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Role of stimulatory GTP-binding protein (Gs) in reduced β-adrenoceptor coupling in the femoral artery of spontaneously hypertensive rats

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1 Arterial relaxant responses to β-adrenoceptor agonists are decreased in spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto rats (WKY). To establish which component of the β-adrenoceptor · adenylate cyclase (AC) system is impaired in the SHR arteries, effects of two activators of AC – cholera toxin (CTX) and forskolin – and of dibutyryl cyclic AMP (db cyclic AMP) were compared between strips of femoral arteries isolated from 13 week-old SHR and age-matched WKY.

2 In the absence of timolol, a β-adrenoceptor antagonist, contractile responses of the strips to noradrenaline (NA) were significantly greater in the SHR than in the WKY. Timolol augmented the contractile responses to NA to a smaller extent in the SHR than in the WKY.

3 After blockade by timolol of β-adrenoceptors, contractile responses of the strips to NA through the activation of α-adrenoceptors were not significantly different between the two strains.

4 Pre-treatment of the strips with CTX, an activator of the stimulatory GTP-binding protein (Gs), produced a slow-onset and long-lived antagonism of the α-adrenoceptor-mediated contractions. The antagonism was much smaller in the SHR than in the WKY.

5 The dose-response curves of the strips from both strains for α-adrenoceptor stimulation with NA determined after pretreatment with CTX were comparable to those determined in the absence of timolol.

6 Forskolin, an activator of the catalytic subunit of AC, and DB cyclic AMP also antagonized the α-adrenoceptor-mediated contractions. However, these antagonisms were not significantly different between the two strains.

7 Isobutyl methylxanthine (IBMX), an inhibitor of cyclic AMP phosphodiesterase, produced a similar antagonism of the α-adrenoceptor-mediated contractions between the two strains.

8 These results suggest that a reduced function of Gs is the main factor responsible for the decreased responsiveness to β-adrenoceptor stimulation in the SHR femoral artery.

Introduction

β-Adrenoceptor-mediated relaxation has been proposed to involve increased cellular adenosine 3′:5′-cyclic monophosphate (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 review, see Anderson & Wilsson, 1977; Hardman, 1981; Kukovetz *et al.*, 1981; Namm, 1982; Krall *et al.*, 1983). A consistent loss in responsiveness of vascular smooth muscles to β-adrenoceptor stimulation has been demonstrated 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). In strips of the SHR femoral artery, the decrease in the β-adrenoceptor responsiveness was reflected in an enhanced vasoconstriction induced by noradrenaline (NA) (Asano *et al.*, 1982). This decreased β-adrenoceptor responsiveness may contribute to the elevation of total peripheral resistance which is present in essential hypertension. However, the precise mechanism responsible for these changes in the SHR femoral artery has not been established. Our previous studies have demonstrated that although the relaxant response to β-adrenoceptor

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stimulation was decreased in the SHR arteries, forskolin, an activator of AC, and dibutyryl cyclic AMP (db cyclic AMP) produced similar relaxations in the arteries from normotensive Wistar-Kyoto rats (WKY) and SHR, suggesting that reduced β-adrenoceptor coupling to AC is mainly involved in the decreased responsiveness to β-adrenoceptor stimulation (Asano *et al.*, 1988).

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 conversion of ATP to cyclic AMP. Cleavage by GTPase of the terminal phosphate group from the bound GTP 'turns off' this process, deactivating C. The elevation of cellular cyclic AMP can be modified by agents which interact directly with Gs, e.g., cholera toxin (CTX), an enterotoxin from *Vibrio cholerae* (Northup *et al.*, 1980), with C, e.g., forskolin, a diterpene isolated from the roots of the plant *Coleus forskohlii* (Seamon & Daly, 1981), or with β-adrenoceptors, e.g., agonists and antagonists.

The main aim of the present study was to establish which component of the β-adrenoceptor · AC system is impaired in the SHR femoral artery. In view of the difficulties in demonstrating the effects of agents which interact with different processes of this system using biochemical methods in this small arterial segment, we have used a pharmacological approach. To this end, we have examined the effects of two activators of AC, CTX and forskolin, on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from SHR and WKY. Our evidence suggests that a reduced function of Gs is the main factor in the decreased responsiveness of the SHR femoral artery to β-adrenoceptor agonists. A preliminary account of these findings was presented to the 60th general meeting of the Japanese Pharmacological Society (Masuzawa *et al.*, 1987).

Methods

Male SHR, 13 weeks of age, and age-matched male WKY were inbred in our laboratory. Systolic blood pressures, measured by the tail-cuff plethysmography, were 119 ± 4 mmHg (WKY, $n = 29$) and 189 ± 9 mmHg (SHR, $n = 29$, significantly different from WKY, $P < 0.001$), respectively. Body weights at this age were not significantly different between the WKY (270 ± 4 g, $n = 29$) and SHR (271 ± 4 g, $n = 29$).

Preparation of femoral arterial strips for tension recordings

The rats were stunned and exsanguinated. The femoral artery (0.7–0.9 mm outside diameter) was quickly dissected and placed in a Petri dish containing oxygenated 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 artery segment was carefully cut into a helical strip. To avoid the possible influence of endothelium-derived relaxing factor, the endothelium of the strip was removed intentionally by gently rubbing the endothelial surface with a cotton pellet. Successful removal of the endothelium was confirmed later by the inability of acetylcholine (10^{-6} M) to induce relaxation (Furchgott & Zawadzki, 1980).

Arterial strips (0.8 mm in width and 7 mm in length) were mounted vertically between hooks in all-glass, 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 Nihon Kohden force-displacement transducer, model TB-612T. The strips were stretched passively to optimal length by imposing a resting tension of 0.6 g, which resulted in the development of maximum isometric tension after stimulation with 60 mM KCl (K^+). Length-passive tension studies failed to demonstrate any difference in the resting tension between the strips from WKY and SHR. This resting tension was 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 added 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.

Contractile response to NA via α-adrenoceptors in the absence and presence of antagonists

Dose-response curves for the contractile effect of NA via α-adrenoceptors were determined by a cumulative addition of the agonist in the presence of 5×10^{-7} M timolol, a β-adrenoceptor antagonist. Timolol was present from 40 min before NA was

tested. In some experiments, dose-response curves for NA were determined in the absence of timolol to examine the interaction between α- and β-adrenoceptors in the femoral artery.

The effects of cholera toxin (CTX) on the contractile response to α-adrenoceptor stimulation with NA were determined in paired arterial strips. Since the reconstituted stock solution of CTX used contains NaN_3, it was necessary to eliminate the influence of NaN_3 on α-adrenoceptor-mediated contractions. To this end, one strip was treated with CTX and after its removal by repeated washing, the dose-response curve for NA was determined in the presence of timolol. Another strip was treated with the equivalent concentration of NaN_3 (in the absence of CTX) in a similar fashion and served as a control. Under these experimental conditions, the influence of NaN_3 on α-adrenoceptor-mediated contractions can be minimized.

The effects of forskolin, db cyclic AMP and isobutyl methylxanthine (IBMX) on the contractile response to α-adrenoceptor stimulation with NA were determined in the following way. Three sequential dose-response curves for NA were determined simultaneously on paired arterial strips in the presence of timolol with an interval of 90 min between each determination. Usually the paired strips were subjected to different treatments. Forskolin, db cyclic AMP or IBMX was applied to one strip: the first curve was taken as a control and effects of forskolin, db cyclic AMP or IBMX were determined on the second and third curves. These agents were present from 40 min before NA was tested. Another strip was the control serving as an indicator of changes in muscle sensitivity during the course of the experiment. When such changes occurred, maximum contractions and pD_2 values were corrected (Asano & Hidaka, 1985).

Statistical analysis

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

Unless specified, results shown in the text and figures are expressed as the mean value $\pm$ s.e. (n = number of preparations). Statistical analysis of the data was conducted 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), timolol maleate (Banyu Pharmaceutical Co., Tokyo, Japan), cholera toxin (CTX; List Biological Laboratories, Campbell, CA), forskolin (Nippon Kayaku Co., Tokyo, Japan), dibutyryl cyclic AMP sodium salt (db cyclic AMP; Sigma), 3-isobutyl-1-methylxanthine (IBMX, Sigma), acetylcholine chloride (Sigma), tris(hydroxymethyl)aminomethane (Tris; Sigma), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA; Sigma) and NaN_3 (Wako Pure Chemical Industries, Osaka, Japan).

CTX (a lyophilized powder), when reconstituted to 1 ml with distilled water, contained 1.0 mg of protein in Tris buffer (50 mM Tris, pH 7.5; 1 mM Na_2EDTA; 3 mM NaN_3; 200 mM NaCl). CTX at this concentration was stored at 4°C and was used for over 3 months with no loss of the antagonistic activity. Stock solutions of forskolin (10^{-3} M) and IBMX (10^{-2} M) were prepared using 50% ethanol with further dilution in distilled water. 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

Arterial contractile responses to NA in the absence and presence of timolol

Following the determination of the maximum contraction developed by 60 mM K^+, dose-response curves for the contractile effect of NA were determined in the absence and presence of timolol, a β-adrenoceptor antagonist (Figure 1). The addition of NA in concentrations ranging from 10^{-9} to 10^{-4} M caused a dose-dependent contraction in strips of femoral arteries from the WKY and SHR in the absence of timolol. However, the responses of the WKY strip to median concentrations of NA were not monophasic. The WKY strip produced a transient contraction followed by a sustained relaxation in response to 10^{-7} M NA. The responses to 10^{-6} and 10^{-5} M NA consisted of three phases; contraction, relaxation and contraction. These relaxations were abolished by incubation of the strip with 5×10^{-7} M timolol, and concomitantly the contractile responses to NA were augmented (Figure 1). On the other hand, in the absence of timolol, the SHR strip showed only a contraction in response to the above concentrations of NA. The contractile responses of the SHR strip to NA were also augmented by timolol (Figure 1).

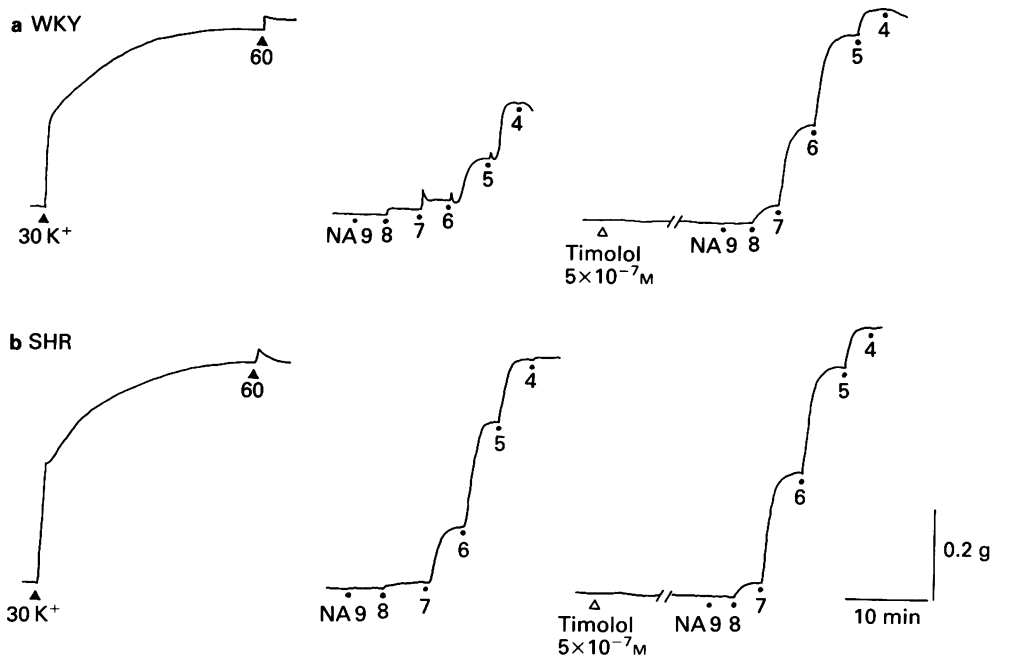

Figure 1 Typical recordings of the contractile response to noradrenaline (NA) in strips of femoral arteries isolated from WKY (a) and SHR (b). After the determination of the maximum contraction induced by 60 mM KCl (K^+), the dose-response curve for NA was determined in the absence of timolol. NA in concentrations ranging from 10^{-9} to 10^{-4} M (expressed as negative log of the molar concentration) was cumulatively added to the bathing solution. The dose-response curve for NA was then repeated in the presence of 5×10^{-7} M timolol. Note the different pattern of contraction at 10^{-7}, 10^{-6} and 10^{-5} M NA in the absence of timolol between the two strains.

Dose-response curves of the strips for the contractile effect of NA are shown in Figure 2. The extent of augmentation induced by timolol of the maximum response to NA was significantly smaller in the SHR (Figure 2b) than in the WKY (Figure 2a). The maximum contraction developed by 10^{-4} M NA in the presence of timolol was not significantly different between the two strains (Figure 2a and b). When the contractile responses to 10^{-4} M NA in the absence of timolol were expressed as a % of the maximum contraction developed by NA in the presence of timolol, there was a greater contraction in the SHR than in the WKY (Figure 2c). The pD_2 value for NA determined in the absence of timolol in the SHR (6.47 ± 0.10, $n = 10$) was not significantly different from the value in the WKY (6.37 ± 0.12, $n = 10$). When the contractile responses to NA determined in the presence of timolol were compared between the WKY and SHR, the dose-response curves were the same for the two strains (Figure 2d). pD_2 values for NA were 6.88 ± 0.10 (WKY, $n = 10$), and 6.86 ± 0.08 (SHR, $n = 10$), respectively. Therefore, the extent of rightward displacement induced by timolol of the dose-response curve for NA was not significantly different between the two strains.

Effects of cholera toxin on α-adrenoceptor-mediated contractions

Effects of pretreatment with CTX on the dose-response curve for NA in the presence of timolol were compared between the strips from WKY and SHR. CTX produced a slow-onset and long-lived effect on the α-adrenoceptor-mediated contractions. The temporal change in the effect of 1 $\mu g\,ml^{-1}$ CTX on the SHR strip is shown in Figure 3. When the dose-response curve for NA was determined in WKY strips 6 h after the 4 h pretreatment with 1 $\mu g\,ml^{-1}$ CTX, the dose-response curve was significantly shifted to the right with a reduction of the maximum response (Figure 4a). However, under the same conditions, the CTX pretreatment produced no significant antagonism on SHR strips (Figure 4b). A shorter pretreatment with CTX showed only a weak effect on α-adrenoceptor-mediated contractions. When the same concentration of CTX was added for a 40 min incubation and removed by washing, the maximum response to NA in WKY strips determined at 6 h was $86.0 \pm 3.5\%$ ($n = 3$) of the control. To demonstrate the dose-response relationship for the effects of CTX, a lower concentration of CTX

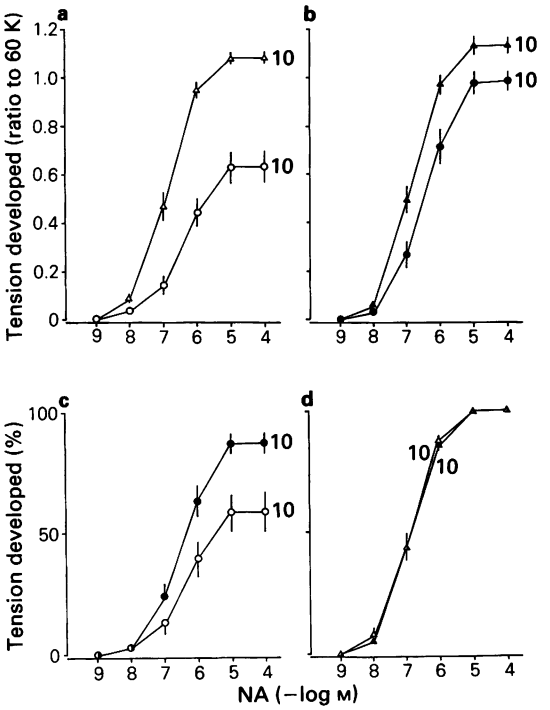

Figure 2 Dose-response curves for noradrenaline (NA) in the absence (○, ●) and presence (△, ▲) of 5×10^{-7} M timolol in strips of femoral arteries isolated from WKY (a) and SHR (b). Experimental conditions were the same as in Figure 1. In (a and b) contractile responses to NA are expressed as a ratio to the maximum contraction induced by 60 mM K^+. Mean values of the maximum contractile tensions induced by 10^{-4} M NA in the presence of timolol in strips of the WKY (a) and SHR (b) were 474 ± 21 mg (1.085 ± 0.023; ratio to the maximum contraction induced by 60 mM K^+, $n = 10$) and 585 ± 32 mg (1.139 ± 0.030, $n = 10$), respectively. Dose-response curves for NA in the absence (c) and presence (d) of timolol were compared between the strips from WKY (○, △) and SHR (●, ▲) on the basis of % response. The maximum contraction induced by 10^{-4} M NA in the presence of timolol was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

($0.3\,\mu g\,ml^{-1}$) was also used for pretreatment in a similar fashion. The pretreatment of WKY strips with $0.3\,\mu g\,ml^{-1}$ CTX caused a significant reduction of the maximum response to NA with a slight rightward displacement of the dose-response curve (Figure 4c). The extent of antagonism induced by CTX was significantly greater in the $1\,\mu g\,ml^{-1}$ CTX-pretreated strips (Figure 4a) than in the $0.3\,\mu g\,ml^{-1}$ CTX-pretreated strips (Figure 4c). Pretreatment with $0.3\,\mu g\,ml^{-1}$ CTX produced no significant antagonism on SHR strips (Figure 4d).

Ten hours after the 4 h CTX pretreatment, dose-response curves of the strips from both strains for NA were antagonized to a greater extent when compared with the antagonism seen at 6 h (Figure 5). The extent of the reduction induced by $1\,\mu g\,ml^{-1}$ CTX of the maximum response to NA was significantly smaller in the SHR (Figure 5b) than in the WKY (Figure 5a). However, the extent of the rightward displacement of the dose-response curve was not significantly different between the two strains (legend for Figure 5). A longer pretreatment with CTX produced no further antagonism of the α-adrenoceptor-mediated contractions. When the same concentration of CTX was added for an 8 h incubation and removed by washing, the maximum response to NA determined at 10 h was $60.7 \pm 5.5\%$ (WKY, $n = 5$) and $92.6 \pm 3.2\%$ (SHR, $n = 5$) of the respective controls. At this time, the extent of antagonism induced by the 4 h pretreatment with $0.3\,\mu g\,ml^{-1}$ CTX was significantly smaller than that induced by $1\,\mu g\,ml^{-1}$ CTX (Figure 5). The antagonism induced by $0.3\,\mu g\,ml^{-1}$ CTX was significantly weaker in the SHR (Figure 5d) than in the WKY (Figure 5c). At 20 h after the 4 h pretreatment with $1\,\mu g\,ml^{-1}$ CTX, NA evoked barely any contraction in the WKY strips, whereas in the SHR strips, the response was half maximal (Figure 6). At this time, the control dose-response curves themselves were significantly reduced.

Thus, CTX produced a slow-onset and long-lived inhibition on α-adrenoceptor-mediated contractions in both strains. Temporal changes in the inhibitory effects of CTX on the maximum response to NA are shown in Figure 7. The difference in the inhibitory effects of CTX between the two strains became more apparent with increasing time.

Since the ability of CTX to antagonize the α-adrenoceptor-mediated contractions ($1\,\mu g\,ml^{-1}$ CTX pretreatment, determined at 10 h; Figure 5a and b) was similar to the decreased β-adrenoceptor responsiveness determined by the interaction between α- and β-adrenoceptors (Figure 2a and b), comparisons were made between these responses and the results are shown in Figure 8. The dose-response curve for NA determined in the absence of timolol was in good agreement with the dose-response curve for α-adrenoceptor stimulation with NA determined after pretreatment with CTX in either the WKY or the SHR. As a matter of course, the dose-response curve for NA in the presence of timolol was well fitted to the control curve before the CTX pretreatment in either the WKY or the SHR (Figures 2d, 5a and b). These results strongly suggest that the decreased responsiveness seen at the level of β-adrenoceptors also exists at the level of Gs.

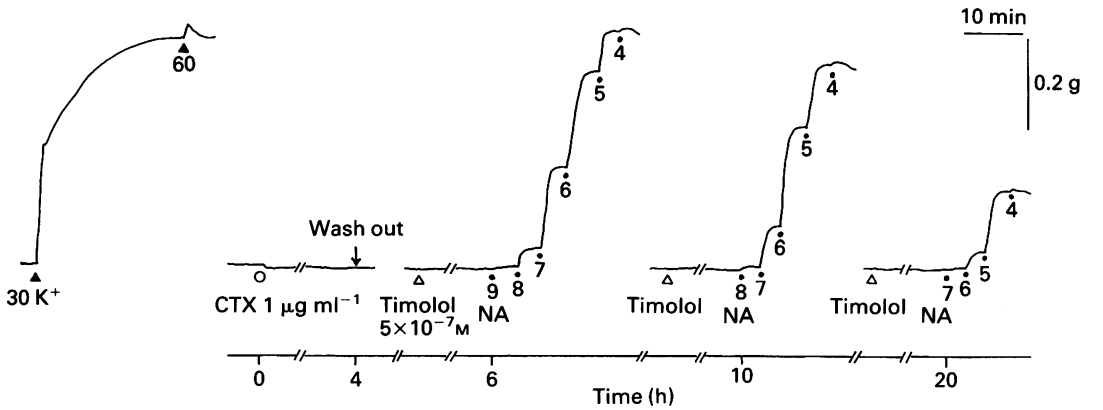

Figure 3 Typical recordings of the temporal change in the effect of cholera toxin (CTX) on α-adrenoceptor-mediated contractions in a strip of femoral artery isolated from SHR. After the determination of the maximum contraction induced by 60 mM K^+, CTX 1 μg ml^{-1} was added for a 4 h incubation. After the removal of CTX by repeated washing for 2 h (6 h after the beginning of CTX pretreatment), the dose-response curve for noradrenaline (NA) was determined in the presence of timolol. Then, after repeated washing, the dose-response curve for NA was repeated at 10 and 20 h after the beginning of CTX pretreatment.

Effects of forskolin and db cyclic AMP on α-adrenoceptor-mediated contractions

The effects of forskolin on the contractile responses to α-adrenoceptor stimulation with NA were compared between the strips from WKY and SHR (Figure 9). Forskolin in concentrations of 1×10^{-7} and 3×10^{-7} M produced a dose-dependent reduction of the maximum response to NA with a rightward displacement of the dose-response curve. The extent of the reduction induced by either 1×10^{-7} or 3×10^{-7} M forskolin of the maximum response to NA was not significantly different between the two strains (Figure 9c and d, respectively). Furthermore, the extent of rightward displacement of the dose-response curve for NA was the same for the two strains (legend for Figure 9).

Further comparisons of the effects of db cyclic AMP on the contractile responses to α-adrenoceptor stimulation with NA were made between the strips from WKY and SHR (Figure 10). The extent of either the reduction of the maximum response to NA or the rightward displacement of the dose-response curve was not significantly different between the two strains (Figure 10).

Effects of IBMX on α-adrenoceptor-mediated contractions

To examine the possibility that altered cyclic AMP phosphodiesterase activities are involved in the decreased β-adrenoceptor responsiveness in the SHR, additional experiments on the effects of IBMX, an inhibitor of cyclic AMP phosphodiesterase, were undertaken in the strips from WKY and SHR (Figure 11). The addition of IBMX in concentrations of 1×10^{-5} and 3×10^{-5} M produced a dose-dependent reduction of the maximum response to NA with a rightward displacement of the dose-response curve. The extent of antagonism induced by IBMX of α-adrenoceptor-mediated contractions was not significantly different between the two strains (Figure 11).

Discussion

The present study evaluated the inhibitory effects of CTX, forskolin and db cyclic AMP on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from SHR and WKY. The major conclusion is that a reduced function of Gs is the main factor in the decreased responsiveness of the SHR femoral artery to β-adrenoceptor stimulation. This is suggested by the following observations: (1) in the absence of β-adrenoceptor antagonists, contractile responses to NA were significantly greater in the SHR than in the WKY, (2) contractile responses to NA through the activation of α-adrenoceptors were the same for the two strains, (3) inhibitory effects of CTX on the α-adrenoceptor-mediated contractions were significantly weaker in the SHR than in the WKY, (4) contractile responses to α-adrenoceptor stimulation with NA determined after CTX-pretreatment were comparable to those determined in the absence of β-adrenoceptor antagonists in either the WKY or SHR, and (5) inhibitory effects of forskolin and db cyclic AMP on the α-

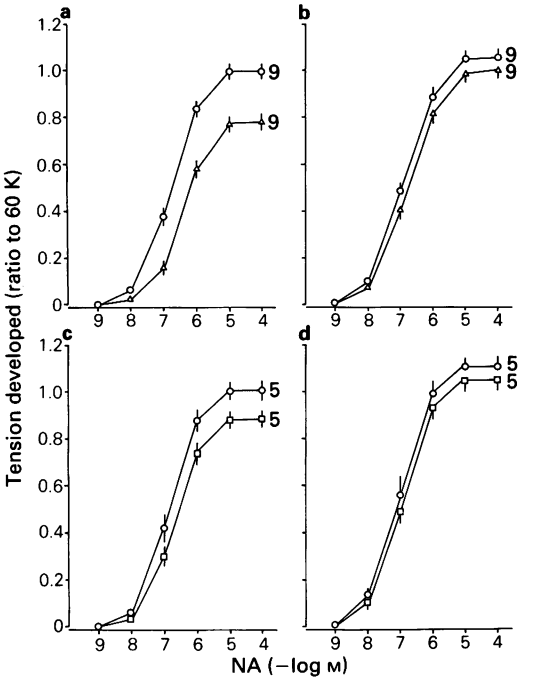

Figure 4 Effects of pretreatment with $1\,\mu g\,ml^{-1}$ (a, b) and $0.3\,\mu g\,ml^{-1}$ (c, d) cholera toxin (CTX) on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a, c) and SHR (b, d). Dose-response curves for noradrenaline (NA) determined in the presence of timolol 6 h after the beginning of CTX pretreatment are shown. In (a and b), (○) control responses and (△) responses after CTX ($1\,\mu g\,ml^{-1}$) pretreatment. In (c and d), (○) control responses and (□) responses after CTX ($0.3\,\mu g\,ml^{-1}$) pretreatment. Contractile responses to NA are expressed as a ratio to the maximum contraction induced by 60 mM K^+. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

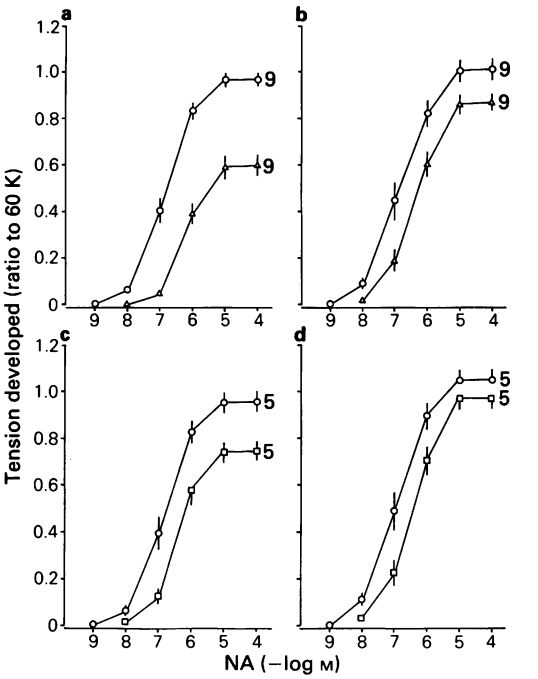

Figure 5 Effects of pretreatment with $1\,\mu g\,ml^{-1}$ (a, b) and $0.3\,\mu g\,ml^{-1}$ (c, d) cholera toxin (CTX) on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a, c) and SHR (b, d). Dose-response curves for noradrenaline (NA) determined in the presence of timolol 10 h after the beginning of CTX pretreatment are shown. In (a and b), (○) control responses and (△) responses after CTX ($1\,\mu g\,ml^{-1}$) pretreatment. In (c and d), (○) control responses and (□) responses after CTX ($0.3\,\mu g\,ml^{-1}$) pretreatment. Contractile responses to NA are expressed as a ratio to the maximum contraction induced by 60 mM K^+. pD_2 values for NA in the control and CTX-pretreated strips in each panel were 6.83 ± 0.10 and 6.26 ± 0.09 (for a), 6.82 ± 0.17 and 6.37 ± 0.13 (for b), 6.78 ± 0.16 and 6.44 ± 0.05 (for c) and 6.90 ± 0.17 and 6.42 ± 0.09 (for d), respectively. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

adrenoceptor-mediated contractions were not significantly different between the WKY and SHR.

A consistent loss in responsiveness of vascular smooth muscles to β-adrenoceptor stimulation has been demonstrated in a variety of hypertensive animals including SHR (Amer, 1973; Amer *et al.*, 1974; Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Asano *et al.*, 1982; Silver *et al.*, 1985). The decreased responsiveness to β-adrenoceptor stimulation was also observed in the dose-response curves of femoral arteries for the contractile effect of NA determined in the absence of β-adrenoceptor antagonists (Figures 1 and 2). When the dose-response curves for NA were determined in the absence of timolol, we observed the responses which consisted of an α-adrenoceptor-mediated contraction and a β-adrenoceptor-mediated relaxation. However, the interaction between the α- and β-adrenoceptors was quite different between the femoral arteries from SHR and WKY. The finding that the contractile responses to NA determined in the absence of timolol were greater in the SHR than in the WKY suggests that the decreased β-adrenoceptor responsiveness in the SHR femoral artery is reflected in an enhanced arterial contraction through the activation of α-adrenoceptors. In the SHR mesenteric artery, in spite of the decreased relaxant responses to β-adrenoceptors, the contractile responses were not

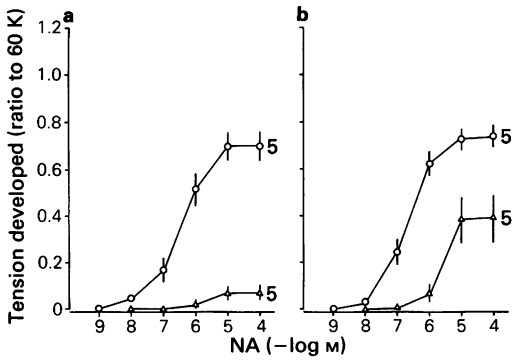

Figure 6 Effects of pretreatment with $1\,\mu g\,ml^{-1}$ cholera toxin (CTX) on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a) and SHR (b). Dose-response curves for noradrenaline (NA) determined in the presence of timolol 20 h after the beginning of CTX pretreatment are shown (△). (○) Control responses. Contractile responses to NA are expressed as a ratio to the maximum contraction induced by 60 mM K^+. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

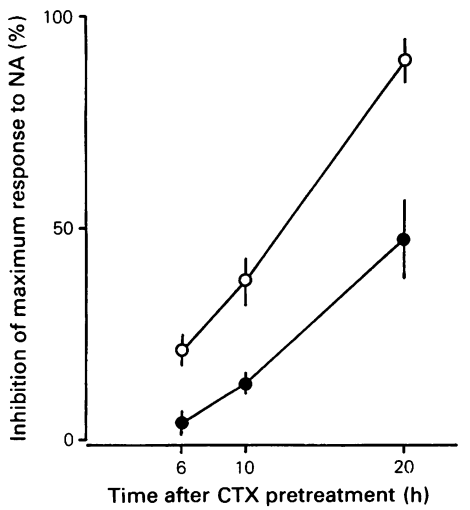

Figure 7 Temporal changes in the inhibitory effects of cholera toxin (CTX) on the maximum response to noradrenaline (NA) in strips of femoral arteries isolated from WKY (○) and SHR (●). The maximum responses to NA determined at 6, 10 and 20 h after the 4 h pretreatment with $1\,\mu g\,ml^{-1}$ CTX are expressed as a % of the maximum response to NA in the respective control curves. These values were obtained from Figures 5, 6 and 7. Vertical lines represent s.e.

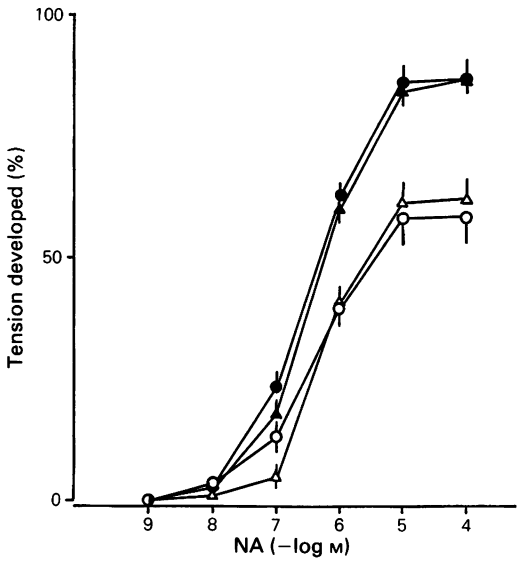

Figure 8 Similarities of the dose-response curves for noradrenaline (NA) in the absence of timolol (○, ●) and the dose-response curves for NA (plus timolol) determined 10 h after the 4 h pretreatment with $1\,\mu g\,ml^{-1}$ cholera toxin (CTX; △, ▲) in strips of femoral arteries isolated from either WKY (○, △) or SHR (●, ▲). Dose-response curves for NA in the absence of timolol were obtained from Figure 2c. The maximum contraction induced by 10^{-4} M NA in the presence of timolol was taken as 100%. Dose-response curves for NA determined 10 h after the 4 h pretreatment with $1\,\mu g\,ml^{-1}$ CTX were obtained from Figure 5a and b. The maximum contraction induced by 10^{-4} M NA in the control curve was taken as 100%. Vertical lines represent s.e.

augmented by the presence of a β-adrenoceptor antagonist (Asano *et al.*, 1982). To this end, the SHR femoral artery is a suitable preparation for investigating the role of decreased β-adrenoceptor responsiveness in the pathogenesis of essential hypertension.

CTX produced a slow-onset and long-lived antagonism of α-adrenoceptor-mediated contractions. The slow-onset and long-lived effect of CTX is consistent with the time course of action of this toxin in other tissues (Richards & Douglas, 1978; Ousterhout & Steinsland, 1981). The onset of antagonism was much slower in the SHR than in the WKY. The slope of the time-dependent inhibition was gentle in the SHR when compared with the WKY (Figure 7). Thus, the ability of CTX to antagonize the contraction was significantly less in the SHR than in the WKY, demonstrating that a decreased responsiveness to CTX exists in the SHR femoral artery. Since the ability of CTX to antagonize the α-adrenoceptor-mediated contractions was similar to the decreased

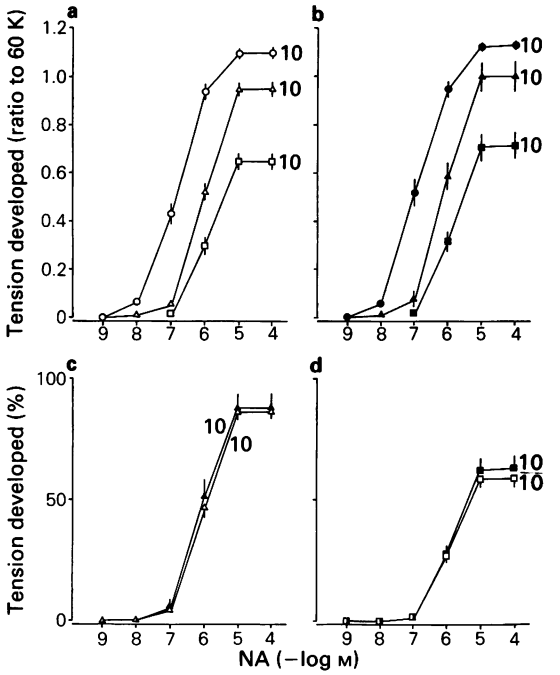

Figure 9 Effects of forskolin on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a) and SHR (b). Contractile responses to noradrenaline (NA), in the absence (control, ○, ●) and presence of forskolin 1×10^{-7} M (△, ▲) and 3×10^{-7} M (□, ■), are expressed as a ratio to the maximum contraction induced by 60 mM K^+. Corrected values of the change in the pD_2 value for NA induced by 1×10^{-7} M forskolin were 0.71 ± 0.08 log scale (WKY) and 0.68 ± 0.06 log scale (SHR), and those by 3×10^{-7} M forskolin were 0.90 ± 0.07 log scale (WKY) and 1.02 ± 0.07 log scale (SHR), respectively. For details, see Methods. (c and d) Dose-response curves for NA determined in the presence of either 1×10^{-7} M (c) or 3×10^{-7} M (d) forskolin were compared between the strips from WKY (△, □) and SHR (▲, ■) on the basis of the % response. The maximum contraction induced by 10^{-4} M NA in the control curve in the presence of timolol was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

β-adrenoceptor responsiveness, as determined by the interaction between α- and β-adrenoceptors (Figure 8), it is strongly suggested that the abnormality seen at the level of β-adrenoceptors also exists at the level of Gs.

Forskolin has been demonstrated to inhibit a variety of contractions in smooth muscles including vascular smooth muscles (Vegesna & Diamond, 1984; Silver *et al.*, 1985; Tsujimoto *et al.*, 1986). The mechanism by which forskolin elevates cellular cyclic

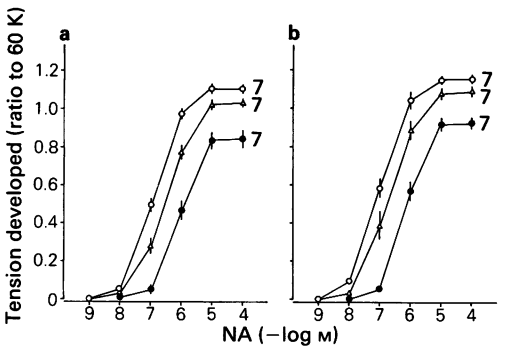

Figure 10 Effects of dibutyryl (DB) cyclic AMP on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a) and SHR (b). (○) Control responses; responses in presence of DB cyclic AMP (△) 3×10^{-5} M and (●) 1×10^{-4} M. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

AMP is assumed to involve a direct interaction with C (Seamon & Daly, 1981; Daly, 1984; Bendar *et al.*, 1984). Therefore, it is likely that the forskolin-induced inhibition of arterial contraction is a consequence of the elevation of cellular cyclic AMP. Although cyclic AMP levels were not actually measured in the present study, we think it is likely that the inhibition by either CTX or forskolin of the femoral arterial contraction is mediated through the elevation of cellular cyclic AMP. The inhibitory effect of 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 ability of forskolin to antagonize

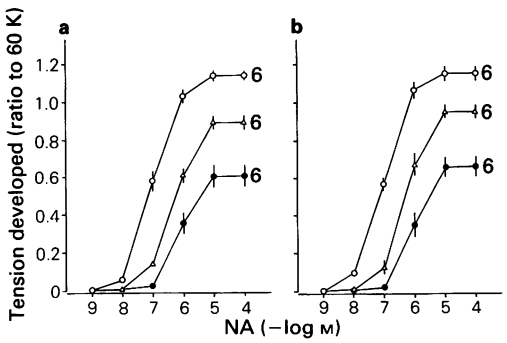

Figure 11 Effects of isobutyl methylxanthine (IBMX) on α-adrenoceptor-mediated contractions in strips of femoral arteries isolated from WKY (a) and SHR (b). (○) Control responses; responses in presence of IBMX (△) 1×10^{-5} M and (●) 3×10^{-5} M. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

the contraction induced by α-adrenoceptor stimulation was the same for the two strains. The results of the experiments with timolol, CTX and forskolin, when taken together, permit the conclusion that components of the β-adrenoceptor · AC system distal to and including AC are probably not responsible for the decreased β-adrenoceptor responsiveness in the SHR femoral artery. This is also suggested by the observation that the inhibitory effects of db cyclic AMP were not significantly different between the SHR and WKY.

Identification of the possible locus (loci) of the impairment might be concluded by estimating changes in cellular cyclic AMP levels. However, several other studies either implicate or eliminate involvement of the components of this system in the decreased β-adrenoceptor responsiveness. The basal levels of cyclic AMP in SHR aortae have been shown to decrease (Amer, 1973; Ramanathan & Shibata, 1974; Sands *et al.*, 1976), increase (Dusseau & Hutchins, 1982; Chatelain *et al.*, 1985) or remain unchanged (Triner *et al.*, 1975), as compared with findings in WKY aortae. Studies by Triner *et al.* (1975) demonstrated a decreased β-adrenoceptor-mediated relaxation that coincided with a reduced production of cyclic AMP by the SHR in response to isoprenaline. Subsequently various studies have demonstrated a decreased responsiveness to β-adrenoceptor stimulation in vascular tissues from SHR (Cohen & Berkowitz, 1976; Asano *et al.*, 1982; Silver *et al.*, 1985). This decreased responsiveness of SHR vascular tissues may reflect a reduced activation of AC in the SHR (Amer, 1973; Triner *et al.*, 1975; Klenerova *et al.*, 1975). The studies by Amer (1973), Triner *et al.* (1975) and Klenerova *et al.* (1975) have demonstrated no change in basal activity of AC in the SHR aorta. These studies also demonstrated that the 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-induced stimulation in the SHR was found to decrease (Amer, 1973) or increase (Triner *et al.*, 1975). Studies by Triner *et al.* (1975) and Donnelly (1978) clearly demonstrated that the levels of the individual cyclic nucleotide phosphodiesterases including cyclic AMP phosphodiesterase were unchanged in SHR aortae as compared with WKY aortae. These studies are in good agreement with the finding in the present study that the inhibitory effects of IBMX on α-adrenoceptor-mediated contractions were the same for the two strains.

From the above biochemical analyses, it is clear that no consistent observations of altered cyclic AMP metabolism characterize the SHR vascular tissues. 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 impairment of the β-adrenoceptor · AC system. From the pharmacological approach to this problem demonstrated in the present study, we propose that a reduced function of Gs is the main factor in the decreased β-adrenoceptor responsiveness in the SHR femoral artery.

However, our work does not provide any specific details concerning the molecular mechanisms involved in the abnormality of Gs in the SHR femoral artery. Recent investigations on the pathogenesis of pseudohypoparathyroidism have proposed a genetic deficiency of Gs as the cause of one type of this disorder (Levine *et al.*, 1980; 1983; Farfel *et al.*, 1980). These investigations have demonstrated that the activity of Gs was reduced to approximately 50% in erythrocytes of patients with one type of this disorder and that this defect is the cause of their resistance to the action of multiple hormones. In the SHR arteries, the decreased responsiveness is also observed in various receptors including H_2-histamine, A_2-adenosine and D_1-dopamine receptors (K. Masuzawa, M. Asano & T. Matsuda, unpublished data). Since the Gs and AC are common to these receptors and β-adrenoceptors, the reduced function of Gs could lead to the resistance to stimulation of these receptors that link to AC. The present pharmacological approach to the Gs function in the SHR femoral artery, including the coupling process of β-adrenoceptors to AC, will have to be strengthened by biochemical analyses in the same tissues, and this is the subject of our current investigations.

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