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Alterations of glucose-dependent and -independent bladder smooth muscle contraction in spontaneously hypertensive and hyperlipidemic rat

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Running title page

1) **Running Title**: Dysfunction of Bladder Contraction in SHHR

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Atr, atropine sulfate; CCh, carbachol; DG, diacylglycerol; DTT, dithiothreitol; EGTA, glycoletherdiaminetetracetic acid; HG, high-glucose; HLR, hyperlipidemic rat; MLCP, myosin light chain phosphatase; OGTT, oral glucose tolerance test: PI, phosphatidylinositol; PIP_2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PSS, physiological salt solution; RLU, relative luminescence units; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; SHHR, spontaneously hypertensive and hyperlipidemic rat; TC, total cholesterol; TG, triacylglycerol.

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ABSTRACT

Alteration of bladder contractility was examined in the spontaneously hypertensive and hyperlipidemic rat (SHHR; age, 9 months; systolic blood pressure, > 150 mm Hg; plasma cholesterol, >150 mg/dL). Carbachol (CCh) induced time- and dose-dependent contractions in Sprague-Dawley (SD; age-matched control) rats and SHHR; however, maximal levels differed significantly (13.3 ± 2.2 and 5.4 ± 1.9 μN/mm^2 following 10 μM CCh treatment, respectively; n=5). This difference, which was maintained in calcium-replaced physiological salt solution (PSS), was suppressed by pretreatment with rho kinase inhibitor (1 μM Y27632); moreover, total activity of rho kinase was also reduced in SHHR bladder. Pretreatment of bladders under high-glucose (HG) conditions (22.2 mM glucose-contained PSS for 30 min) led to enhancement of CCh-induced contraction solely in control animals. Under HG conditions, both protein kinase C (PKC) activity and production of diacylglycerol (DG) derived from incorporated glucose declined in SHHR bladder; however, sustained elevation of plasma glucose level was not detected in SHHR. These results suggested that bladder contractility dysfunction in SHHR is attributable to alteration of rho kinase activity and the DG-PKC pathway. This dysfunction may occur prior to chronic hyperglycemia onset in progressive hypertension and hyperlipidemia.

Introduction

The numbers of individuals displaying hypertension, hyperlipidemia, cardiovascular diseases and/or diabetes are on the rise in many countries. In general, onset of hypertension and hyperlipidemia are evident in early pathology. Deterioration associated with these diseases leads to acute angiopathy and/or chronic tissue dysfunction, e.g., diabetes (McDermott, 2006). Consequently, these severe diseases may be life-threatening. These serious dysfunctions were noted in lifestyle-related diseases; however, dysfunctions of the urinary organs including dysuria and frequent urination negatively impact social situations (Bremnor and Sadovsky, 2002; Kowalczyk, 2003). It is generally accepted that normalization of urinary dysfunction is beneficial in order to maintain patient quality of life.

Hypertension, a major lifestyle-related disease, has been investigated by many groups (Sonkusare et al., 2006; Mehta and Griendling, 2007). An association between autonomic nerve dysfunction (Velez-Roa et al., 2004; Mancia et al., 2007) or renal disease (Williams et al., 2007) and hypertension was suggested; additionally, alterations of urinary bladder function were also reported (Schneider et al., 2005). Bladder smooth muscle tissue isolated from spontaneously hypertensive rat (SHR) was indicative of over-contraction (Persson et al., 1998). These findings suggested that enhancement of bladder contraction causes various urinary dysfunctions. Over-expression or sensitization of muscarinic receptors was noted as the mechanism (Schneider et al., 2005). Moreover, intracellular calcium sensitization (Sherer et al., 2000; Rajasekaran et al., 2005) and correlation of autonomic nerve alteration (Steers et

al., 1999) were also hypothesized.

In some patients of hypertension, hyperlipidemia, cardiovascular diseases and diabetes indicate not a single disease, but multiple diseases. Recently, multiple types are increasing. This situation creates diversification and complicates classification of tissue dysfunctions. In particular, the possibility exists that hypertension may be accompanied by hyperlipidemia. This combined hypertension and hyperlipidemia is believed to lead to arteriosclerosis and/or other serious cardiovascular diseases. Not unexpectedly, bladder responses may be characteristically altered under conditions of hypertension and hyperlipidemia. However, alteration of the response has not been demonstrated.

In 2003, spontaneously hypertensive and hyperlipidemic rat (SHHR) was generated from SHR and hyperlipidemic rat (HLR) (Kumai et al., 2003; Amagasa et al., 2005). SHHR is characterized by systolic blood pressure of approximately 150 mmHg and plasma cholesterol concentration exceeding 150 mg/dL. Moreover, plasma catecholamine levels and low-density lipoprotein expression were higher in SHHR than in Sprague-Dawley (SD) rats. In the aorta, both endothelial degeneration and lipid deposits were also reported (Kumai et al., 2004). Based on these findings, we concluded that SHHR is a novel beneficial tool for application as a multiple-hypertensive and hyperlipidemic model.

The objective of this study was to evaluate differences in isolated bladder smooth muscle contractility between SD rat and SHHR bladders and to examine the correlation between the intracellular signaling pathway and bladder smooth muscle dysfunction.

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Materials and Methods

Reagents. D-[^{14}C]glucose was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). Carbachol (CCh), atropine sulfate (Atr) and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo-(2,3-a)pyrrolo(3,4-*c*)-carbazole (Gö6976) were obtained from Sigma-Aldrich (St. Louis, MO). (R)-(+)-trans-N-(4-pyridyl)-4- (1-aminoethyl)-cyclohexanecarboxamide (Y27632) was procured from Wako Pure Chemical Co. (Osaka, Japan). Calphostin C (isolated from *Cladosporium cladosporioides*) and rottlerin (mallotoxin) were acquired from Calbiochem-Novabiochem (San Diego, CA). All other reagents, which were of the highest purity, were purchased from Sigma-Aldrich, except as noted. Calphostin C and Gö6976 were dissolved in dimethyl sulfoxide; no effects of vehicle were noted when total vehicle concentration was 0.03% or less.

Experimental Models. Spontaneously hypertensive and hyperlipidemic rat (SHHR; male, 7-9 months of age) and age-matched control (SD) rats were used in this study. The animals were prepared as described previously (Kumai et al., 2003). Rats were housed at constant room temperature (23 ± 1 ^{o}C) with a 12-h light and dark cycle. The rats were fed standard rat chow including 5% fat (CE-2, Clea, Tokyo, Japan). Food and water were available ad libitum and the rats grew satisfactorily. All procedures were performed according to the quilting principles for the care and use of laboratory animals for the Japanese Pharmacological Society.

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Blood Collection and Plasma Biochemical Assays. Blood specimens, which were obtained from the inferior vena cava under pentobarbital anesthesia, were mixed with a 3.2% sodium citrate solution at a ratio of 9:1. The citrated plasma supernatant was utilized for chemical assays. Total cholesterol (TC) and triacylglycerol (TG) levels were measured as described previously (Kumai et al., 2003).

Bladder Smooth Muscle Preparation. The urinary bladder was isolated from pentobarbital-anesthetized (35 mg/Kg) rats. Bladders were rinsed in physiological salt solution (PSS); subsequently, fat and connective tissue were removed from both sides. PSS, which was supplemented with 118 mM NaCl, 5.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.4 mM NaH_2PO_4, 21.4 mM $NaHCO_3$ and 11.1 mM glucose, was aerated with 95% O_2 and 5% CO_2 at 37^oC. Prior to measurements, the wet weight of each tissue was determined.

Isometric Force Measurement. Each tissue was positioned vertically in a temperature-controlled 5-ml organ bath. One end of the tissue was connected to a strain gauge transducer (Type T-7-8-240, Orienteck, Tokyo, Japan) to monitor contractile responses. Bladder contractions were normalized to cross-sectional area with the following equation:

Cross-sectional area (mm^2) = (2 × wet weight (mg)) / (1.06 × circumference (mm)), where 1.06 is smooth muscle tissue density (mg/mm^3).

Resting tension was set at 10 mN, where the tissue length was established in the optimal range for force generation.

Measurement of Relative Rho Activity. Bladders were stimulated under various

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conditions similar to force measurement under resting tension (10 mN). Subsequently, tissues were powdered at liquid nitrogen temperature. The samples were re-suspended in a homogenization buffer containing 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.2), 250 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM glycoletherdiaminetetraacetic acid (EGTA), 1 μg/mL pepstatin, 1 μg/mL leupeptin and 50 μg/mL soybean trypsin inhibitor (Buffer A); homogenates were centrifuged (1,000 × g for 5 min) to remove nuclei. The supernatant was decanted, after which pellets were washed once with sucrose-free Buffer A (Buffer B). Next, the combined supernatants were re-centrifuged (20,000 × g for 30 min). The pellets were re-suspended in Buffer B. Finally, total membrane fractions were collected by centrifugation of the supernatant (100,000 × g for 60 min) and re-suspended in Buffer B. The supernatant served as the cytosolic fraction. Total Rho A activities were measured with a specific Rho A activation assay kit (G-LISA^{TM}; Cytoskeleton. Inc., Denver, CO). Membrane and cytosolic fractions (25 μg protein) were assayed and results were detected as relative luminescence units (RLU) over background signal (background incubation with assay reagents alone instead of cellular fractions).

Measurement of Total Mass of Diacylglycerol (DG). The total mass of DG in each tissue was measured in a manner similar to that described in a previous report (Nobe et al., 1993). Isolated tissues were treated under various conditions in 200 μL of PSS. The reaction was terminated upon the addition of chloroform/methanol (1:2 by vol, 750 μL). Tissues were homogenized; subsequently, water and chloroform (200 μL of each) were added. The mixture was shaken, followed by centrifugation at

1,000 × g. The lower phase was removed and dried under N_2 gas. The residue was re-dissolved in chloroform (concentration; 2 μL/mg wet weight tissue). This sample was spotted on a TLC plate (Merck, Silica gel 60 with concentrating zone). DG separation was effected with diethylether/heptane/acetic acid (75:25:1 by vol). The plates were dried and stained with 0.03% coomassie brilliant blue solution containing 30% methanol and 100 mM NaCl for 30 min; plates were de-stained for 5 min in dye-free staining solution. Each TLC plate was scanned; moreover, the density of each band was calculated using NIH image software. Total mass of DG was determined from a dioreoyl-glycerol standard curve. Results are expressed as ng/mg wet weight tissue.

D-[^{14}C]glucose Incorporation into DG. Tissues were pre-labeled with 33 mCi/mL D-[^{14}C]glucose contained within normal PSS and high glucose-PSS (HG-PSS; twice the glucose concentration of normal PSS) at 37 ^{o}C for 60 min (Inoguchi et al., 1994; Lee et al., 2004). CCh and/or additional reagents were introduced following this treatment. Following termination of these reactions, total lipids were extracted as described above. DG was separated on silica gel G thin-layer plates and developed in hexane/ether/acetic acid (60:40:1). Spots on the silica gel were removed by scraping; subsequently, radioactivity of [^{14}C]DG was measured on a liquid scintillation counter.

Data Analysis. Values are presented as means ± SEM obtained from at least 4-6 animals. Statistical analyses for simple comparisons were performed utilizing Student's t-test. Multiple comparisons were conducted with ANOVA for repeated measures followed by the Student-Newman-Keuls (SNK) test.

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Results

Basic Characteristics of SHHR. A significant difference in body weight was not observed in SHHR at 9 months of age relative to SD rat (Table 1). As is typical with SHHR, changes in blood pressure and lipid levels were examined. Blood pressure level in SHHR was higher than that in controls (115% of SD rat). After a 12-h fast, total cholesterol and triglyceride levels were also significantly higher (337.5 and 172.4% of SD rat, respectively). However, meaningful differences in plasma glucose levels between these animals were not detected.

Alteration of CCh-induced Isometric Force Responses in SHHR and SD Rat Bladder. Resting levels of isometric force in SD rat and SHHR bladders averaged 0.96 ± 0.02 and 0.98 ± 0.04 mN/mm^2 (n = 5), respectively. Cumulative addition of CCh induced significant increases in isometric force. Typical responses of individual SD rat and SHHR bladders are shown in Fig. 1A; the averaged concentration-response relationships from these experiments for both bladder types are summarized in Fig. 1B. Maximal increase in force response in SD rat bladder in the presence of 30 μM CCh was 13.14 ± 0.21 mN/mm^2 (n = 5). At higher CCh levels, force development declined. Decreases at high concentrations of CCh (30 μM) have been reported (Lundbeck and Sjogren, 1992). The basis for this decline is not known with certainty; however, it may be attributable to G-protein-mediated inhibition associated with high levels of muscarinic receptor activation. In SHHR, the maximal increase in isometric force was

depressed considerably (6.93 ± 0.30 mN/mm^2; n = 5). Significant decreases were also evident between 1.0-100 μM CCh. EC_{50} values for SD rat and SHHR bladders were 2.5 and 7.5 μM, respectively. The dose-response curve for SHHR displayed a substantial rightward shift in comparison to that of SD rat. The responses to CCh were responsible for this shift following a rise in PSS for 15 min.

The muscarinic receptor antagonist atropine (Atr; 10 nM, 10-min pre-incubation) inhibited the CCh-induced increase in isometric force in SD rat and SHHR (Fig. 1B). Dose-response curves were shifted to higher concentrations of CCh. pA_2 values of 10 nM Atr in SD rat and SHHR were 8.48 and 8.72, respectively.

Effects of $CaCl_2$ replacement on CCh-induced Increase in Isometric Force in SD Rat and SHHR Bladders. The depressed responses of SHHR are consistent with a decrease in calcium channel activity. $CaCl_2$-replaced PSS (nominally Ca^{2+}-free PSS) was utilized with respect to this approach; summarized data are presented in Fig. 2. In SD rat bladder, pretreatment with Ca^{2+}-free PSS for 5 min did not alter isometric force resting level. In contrast, the response was severely blunted by cumulative CCh stimulation; isometric force at 30 μM CCh was 45.0 ± 3.82% (n = 5) of that of the control (response in normal-PSS). In SHHR bladder, pretreatment with Ca^{2+}-free PSS also markedly reduced the isometric force response to CCh without affecting the resting level. CCh (30 μM)-induced force development in Ca^{2+}-free PSS was 3.56 ± 0.64 mN/mm^2 (n = 5) and the inhibition ratio (45.7 ± 13.8%) was similar to that of SD rat. Significant rates of reduction relative to SD rat were detected at concentrations of 10-30 μM CCh.

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Effect of Rho-Rho Kinase Pathway on CCh-induced Increase in Isometric Force in SD Rat and SHHR Bladder. In order to identify rho-rho kinase-mediated pathway involvement with respect to the differences between SD rat and SHHR, a specific rho kinase inhibitor, Y27632, was introduced (Fig. 3). In the SD rat, pretreatment with 1 μM Y27632 significantly reduced the isometric force attributable to CCh; moreover, the resting level was unaffected. Isometric force induced by 30 μM CCh in the presence of Y27632 was 7.45 ± 0.21 mN/mm^2 (54.5% of control response; n = 5). However, inhibitory effects of Y27632 were not evident in SHHR bladder. Dose-response curves for CCh in the presence or absence of Y27632 overlapped. Isometric force levels induced by 30 μM CCh were 5.88 ± 0.42 and 6.74 ± 0.39 mN/mm^2 (n = 5), respectively. Differences between SD rat and SHHR bladder were suppressed by Y27632 pretreatment.

Total rho A activity was measured to confirm a relationship between the rho-rho kinase pathway and alteration of bladder contraction in SD rat and SHHR (Fig. 4). Rho A activity in the total membrane fraction was determined with a specific rho A assay kit. In SD rat bladder, the resting level of rho A activity was 141.7 ± 12.4 RLU (n = 5). This level increased 14-fold following treatment with 30 μM CCh (2003.0 ± 36.2 RLU; n = 5), however, that was surpressed in SHHR bladder. Rho A activities under resting and CCh treatment conditions were 83.7 ± 11.1 and 150.3 ± 26.2 RLU (n = 5), respectively.

In order to demonstrate alteration of rho A activity in hyperglycemia, CCh-induced changes in rho A activity were measured under high-glucose (HG) conditions.

Diabetes, which is characterized by elevated extracellular glucose levels, was created by pretreatment of the bladder with HG-PSS at 37 ^{o}C for 30 min. This condition was introduced in our previous studies. Under HG conditions, resting and CCh-induced activation of membrane rho A was not affected in SD rat (1974.6 ± 49.6 RLU; n = 5). Activity in SHHR bladder also was unaffected.

Alteration of Bladder Contractility under HG Conditions in SD Rat and SHHR Bladder. CCh-induced force development in HG-PSS was assessed employing a strategy similar to that of the rho A assay under HG conditions (Fig. 5). Pretreatment of SD rat bladder with HG-PSS for 30 min did not alter the non-stimulated resting level (1.05 ± 0.07 mN/mm^2; n = 5). However, the CCh-induced dose-dependent increase in force development displayed significant enhancement. Apparent enhancement in comparison to the responses in normal-PSS was detected following treatment with 0.3-30 μM CCh. Submaximal response induced by 30 μM CCh was 13.8 ± 0.23 mN/mm^2 (n = 5). EC_{50} value in HG-PSS was 2.1 μM. In SHHR bladder, the CCh-induced increase in force development was not affected by extracellular glucose accumulation. Force developments induced by 30 μM CCh in normal- and HG-PSS were 7.09 ± 0.19 and 7.52 ± 0.54 mN/mm^2 (n = 5), respectively.

Alteration of Intracellular DG Formation under Normal and HG Conditions. The roles of DG in phosphatidylinositol (PI) turnover-mediated contraction have been identified; thus, in order to establish alteration of the intracellular signaling system in SD rat and SHHR, levels of DG, a major contractile factor, were measured (Fig. 6). In SD rat bladder, the endogenous DG level in the resting state was 138.5 ± 11.1 ng/mg

wet weight tissue (n = 5) (Fig. 6A). This level rose markedly in the presence of 30 μM CCh to 246.3 ± 14.0 ng/mg wet weight tissue (n = 5). Under HG conditions, the resting level of DG increased significantly to 271.6 ± 12.7 ng/mg wet weight tissue (n = 5). DG increased to 338.4 ± 10.7 ng/mg wet weight tissue (n = 5) following stimulation with 30 μM CCh. In SHHR bladder, resting and CCh-treated values of total mass of DG in normal PSS were 134.1 ± 10.9 and 186.4 ± 7.36 ng/mg wet weight tissue (n = 5), respectively. A meaningful increase in terms of CCh stimulation was not evident. Moreover, enhancement in HG-PSS in SHHR was blunted; resting and CCh-treated values were 150.4 ± 15.2 and 194.9 ± 11.0 ng/mg wet weight tissue (n = 5), respectively. Significant differences between normal- and HG-PSS were not observed.

To establish the association between HG treatment and endogenous DG formation, incorporation of [^{14}C]glucose into the DG fraction was measured (Fig. 6B). Isolated tissues were incubated with [^{14}C]glucose under several conditions; subsequently, intracellular [^{14}C]DG was analyzed. In the SD rat in the resting state, [^{14}C]DG level was 427.7 ± 37.1 cpm/mg wet weight tissue (n = 5). This value was not affected by 30 μM CCh (463.5 ± 28.7 cpm/mg wet weight tissue; n = 5). However, treatment with HG-PSS significantly increased the resting level of [^{14}C]DG (776.1 ± 38.1 cpm/mg wet weight tissue; n = 5). This increase was maintained by CCh stimulation. In the SHHR bladder, [^{14}C]DG formation in normal PSS was similar to the response in the SD rats. Resting and CCh-treated responses were 387.3 ± 16.8 and 338.5 ± 40.0 cpm/mg wet weight tissue (n = 5), respectively. However, enhancement of the resting level in HG-PSS was not apparent (353.6 ± 30.5 cpm/mg wet weight tissue; n = 5). Under HG

conditions, CCh-induced enhancement of the [^{14}C]DG level was not detected (382.5 ± 25.4 cpm/mg wet weight tissue; n = 5).

Effects of Protein Kinase C (PKC) Inhibitors on CCh-induced Bladder Contractility under HG Conditions in SD Rat and SHHR. In order to identify a relationship between PKC and bladder contraction in SD rat and SHHR, three types of PKC inhibitors were introduced. Calphostin C is a general-type (non-isoform-specific type) PKC inhibitor. Gö6976 and rottlerin, a calcium-dependent and -independent PKC inhibitor, respectively, were selected (Fig. 7-inset). To assess the specific inhibitory effects of the PKC inhibitors, time- and dose-dependence of the inhibitors were measured (data not shown). Based on these preliminary trials, each condition was adopted as the minimum concentration in the critical inhibitory range. Treatment with 30 μM CCh under HG conditions apparently increased SD rat bladder contraction in a manner similar to that of Fig. 1. Both Calphostin C and Gö6976 significantly reduced CCh-induced force development (57.8 ± 3.6 and 50.9 ± 2.7% of control response; n = 5). In contrast, rottlerin did not affect the response (92.6 ± 9.5% of control response; n = 5). Similar trials were performed under HG conditions. In the non-stimulated resting state in HG-PSS, force level was indistinct from the corresponding level in normal-PSS (1.1 ± 0.1 mN/mm^2; n = 5); however, significant enhancement of force development was evident following treatment with 30 μM CCh (12.8 ± 0.3 mN/mm^2; n = 5). A similar pattern in terms of isometric force changes in the presence of Calphostin C was detected under HG conditions in SD rat bladder. However, marked inhibition was observed in the presence of rottlerin (44.6 ± 3.4% of

control response; n = 5); Gö6976 did not alter the force response.

The inhibitory effects of rottlerin on the CCh-induced dose-dependent enhancement of force development were also measured (Fig. 7A). In a manner similar to that of Fig. 5B, CCh-induced dose-dependent increase in force development was confirmed under normal glucose conditions. This response exhibited meaningful enhancement during stimulation with 0.3-100 μM CCh under HG conditions. Pretreatment with 1 μM rottlerin significantly reduced the isometric force in the presence of 1-100 μM CCh, although resting level was unaffected. This inhibitory effect was apparent only in HG-PSS. Dose-response curves for CCh in the presence of rottlerin in normal- and HG-PSS were striking.

In SHHR bladder, enhancement of force development induced by 30 μM CCh under normal-glucose conditions was confirmed (resting and maximal levels were 1.0 ± 0.02 and 4.6 ± 0.2 mN/mm^2, respectively; n = 5). Pretreatment of tissues with Calphostin C reduced CCh-induced responses (5.5% of control; n = 5); however, meaningful attenuation of force development was not evident in the presence of rottlerin (over 70% of control responses remained). Pretreatment of bladders with Gö6976 significantly reduced force development (1.2 ± 0.1 mN/mm^2, respectively; n = 5). This inhibitory effect resembled that of Calphostin C. Under HG conditions, effects of CCh and PKC inhibitors were measured; moreover, patterns similar to the responses in normal-PSS emerged. Extracellular glucose-dependent alteration of force development was not detected. The dose-response curve for CCh treatment in SHHR bladder was not impacted by extracellular glucose levels as in Fig. 5B (Fig. 7B). Pretreatment of

SHHR bladder with 1 μM rottlerin did not influence the CCh-induced dose-response curves.

In order to confirm the relationship between force development and PKC activity in the presence or absence of rottlerin, the effect of rottlerin on PKC activity in total membrane fractions was measured (Fig. 8). In SD rat, PKC activity in the membrane fraction of the non-stimulated resting state was 6.2 ± 0.6 pmol/min/mg protein (n = 5). This activity increased markedly upon treatment of tissue with 30 μM CCh (20.1 ± 1.4 pmol/min/mg protein; n = 5). The calcium-independent PKC inhibitor, rottlerin, slightly reduced CCh-induced PKC activation; however, this result was not significant. Similar trials were performed under HG conditions. In the non-stimulated resting state in HG-PSS, PKC activity in the membrane fraction (17.6 ± 1.8 pmol/min/mg protein; n = 5) was significantly greater in comparison with the corresponding value in normal PSS. This increase was maintained during CCh stimulation (20.2 ± 0.9 pmol/min/mg protein; n = 5). However, rottlerin (1 μM; 5 min) suppressed PKC activity in HG-PSS (8.9 ± 0.4 pmol/min/mg protein; n = 5).

In SHHR bladder, resting activity of PKC in normal-PSS was similar to the corresponding value in SD rat. PKC activity was 4.9 ± 0.5 pmol/min/mg protein (n = 5). CCh treatment enhanced PKC activity in SHHR (9.3 ± 0.7 pmol/min/mg protein; n = 5). However, this CCh-induced increase in PKC activity was significantly smaller than that in SD rat (32% of the increase in SD rat). Pretreatment with rottlerin did not influence the CCh-induced response (7.0 ± 0.5 pmol/min/mg protein; n = 5). Under HG conditions, enhancement of resting PKC activity apparent in the SD rat was not

observed (6.5 ± 0.7 pmol/min/mg protein; n = 5). CCh-enhanced PKC activity was not detected (7.5 ± 0.4 pmol/min/mg protein; n = 5); moreover, rottlerin exerted no effect on the response (7.0 ± 0.4 pmol/min/mg protein; n = 5).

Discussion

Alteration of bladder smooth muscle contraction consistent with the combined hypertensive and hyperlipidemic model was examined in the current investigation.

Pathological stages are important in studies employing experimental models. As shown in the Table 1, SHHR (7-9 months of age) exhibited simultaneous increases in systolic blood pressure and plasma cholesterol levels in comparison with values of normal SD rat. It is generally accepted that obesity may lead to lipidosis and/or desensitization to insulin (Hand et al., 1989; Biourge et al., 1997); 9-month-old SHHR did not display this stage. Furthermore, sustained elevation of fasting blood glucose level was not evident in SHHR and SD rat. Meaningful elevation of glucose level was detectable via the oral glucose tolerance test (OGTT) at 7 months of age. These results indicated that the selected SHHR stage (9 months of age) critically demonstrated both hypertension and hyperlipidemia; however, obesity and diabetic hyperglycemia were absent. These data are indicative of the onset of these conditions in the early stage of the model. The significance of this investigation with respect to the early stages pertains to the provision of novel findings in terms of prevention and early care regarding multiple hypertension and hyperlipidemia mediated by an understanding of

the initial dysfunction of the tissue.

Treatment of bladder smooth muscle tissue with CCh induced time- and dose-dependent contraction in both SD rat and SHHR (Fig. 1A). However, the response in SHHR was significantly lower than that in SD rat (Fig. 1B). Enhancements of contraction in diabetic models (Gupta et al., 1996; Waring and Wendt, 2000) and spontaneous hypertensive rat (SHR) (Rajasekaran et al., 2005) have been described. These inverted dysfunction in SHR based on the SHHR; consequently, reduction of contraction in SHHR is a characteristic of bladder dysfunction in combined hypertension and hyperlipidemia. Increased bladder output was also documented (Drake et al., 2006), and it may be correlated with dysuria and frequent urination in hypertensive patients. In terms of previous findings regarding enhanced bladder contractility in hypertension, why is bladder contraction reduced in SHHR?

Based on the results that SHHR bladder indicated inverted dysfunction from the SHR, we surmised that reduction of contractility might be governed by original mechanisms. We initially measured alteration of muscarinic receptor function (Fig. 1B) and intracellular calcium dependency (Fig. 2). In these experiments, pA_2 values in SD rat and SHHR were the similar (8.48 and 8.72, respectively). We concluded that alteration of muscarinic receptor function was not a major reason for reduced contraction.

CCh-induced contraction in SD rat and SHHR bladders were significantly attenuated (approximately 50% of each response in normal-PSS). However, differences between SD rat and SHHR remained. These results indicated that alterations of muscarinic

receptor and extracellular calcium dependencies in SD rat and SHHR were not involved with the reduction of contraction. We therefore focused on rho and the rho kinase-mediated pathway in terms of a calcium-independent smooth muscle regulatory mechanism. This pathway plays an important role in bladder smooth muscle contraction as well as in other types of smooth muscle contraction (Nobe and Paul, 2001; Tsai and Jiang, 2006). A specific rho kinase inhibitor, Y27632, significantly inhibited CCh-induced SD rat bladder contraction (Fig. 3). The dose-response curve derived from Y27632 inhibition in SD rat was similar to the control dose-response curve in SHHR. However, addition of Y27632 did not alter contraction in SHHR. These data indicated that the rho-rho kinase pathway was involved in CCh-induced bladder smooth muscle contraction in SD rat; additionally, activity of the pathway was reduced in SHHR. This reduction may lead to bladder dysfunction in SHHR. To confirm inactivation of the rho-rho kinase pathway, cellular rho activities were measured (Fig. 4). From the CCh-induced increase in rho activation was suppressed only in SHHR, it was suggested that suppression of rho activation leads to reduced bladder smooth muscle responsiveness mediated by rho kinase inactivation.

Reduction of contractile responses in bladder smooth muscle isolated from hypertensive and hyperlipidemic rats was observed for the first time in the current study. This phenomenon does not involve alteration of muscarinic receptor or extracellular calcium dependency, e.g., SHR; however, participation of the rho-rho kinase pathway in this dysfunction was strongly suggested.

In SHHR, hypertension and hyperlipidemia are aggravated with age. After 7

months of age, marked increases in blood glucose level were also detected in OGTT. Although the selected stage of combined hypertension and hyperlipidemia in SHHR did not follow with respect to chronic hyperglycemia (Table 1), the relationship between extracellular glucose accumulation and CCh-induced contractile responses in bladder was assessed.

HG conditions were introduced in our previous study involving diabetic vascular smooth muscle measurement (Nobe et al., 2003; Nobe et al., 2004). CCh-induced SD rat bladder contraction was significantly enhanced under HG conditions, but it was not evident in SHHR (Fig. 5). This finding indicated that SHHR bladder lacks glucose sensitivity. In diabetic bladder dysfunction under HG conditions, Stevens *et al.* noted that bladder over-contraction is involved in diabetic dysfunction (Stevens et al., 2006). Reduced bladder contraction was also reported (Su et al., 2004). Our results in SHHR may support future possibilities. As an important point in the current investigation, it was indicated that reduction of bladder smooth muscle contraction in SHHR occurred prior to sustained elevation of plasma glucose level. It was thought that glucose sensitivity was suppressed prior to the rat's progression to the stage of chronic hyperglycemia. This desensitization, which was described initially in this study, might be critical in terms of a thorough understanding of bladder dysfunction in combined hypertension and hyperlipidemia.

Dysfunction of SHHR bladder contraction in normal PSS arose due to inactivation of the rho-rho kinase pathway; consequently, rho activity in the membrane fraction was measured under HG conditions (Fig. 4). These results suggested that suppression of

glucose sensitivity in SHHR bladder is not correlated with activity of the rho-rho kinase pathway. We previously reported that enhancement of smooth muscle contraction under HG conditions involves the DG-PKC pathway in some vascular tissue as an additional regulatory factor of bladder contraction (Nobe et al., 2003; Nobe et al., 2004). It is generally accepted that CCh-induced bladder smooth muscle contraction is mediated by phosphatidylinositol-bisphosphate (PIP_2) hydrolysis and DG formation in a manner similar to that of other types of excitable cells (Nord, 1996; Nofer et al., 2000). Individual PKC isoforms might undergo activation by a specialized DG species (Kanoh et al., 1990). Moreover, some activated-PKC isoform types accelerate PI turnover via activation of DG kinase.

Accumulation of endogenous DG during CCh stimulation was observed in SD rat bladder (Fig. 6A). Under HG conditions, incorporated glucose is utilized by the glycolytic pathway as well as in DG formation via a *de novo* synthesis pathway (Mancia et al., 2007). HG treatment induced an elevation in resting DG level, which was maintained during CCh stimulation. DG formation derived from incorporated glucose also increased (Fig. 6B). These findings suggested that sustained basal DG level under HG conditions in SD rat bladder results from an increase in the formation of DG derived from glucose. This enhanced DG level might contribute to over-activation of PKC and DG kinase. PKC activity in the membrane fraction was significantly enhanced under HG conditions (Fig. 8). Therefore, these data revealed that HG enhances intracellular DG level via a *de novo* synthesis pathway in SD rat bladder.

Is PKC activation under HG conditions distinguishable from PKC activation in

normal-PSS? De Witt *et al.* described rottlerin inhibition of calcium-independent isoforms of PKC (nPKC-δ, -ε, -η, -θ) (De Witt et al., 2001). Pretreatment with rottlerin significantly attenuated CCh-induced SD rat bladder contraction (Fig. 7A) in HG-PSS. Glucose-dependent activation of PKC in the membrane fraction was also inhibited (Fig. 8). The inhibitory effects of rottlerin were similar to the effect of general (isoform-nonselective) PKC inhibitor types, namely, Calphostin C (Fig. 7A-inset). Moreover, rottlerin altered neither contractile response nor PKC activity under normal glucose conditions (Fig. 7A, 8). These results suggested that DG is unnaturally formed from incorporated glucose under HG conditions, which leads an activated calcium-independent isoform of PKC. This activation may induce glucose-dependent over-contraction in SD rat bladder. It is a normal physiologic response in bladder function under HG conditions; absence of this response may be associated with bladder dysfunction.

In SHHR bladder, enhancement of total DG level was not observed in HG-PSS; furthermore, DG formation from incorporated glucose was undetectable in HG-PSS (Fig. 6). A calcium-independent isoform of PKC activation was not detected due to the absence of glucose-dependent DG (Fig. 6B, 8). Consequently, contractile response in SHHR bladder under HG conditions was unaltered by pretreatment with rottlerin (Fig. 7B). These data suggested that suppression of glucose-dependency in SHHR bladder is responsible for the reduction of DG formation from incorporated glucose, which may be attributable to inactivation of the glucose transporter in bladder smooth muscle.

We examined bladder responsiveness in a combined hypertensive and

hyperlipidemic rat. Prior to any indication of hyperglycemia in this model, glucose utilization diminished and reduction of bladder smooth muscle contraction occurred. Under this pathological condition, we hypothesized that improvement with respect to reduced formation of DG from glucose and/or reduced activity of calcium-independent PKC isoforms contribute to the normalization of bladder function.

Diminished bladder smooth muscle contraction was detected initially under combined hypertensive and hyperlipidemic conditions in this investigation. The reduction of bladder contractility in SHHR involved inactivation of the rho-rho kinase pathway. The absence of extracellular glucose-dependency was observed prior to hyperglycemia indications in SHHR. The occurrence of alteration of contractile response prior to hyperglycemia onset was critical with respect to a thorough understanding of bladder dysfunction. Mechanistically, this phenomenon suggested that this alteration mediates a reduction in calcium-independent PKC activity followed by formation of DG from incorporated glucose. The current findings afforded novel information with respect to establishment of pharmacotherapy for bladder dysfunction.

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Footnotes

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Legends for Figures

Fig. 1. Effects of carbachol (CCh) on isometric force in bladder from SD rat and SHHR. CCh-induced changes in isometric force (mN/mm^2) were measured as described in Methods. A, typical changes observed in a single bladder preparation (left panel, SD rat; right panel, SHHR). B, concentration-response relationships for CCh-induced isometric force responses in bladder from SD rat (circles) and SHHR (squares). Bladder tissues were pre-incubated in the presence (closed) or absence (open) of the muscarinic receptor antagonist (10 nM Atr) for 10 min; subsequently, indicated contractions of CCh were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # indicate values significantly different from SD rat and CCh responses, respectively, at $p<0.01$.

Fig. 2. Effects of calcium-free PSS on CCh-induced isometric force in bladder from SD rat and SHHR. CCh-induced changes in isometric force (mN/mm^2) were measured as described in Methods. A, typical changes observed in a single bladder preparation (upper panel, SD rat; lower panel, SHHR). B, concentration-response relationships for CCh-induced isometric force responses in bladder from SD rat (circles) and SHHR (squares). Bladder tissues were pre-incubated in normal (left panel, open symbol) or calcium-free (right panel, closed symbol) PSS for 10 min; subsequently, indicated contractions of CCh were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # indicate values significantly

different from SD rat and responses in normal PSS, respectively, at $p<0.01$.

Fig. 3. Effects of rho kinase inhibitor on CCh-induced isometric force in bladder from SD rat and SHHR. CCh-induced changes in isometric force (mN/mm^2) were measured as described in Methods. A, typical changes observed in a single bladder preparation (upper panel, SD rat; lower panel, SHHR). B, concentration-response relationships for CCh-induced isometric force responses in bladder from SD rat (circles) and SHHR (squares). Bladder tissues were pre-incubated in the presence (closed) or absence (open) of the rho kinase inhibitor (1 μM Y27632) for 10 min; subsequently, indicated contractions of CCh were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # indicate values significantly different from SD rat and CCh responses, respectively, at $p<0.01$.

Fig. 4. Alteration of Rho A activity in normal- and high-glucose PSS. Bladders were isolated from SD rat (bright bars) and SHHR (dark bars). Tissues were pre-incubated with normal- and high-glucose (HG) PSS at 37 ^oC for 30 min. Subsequently, 30 μM CCh (CCh) was added for 5 min. Following termination of the reaction, Rho A activities were measured with G-LISA™ Rho A assay kits. Results are expressed as relative luminescence units (RLU). Each value represents the mean ± SEM of five independent determinations. * indicates values significantly different from non-stimulated resting state at $p<0.01$.

Fig. 5. Effects of HG-PSS on CCh-induced isometric force in bladder from SD rat and SHHR. CCh-induced changes in isometric force (mN/mm^2) were measured as described in Methods. A, typical changes observed in a single bladder preparation (upper panel, SD rat; lower panel, SHHR). B, concentration-response relationships for CCh-induced isometric force responses in bladder from SD rat (circles) and SHHR (squares). Bladder tissues were pre-incubated in normal- (open) and HG- (closed) PSS at 37 ^oC for 30 min; subsequently, indicated contractions of CCh were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # indicate values significantly different from normal PSS and SD rat, respectively, at $p<0.01$.

Fig. 6. Alteration of intracellular DG formation in bladder from SD rat and SHHR. Bladders were isolated from SD rat (bright bars) and SHHR (dark bars). Tissues were pre-incubated with normal- and high-glucose (HG) PSS at 37 ^oC for 30 min. Subsequently, 30 μM CCh (CCh) was added for 5 min. Following termination of the reaction, total mass of DG was measured as described in Methods (A). In order to assess DG formation from incorporated glucose, [^{14}C]glucose was added to pre-incubation solutions. Next, [^{14}C]DG levels were measured as described in Methods (B). Each value represents the mean ± SEM of five independent determinations. * and # indicate values significantly different from non-stimulated resting state and normal PSS, respectively, at $p<0.01$.

Fig. 7. Effects of PKC inhibitors on CCh-induced isometric force in bladder from SD rat and SHHR. CCh-induced changes in isometric force (mN/mm^2) were measured in SD rat (A) and SHHR (B) bladders as described in Methods. Tissues were incubated with normal- (open, bright column) and HG-PSS (closed, dark column) at 37 ^oC for 30 min. Subsequently, bladder tissues were pre-incubated in the presence (squares) or absence (circles) of the calcium-independent PKC inhibitor (1 μM Rottlerin) for 10 min; subsequently, indicated contractions of CCh were introduced. Other types of PKC inhibitors were tested in a similar manner (*inset panels*). Each treatment was conducted per the following panel: A, resting; B, 30 μM CCh; C, 1 μM Calphostin C-pretreated CCh; D, 1 μM Rottlerin-pretreated CCh; E, 1 μM Gö6976-pretreated CCh. Each value represents the mean ± SEM of at least five independent determinations. *, $p< 0.01$ versus normal-glucose PSS. #, $p<0.01$ versus CCh responses. †, $p<0.01$ versus non-stimulated resting level.

Fig. 8. Alteration of PKC activity in SD rat and SHHR bladders. Bladders were isolated from SD rat (bright bars) and SHHR (dark bars). Tissues were pre-incubated with normal- and high-glucose (HG) PSS at 37 ^oC for 30 min. Subsequently, 30 μM CCh (CCh) was added for 5 min. PKC inhibitor (1 μM Rottlerin) was introduced 10 min before CCh stimulation. Following termination of the reaction, total membrane fractions (100,000 x *g* pellet) were prepared as described in Methods. Total PKC activities were measured with PKC fluorescence assay kits (Invitrogen, CA). Each value represents the mean ± SEM of five independent determinations. *, $p< 0.01$

versus non-stimulated resting level. #, $p<0.01$ versus CCh responses. †, $p<0.01$ versus normal-glucose PSS.

Table 1

Basic characteristics of SHHR

Animals	n	BW (g)	BP (mmHg)	TC (mg/dL)	TG (mg/dL)	PG (mg/dL)
SD rat	**6**	**567.3 ± 29.5**	**138.2 ± 6.66**	**63.2 ± 1.2**	**121.6 ± 13.5**	**103.2 ± 3.23**
SHHR	**6**	**537.2 ± 20.0**	**159.6 ± 0.32***	**172.6 ± 6.6***	**233.1 ± 33.8***	**95.6 ± 11.2**

Body weight (BW) and blood pressure (BP) were measured in 9-month-old SD rat and SHHR. Plasma total cholesterol (TC), triacylglycerol (TG) and 12 hr-fasted blood glucose (PG) levels were measured as described in "Methods". * $p<0.05$ vs SD rat (t-test).

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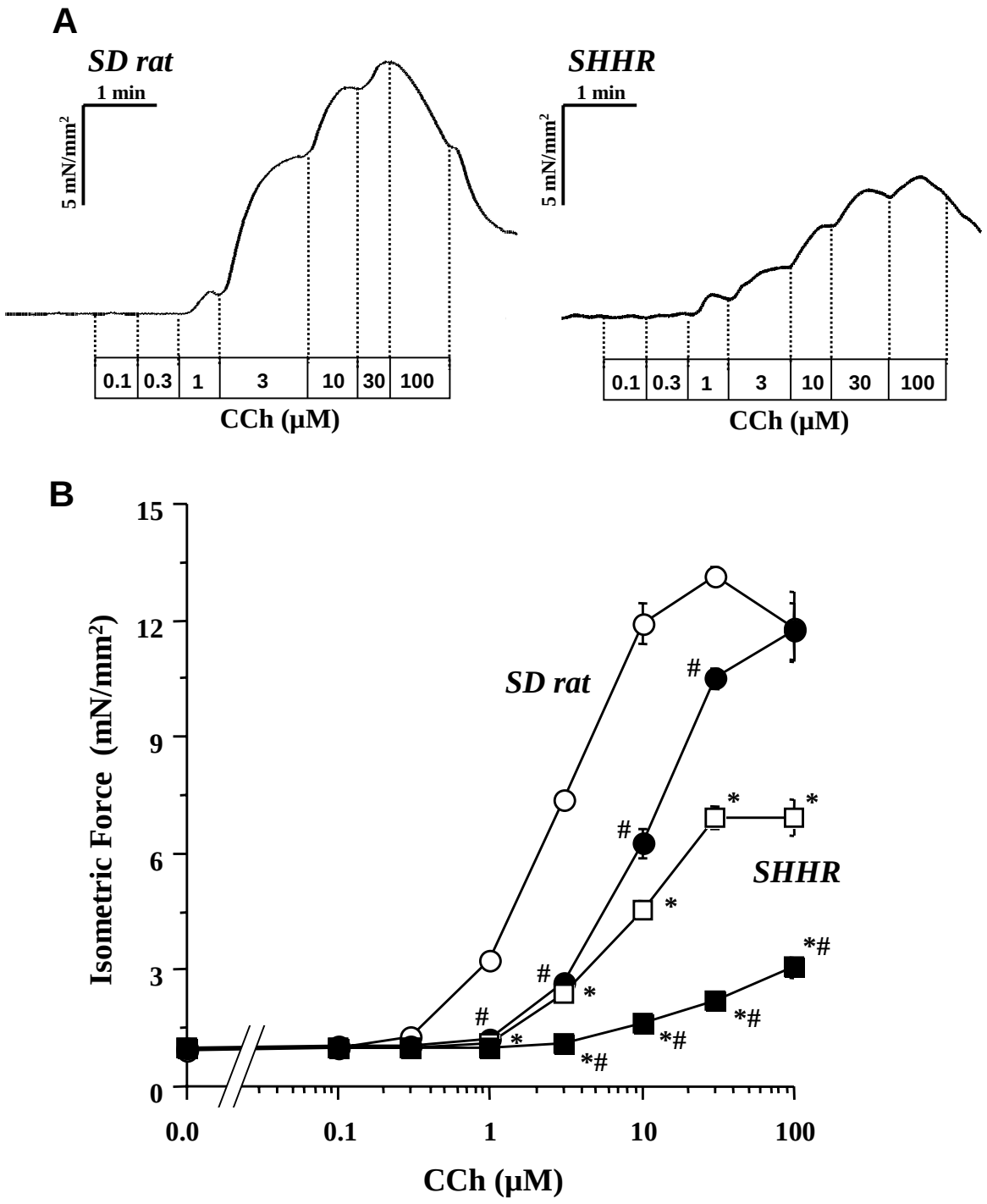

Fig. 1

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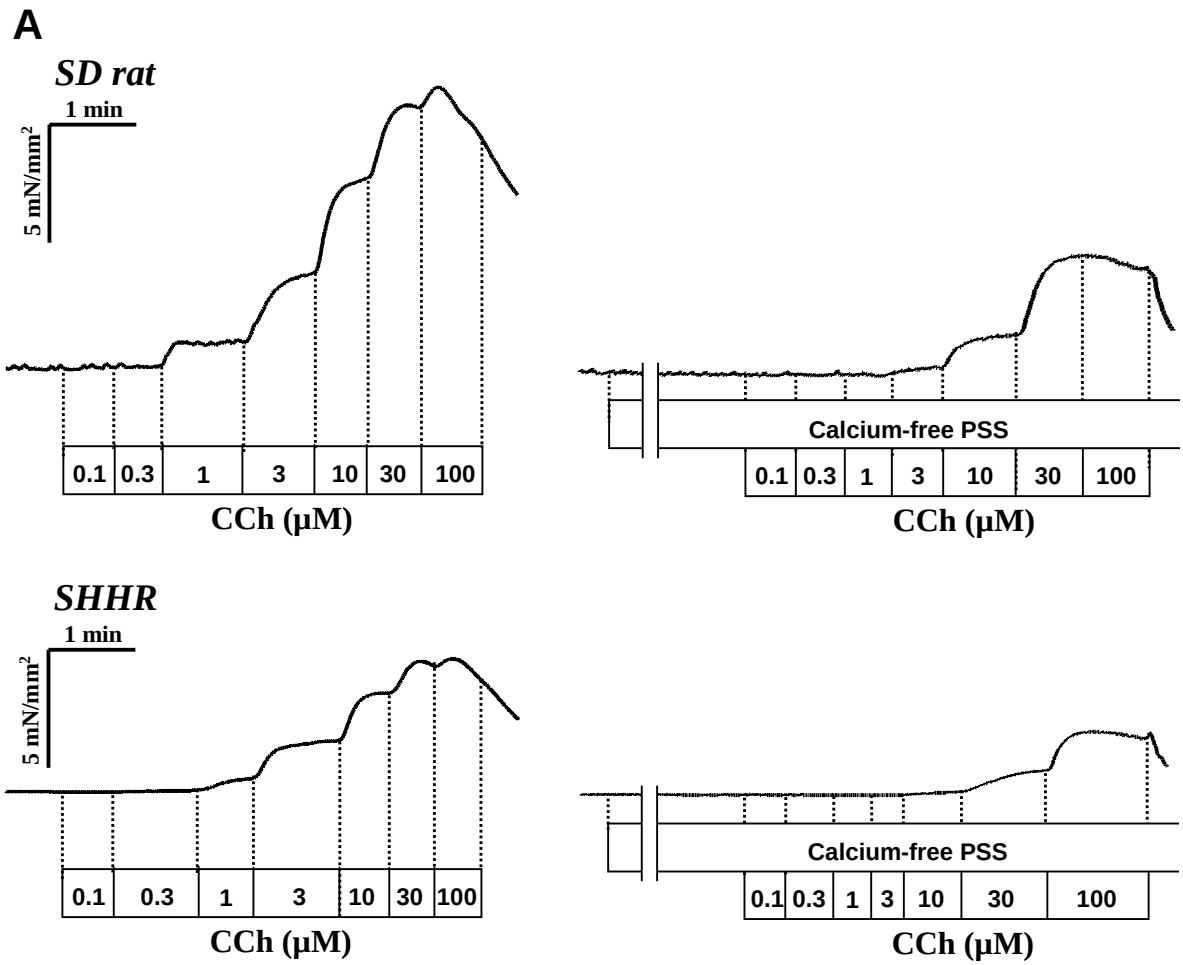

Fig. 2

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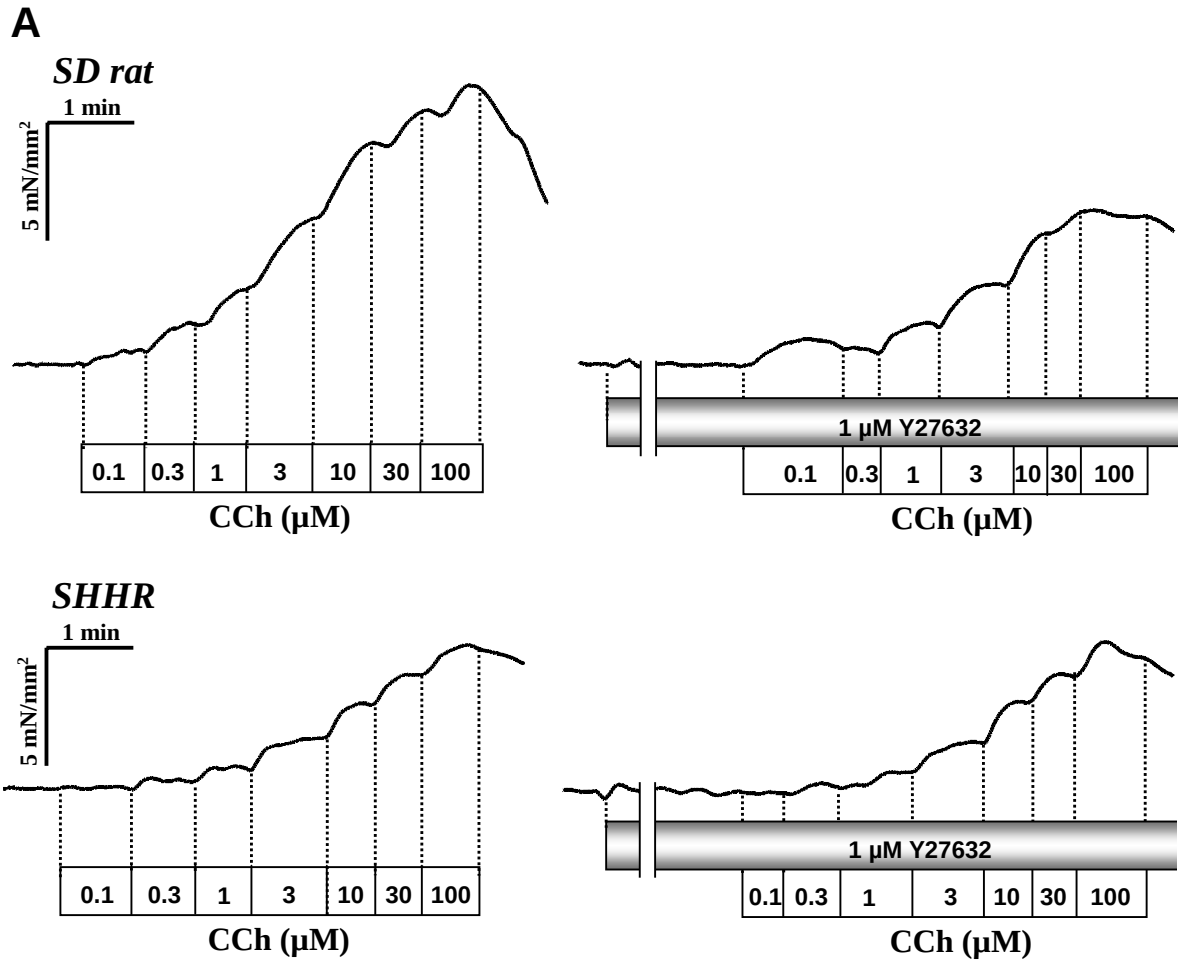

Fig. 3

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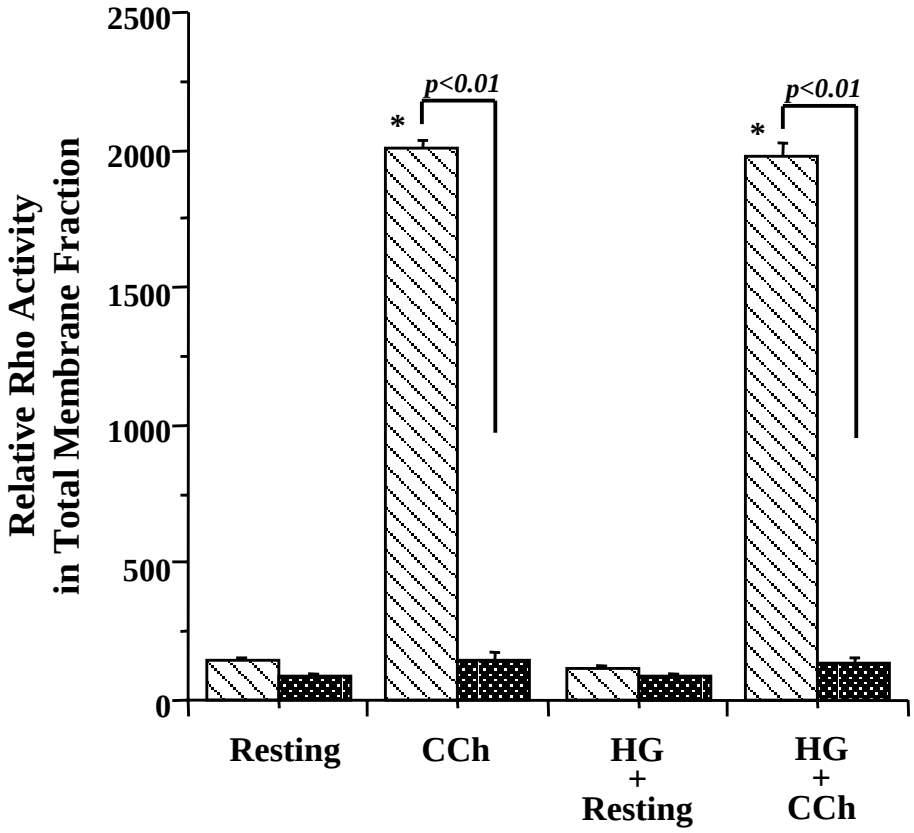

Fig. 4

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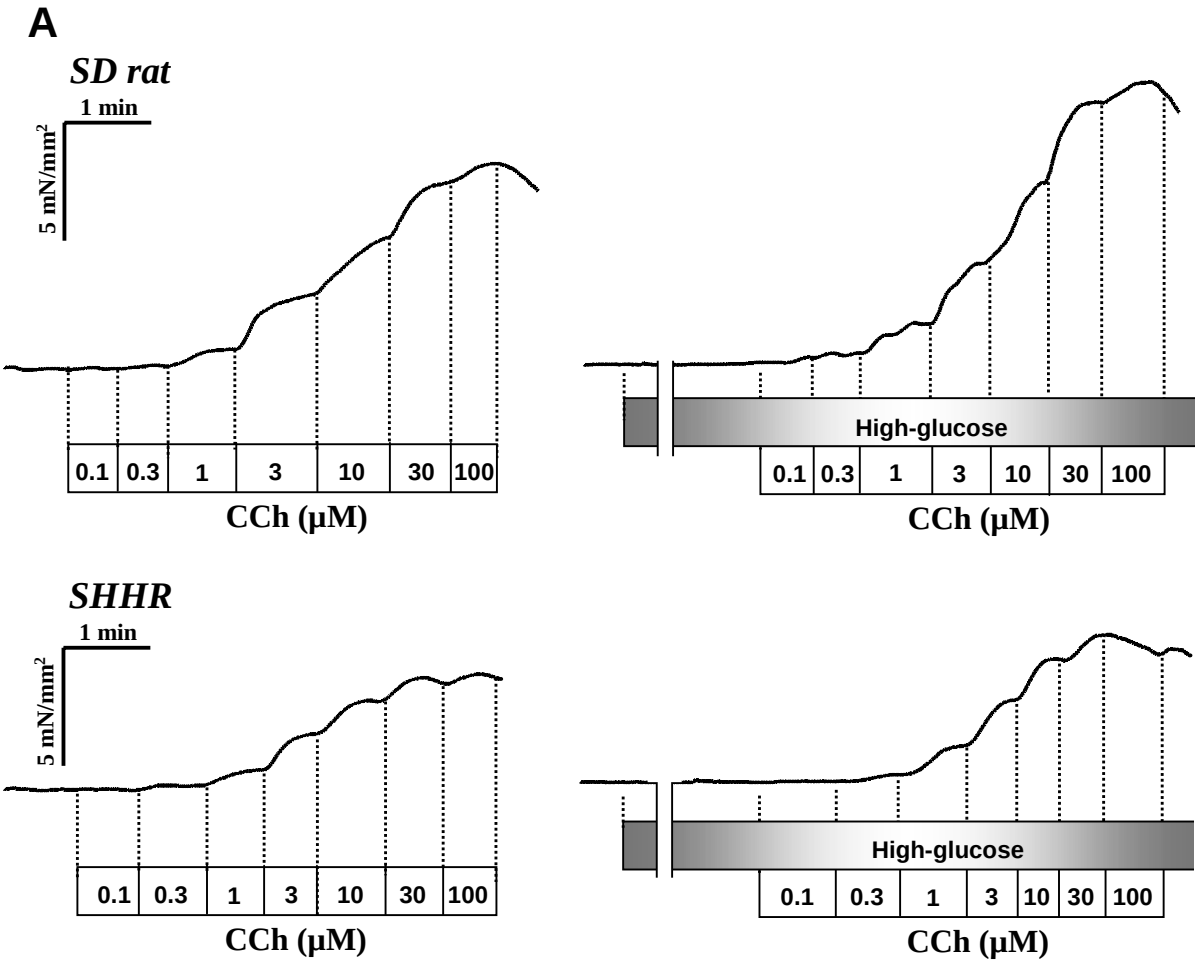

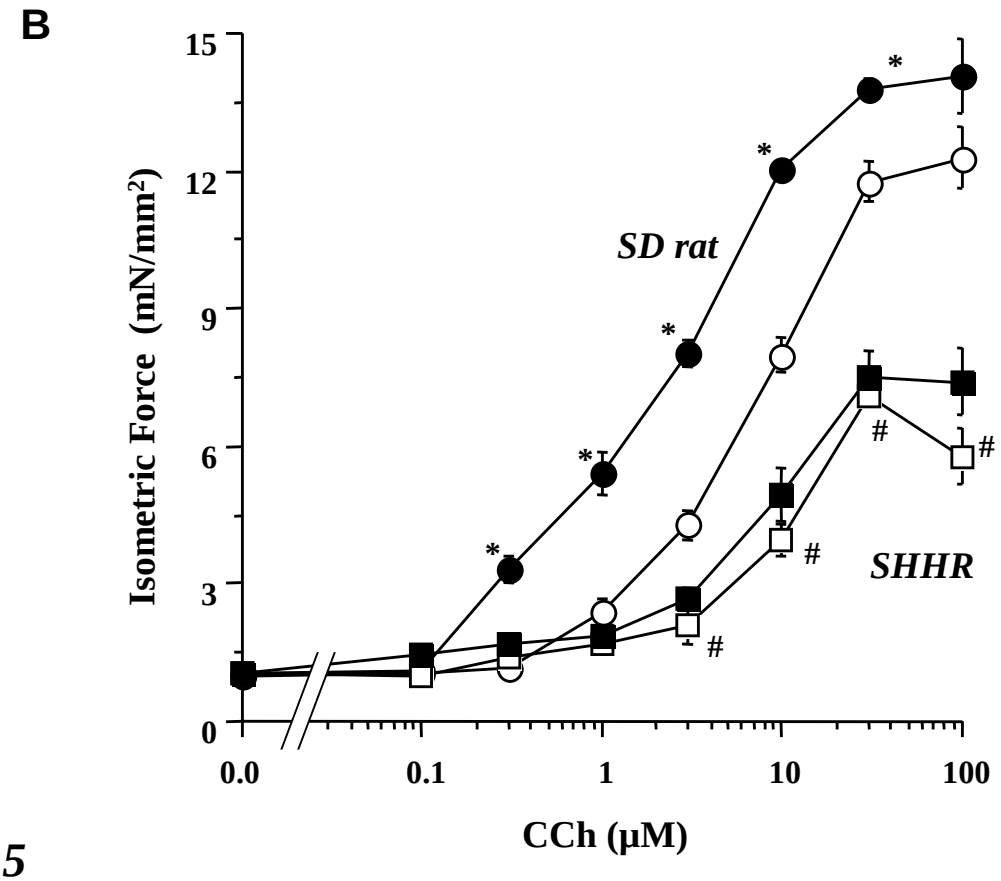

Fig. 5

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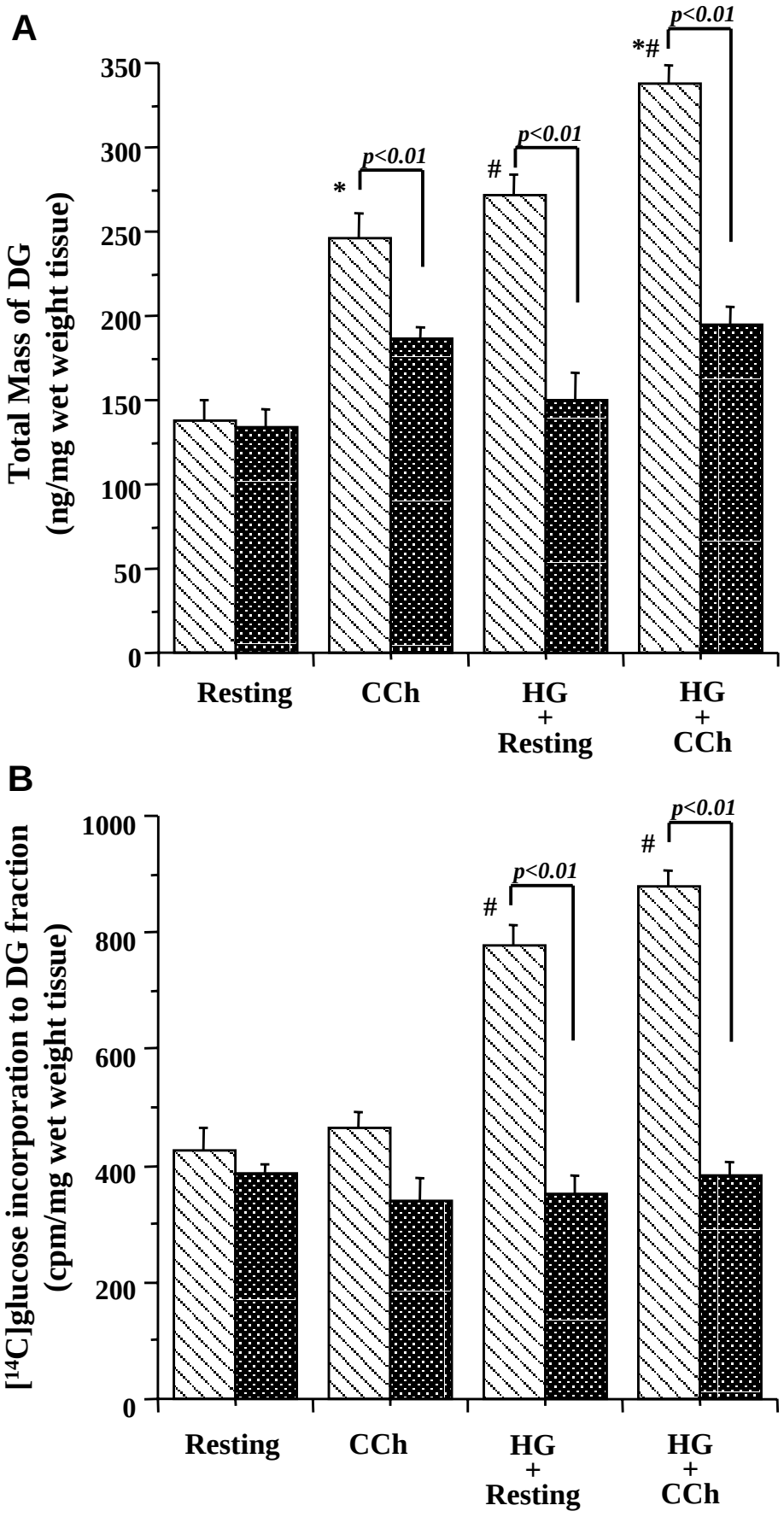

Fig. 6

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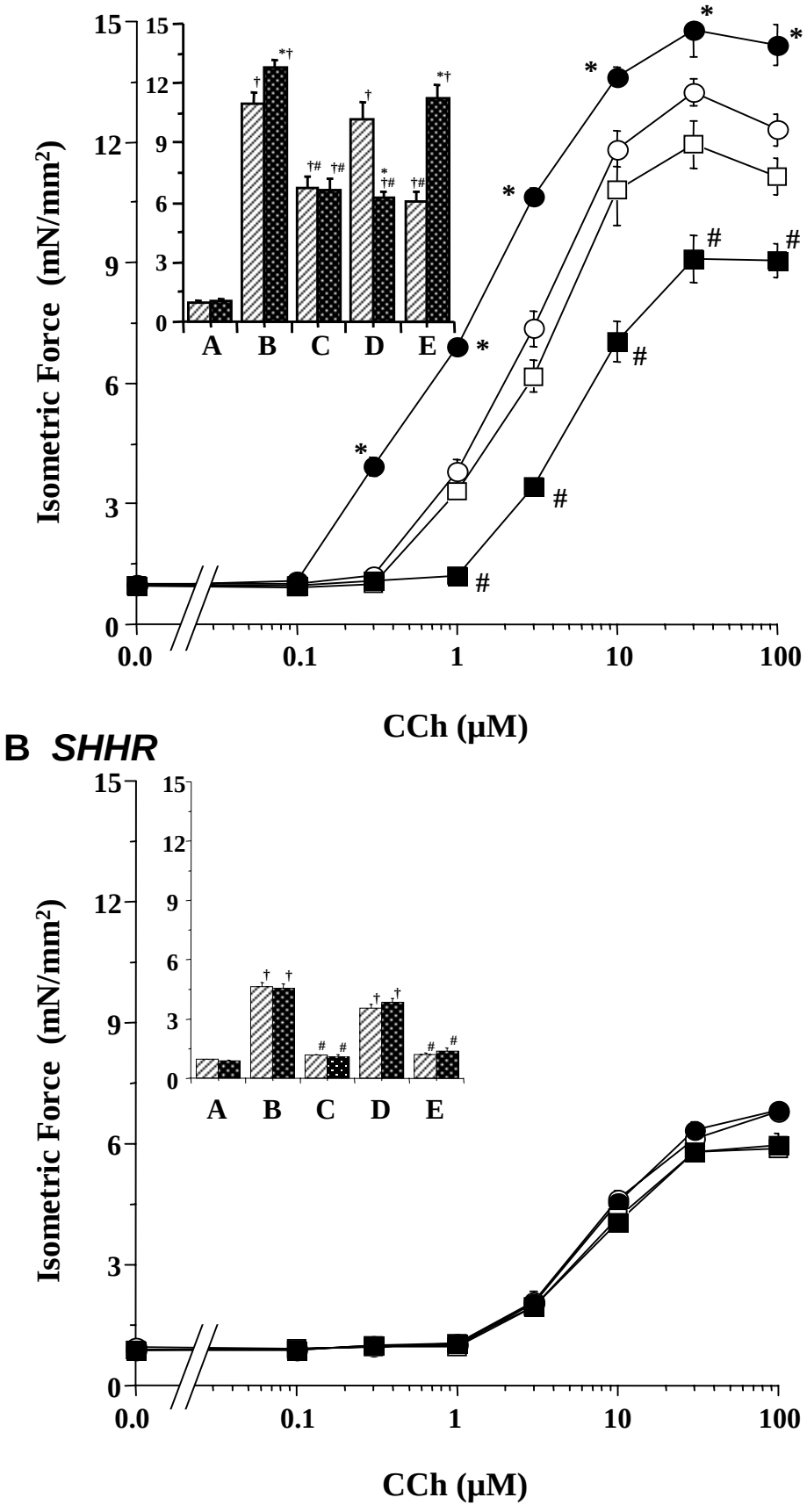

Fig. 7

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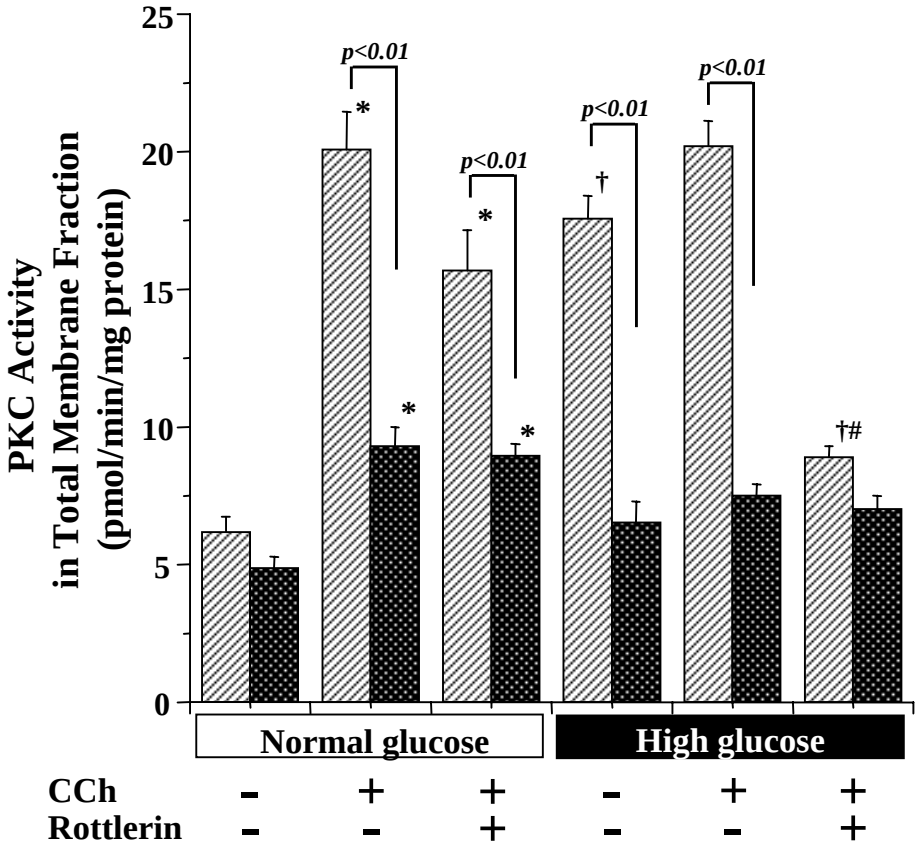

Fig. 8