaxation in the mesenteric artery as in the femoral artery supports the concept that β-adrenoceptor-mediated vasodilatation predominates in blood vessels of skeletal muscle, as compared with other vessels of the abdominal viscera. Indeed, β-adrenoceptor stimulation modulates NA-induced contraction in the femoral artery but not in the mesenteric artery or the aorta (Asano *et al.*, 1982). Therefore, the β-adrenoceptors of the femoral artery are physiologically important, as compared to those of the mesenteric artery and the aorta. This is the reason why we used the femoral artery.

Identification of the possible locus (loci) of the decreased responsiveness to β-adrenoceptor stimulation in the SHR might be determined by estimating changes in cellular cyclic AMP levels. The studies by Amer (1973), Triner *et al.* (1975) and Klenerova *et al.* (1975) demonstrated no change in basal activity of AC in the SHR aorta. These studies also demonstrated that the extent of stimulation by β-adrenoceptor agonists of AC activity was significantly less in the SHR than in the WKY. However, the stimulation of AC activity by NaF, an activator of Gs (also other G proteins), is controversial: NaF has been demonstrated to decrease (Amer, 1973) or increase AC activity (Triner *et al.*, 1975) in the SHR. The studies by Triner *et al.* (1975) demonstrated a decreased β-adrenoceptor-mediated vascular relaxation that coincided with a reduced production of cyclic AMP by the SHR in response to isoprenaline. From these biochemical analyses in aorta, it is clear that no consistent observations of altered cyclic AMP metabolism characterize the SHR arteries. Although the steps which link β-adrenoceptor stimulation to physiological responses are many, the major goal of these studies is to define the possible locus of the decreased responsiveness to

β-adrenoceptor stimulation in the SHR arteries. From the pharmacological approach to this problem demonstrated in the present study, we propose that a reduced coupling of the β-adrenoceptors to AC is mainly involved in the decreased β-adrenoceptor responsiveness which is present in the SHR arteries.

However, the present study does not provide any specific details concerning the molecular mechanisms involved in the abnormality of the β-adrenoceptor · AC system of the SHR femoral artery. β-Adrenoceptor-mediated vascular relaxations have been demonstrated to decrease with increasing age (Fleisch *et al.*, 1970; Cohen & Berkowitz, 1974; Fleisch, 1980; Duckles & Hurlbert, 1986). Tsujimoto *et al.* (1986) have examined the mechanism responsible for this phenomenon using rat mesenteric arteries from young (5–6 weeks) and older (10–12 months) rats. The decreased relaxation is specific for the β-adrenoceptor agonist isoprenaline, since the relaxations induced by acetylcholine and nitroglycerin were similar in the two groups. The decreased β-adrenoceptor responsiveness was not explained by a change in β-adrenoceptor number in these arteries. The maximum stimulation of cyclic AMP accumulation by the β-adrenoceptor agonist was lower in the older arteries, whereas forskolin activated cyclic AMP accumulation equally in the two groups (Tsujimoto *et al.*, 1986). It is of interest to note that the effects of the relaxant agents on the young and older rats (Tsujimoto *et al.*, 1986) are similar to the effects of these agents on the WKY and SHR arteries (the present study). The present pharmacological approach to the β-adrenoceptor · AC system of the SHR arteries will have to be strengthened by biochemical analyses, e.g., β-adrenoceptor number, the function of Gs, AC activity and cyclic AMP accumulation, in the same tissues, and this is the subject of our current investigations.

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