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# Role of $Na^+/H^+$ exchanger in acetylcholine-mediated pulmonary artery contraction of Spontaneously hypertensive rats

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**Abstract**

Compared to sympathetic nervous system, the role of parasympathetic innervation on tone development, especially under diseased conditions, of the pulmonary artery is relatively unknown. In this study, the contractile effect of acetylcholine and the type(s) of muscarinic (M) receptor involved in the pulmonary artery (1st intralobar branch; endothelium-denuded, under resting tension) of the normotensive Wistar–Kyoto (WKY) and age-matched (male, 22–26 weeks old) Spontaneously hypertensive rats (SHR) were investigated. Cumulative administration of acetylcholine ($\geq 0.1$ μM) caused a concentration-dependent increase in tension (antagonised by *p*-fluoro-hexahydro-sila-difenidol and 4-diphenylacetoxy-*N*-methylpiperidine, both are selective muscarinic $M_3$ receptor antagonists) and the magnitude of maximum contraction (expressed as % of 50 mM $[K^+]_o$-induced contraction) was markedly enhanced in the presence of neostigmine (10 μM, an anti-cholinesterase) (acetylcholine 30 μM, SHR: 72% vs. 35%; WKY: 32% vs. 20%). In SHR only, acetylcholine-elicited contraction was suppressed by 1-[β-[3-(4-Methoxyphenyl)-propoxyl]-4-methoxyphenethyl]-1H-imidazole (SK&F 96365, 1 μM), amiloride (500 μM), ethyl-isopropyl-amiloride (EIPA, 10 μM), 2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea (KB-R 7943, 5 μM), 2,4-dichlorobenzamil (10 μM), and an equal molar substitution of $[Na^+]_o$ ($\leq 30$ mM) with choline or *N*-methyl-D-glucamine. In nominally $[Ca^{2+}]_o$-free, EGTA (0.5 mM)-containing Krebs' solution, acetylcholine ($\geq 3$ μM) only elicited a small contraction. In conclusion, muscarinic $M_3$ receptor activation is responsible for the pulmonary artery contraction induced by acetylcholine, with a greater magnitude observed in SHR. The exaggerated contraction in SHR is probably due to an influx of $[Na^+]_o$ through the $Na^+/H^+$ exchanger and the store-operated channels (SOC) into smooth muscle cells. Elevation of cytosolic $[Na^+]_i$ subsequently leads to an influx of $[Ca^{2+}]_o$ through the reverse mode of the $Na^+/Ca^{2+}$ exchanger seems to play a permissive role in mediating the exaggerated contractile response of acetylcholine recorded in the SHR.


*Keywords:* Acetylcholine; Pulmonary artery; $Na^+/H^+$ exchanger; Wistar–Kyoto, rat (WKY); Spontaneously hypertensive, rat (SHR)

## 1. Introduction

The autonomic nervous system modifies pulmonary blood flow under physiological conditions and may be involved in the pathophysiology of pulmonary vascular diseases (Wood, 1958). However, compared to adrenergic nerves, the functional significance of cholinergic nerves (Dinh Xuan et al., 1989) and the muscarinic receptor subtype(s) involved (McCormack et al., 1988; Choy et al., 2002) in the regulation of the tone of pulmonary circulation is less clear.

Five subtypes (m1 to m5) have been identified using molecular biological techniques, and muscarinic $M_1$ to $M_4$ receptors can be differentiated pharmacologically (Hulme et al., 1990). Muscarinic receptors mediating endothelium-dependent relaxation are muscarinic $M_3$ receptors in pulmonary arteries (McCormack et al., 1988; Choy et al., 2002) but the precise identity of muscarinic receptor subtype(s) responsible for the contractile effects of acetylcholine is relatively unknown. In rabbit, the muscarinic receptors mediating the increase in pulmonary vascular resistance have been suggested to be muscarinic $M_1$-like (El-Kashef and Catravas, 1991), $M_2$-like (Jaiswal et al., 1991) or $M_3$-

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like receptors (Altiere et al., 1994). Both muscarinic $M_1$ and $M_2$ receptors are involved in canine pulmonary vascular beds (El-Kashef et al., 1991), whereas muscarinic $M_3$ receptors are responsible for acetylcholine-mediated contraction in human pulmonary arteries (Norel et al., 1996).

It has been demonstrated that acetylcholinesterase is present in the pulmonary artery (Hebb, 1969; Bradley et al., 1970; Altiere et al., 1994). The presence of acetylcholinesterase creates a non-equilibrium condition that can alter the shape and/or slope of the Schild plot, resulting in erroneous estimates of the p$A_2$ value for an antagonist. In the literature, most studies except two (Altiere et al., 1994, and our group; Choy et al., 2002) did not include any acetylcholinesterase inhibitors in the bathing fluid when characterisation of muscarinic receptors was performed using acetylcholine (McCormack et al., 1988; El-Kashef and Catravas, 1991; Jaiswal et al., 1991; El-Kashef et al., 1991; Norel et al., 1996; Duckles, 1988; Eglen et al., 1990; Hohlfeld et al., 1990; Jaiswal and Malik, 1991; Hoover and Neely, 1997). In our previous study (Choy et al., 2002), we have observed that the in vitro pulmonary vascular effect of carbachol (a non-hydrolyzable analogue of acetylcholine) could not mimic the effect of acetylcholine (with neostigmine present). Hence, the first aim of this study was to determine the muscarinic receptor subtype(s) that mediates the contractile response of the endogenous neurotransmitter acetylcholine (with neostigmine, an anti-cholinesterase) in the pulmonary artery (endothelium-denuded) of the normotensive Wistar–Kyoto (WKY) rats.

On the other hand, numerous studies have demonstrated that under hypertensive conditions there is an enhanced contractile response to various vasoactive substances, e.g. noradrenaline and angiotensin II (Fronhoffs et al., 1999). To the best of our knowledge, there is no report in the literature examining the contractile effect of acetylcholine in the pulmonary circulation especially under hypertensive conditions. Therefore, we employed the Spontaneously hypertensive rat (SHR, an animal model of essential hypertension) (Trippodo and Frohlich, 1981) to evaluate the modulatory effect, if any, of hypertensive state on the response of the pulmonary artery to acetylcholine challenge. It has been reported that the pulmonary circulation of SHR (age $\geq$ 14 weeks) developed morphological changes with an elevated blood pressure and ventricular hypertrophy that resemble to man who has pulmonary hypertension (Janssens et al., 1994; Aharinejad et al., 1996; Camilión de Hurtado et al., 2002).

The role of common salt ($Na^+$) in the aetiology of different forms of hypertension has been documented. A high dietary $Na^+$ has been suggested associated with increases in vascular reactivity to different vasoconstrictors (Ouchi et al., 1988; Egan et al., 1991; Adegunloye and Sofola, 1997; Watts, 1998). So far, most previous studies were only concentrated on the effect of $[Na^+]_o$ elevation (above the steady-state physiological level of $[Na^+]_o$ in plasma) on the vascular reactivity of various blood vessels. However, there is no evidence to show that the plasma concentration of $[Na^+]_o$ in hypertensives is altered. Irrespective of the route of entry, an influx of $[Na^+]_o$ into the cell cytosol resulted in depolarisation and hence a greater contraction is expected. Hence, the third aim of this study was to evaluate the importance of $[Na^+]_o$ (using equal molar substitution of $[Na^+]_o$ with choline and *N*-methyl-D-glucamine) in mediating the enhanced/altered vascular response to acetylcholine of the pulmonary artery of SHR. In addition to $[Na^+]_o$, the role of $[Ca^{2+}]_o$ and protein kinase C activation in mediating acetylcholine-elicited contraction was evaluated.

## 2. Materials and methods

### 2.1. Tissues preparation

The Spontaneously hypertensive rats (SHR) and normotensive Wistar–Kyoto (WKY) rats were bred at The Chinese University of Hong Kong from three original pairs (three SHR and three WKY rats) purchased from the Animal Resources Centre (Australia). Both the WKY and SHR (male) were 22–26 weeks old and weighed 325 ± 13 and 341 ± 18 g, respectively. Rats were housed under a 12:12 h light–dark cycle and were given standard rat chow and water ad libitum before they were killed. Systolic arterial blood pressure was registered using an automatic sphygmomanometer with a tail-cuff method device. The average systolic blood pressure measured was: 132 ± 8 mm Hg for WKY and 253 ± 7 mm Hg for SHR ($P < 0.05$, $n = 5$). Rats were sacrificed by cervical dislocation and the pulmonary artery (1st intralobar branch, O.D. ~ 800 μm) isolated and excess fat and connective tissue removed under the dissecting microscope. Only one pulmonary artery ring was obtained from each rat and was cut ~ 1 mm in length. Tissues were mounted in a 5-ml vertical organ-bath containing Krebs' solution (gassed with 95% $O_2$–5% $CO_2$; pH 7.4, 37 ± 1 °C; indomethacin 1 μM) of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5 (Choy et al., 2002). Choline and *N*-methyl-D-glucamine were used as $[Na^+]_o$ substitute where needed.

Unlike most studies in which the activity of $Na^+/H^+$ exchanger was determined in bicarbonate-free, HEPES-containing solution (intracellular pH inside the smooth muscle cells increased without physiological $HCO_3^-$ and may alter the vascular reactivity), our experiments were performed in a more physiological condition using the bicarbonate-containing physiological salt solution. It has been shown that the $Na^+/H^+$ exchanger is active even in the physiological bicarbonate-containing buffer (Quinn et al., 1991).

As we are only interested in the contractile effect of acetylcholine on the pulmonary artery vascular smooth muscle, all experiments were performed in endothelium-denuded preparations. Endothelium was carefully removed

by gentle rubbing the intima of the blood vessels with a wire. The absence of the functional endothelium was confirmed, at the beginning of the each experiment, by the failure of acetylcholine (10 μM) in producing relaxation of phenylephrine (1 μM)-precontracted preparation. Moreover, nicotinic receptors are present in the pulmonary artery (unpublished data) and hexamethonium (1 μM, a nicotinic receptor blocker) was included in Krebs' solution for all experiments. The Animal Ethics Research Committee of the Chinese University of Hong Kong approved experiments performed in this study (approval number: 00/001/DG).

### 2.2. Isometric tension determination

Two stainless steel hooks were inserted into the lumen of the isolated blood vessel, one fixed and the other connected to a force–displacement transducer (Grass FT 03). Isometric tension measurement was performed using the MacLab Chart v 3.6 programme. Data were stored in the hard disk of a Macintosh computer for subsequent analysis. The pulmonary artery ring was equilibrated under the optimum resting tension of 10 ± 2 mN (produced maximal responses to contractile agents used), as previously reported (Zhao et al., 1996; Kwan et al., 1999; Choy et al., 2002) in the bath solution for 90 min. During the equilibration period, the preparation was washed with drug-free Krebs' solution every 20 min and the resting tension was readjusted, if necessary, before commencing the experiments.

To normalise the responses in the WKY and SHR, acetylcholine-induced contraction (in control and in the presence of inhibitors/blockers) observed in individual preparation was expressed as % of contraction evoked by 50 mM $[K^+]_o$ recorded in the same tissue (the concentration of $[K^+]_o$ with which the maximum contraction was observed in both strains of rat) (Choy et al., 2002) without adjusting osmolarity of the bathing solution. Results obtained from the pulmonary artery of the WKY rats were compared with SHR. To avoid the possible de-sensitisation of the pulmonary artery preparations to acetylcholine challenge, only two concentration–response curves of acetylcholine were constructed in each preparation. The first curve served as the control with a maximum concentration of 30 μM acetylcholine employed. Individual muscarinic receptor antagonist was evaluated at four to five different concentrations in separate pulmonary artery preparations. Where stated, the concentration of antagonists/blockers used in this study was the effective concentration of individual blocker previously reported in the literature. Antagonist was allowed to equilibrate for at least 30 min with the preparation and present throughout the construction of the second concentration–response curve of acetylcholine. Experiments using light-sensitive compounds were performed in a dimly lit room. Effect of solvent (dimethyl sulphoxide, ≤ 0.01% vol./vol.) was also examined and it was found to have no apparent effect on acetylcholine-induced pulmonary artery contraction of the WKY ($n = 5$) and SHR ($n = 6$).

### 2.3. Chemicals

Physiological salts (GR grade) for preparing Krebs' solution were purchased from Merck (Germany). Acetylcholine chloride, neostigmine bromide, hexamethonium bromide, indomethacin, choline chloride, atropine sulphate, amiloride hydrochloride, 5-(*N*-ethyl-isopropyl)-amiloride, choline chloride, *N*-methyl-D-glucamine, caffeine, ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetra-acetic acid (EGTA) and ouabain were purchased from Sigma (St. Louis, MO, USA). Pirenzepine dihydrochloride, methoctramine hydrochloride, *p*-fluoro-hexahydro-sila-difenidol (*p*-FHHSiD), tropicamide, 4-hydroxy-2-butynyl-trimethylammonium-*m*-chlorocarbanilate chloride (McN-A-343), 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and oxotremorine sesquifumarate were obtained from Research Biochemicals (Natick, USA). Green Mamba toxin 3 (MT-3) and tetrodotoxin were purchased from Alomone Labs. (Jerusalem, Israel) and Green Mamba toxin 7 (MT-7) was obtained from Peptide Institute (Osaka, Japan). 2,4-Dichlorobenzamil was obtained from Biomol Research Labs. (Pennsylvania, USA). 2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R 7943) was obtained from Tocris Cookson (Bristol, UK), 1-[β-[3-(4-Methoxyphenyl)-propoxyl]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SK&F 96365) and bisindolylmaleimide I (BIM I) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA).

### 2.4. Statistical analysis

Data are presented as mean ± S.E.M. Log concentration–response curves of acetylcholine were generated and half-maximal responses ($EC_{50}$), and Hill coefficient was estimated using Prism (GraphPad, USA). Dose ratios (DR) were calculated at $EC_{50}$ concentration for each concentration of muscarinic antagonist used. Schild plots were generated by plotting log (DR-1) vs. log concentration of antagonist ([B]), using the equation log (DR-1) = log [B] + $pA_2$. Linear regression analysis of the Schild plot was performed for calculating the slopes and intercepts. The $pA_2$ value, which is defined as negative logarithm of the antagonist concentration that produces a twofold rightward shift in the agonist concentration–response curve (Arunlakshana and Schild, 1959), was derived from the *x*-intercepts of the Schild plots for each antagonist.

Statistical analysis was performed using Student's *t*-tests (paired and unpaired) and analysis of variance, where appropriate. $P < 0.05$ was considered significant. Where stated, *n* values ($n = 5–9$) represented the number of rats used in each set of experiment in this study, except in experiments using Green Mamba toxin 3 (MT-3) and Green Mamba toxin 7 (MT-7), $n = 4$ for each concentration of toxin tested.

## 3. Results

### 3.1. Modulatory effect of neostigmine

Cumulative application of acetylcholine caused a concentration-dependent vasoconstriction of endothelium-denuded pulmonary artery of the WKY and SHR (under resting tension) with a maximum contraction at 30 μM acetylcholine of 19.65 ± 6.33% ($n = 6$) and 34.62 ± 5.52% ($n = 9$) (expressed as % of 50 mM $[K^+]_o$-induced contraction), respectively ($P < 0.001$) (Fig. 1). Administration of neostigmine (10 μM, an anti-cholinesterase to prevent the metabolism of acetylcholine) resulted in a significant upward shift of acetylcholine concentration–response curve. The maximum contraction of acetylcholine (occurred at 30 μM) was significantly enhanced (WKY: 32.37 ± 4.22%; SHR: 71.63 ± 6.75%) ($P < 0.001$ vs. the respective controls) ($n = 6–9$) (Fig. 1). There was no significant difference of the estimated $EC_{50}$ values between the WKY and SHR, irrespective of the inclusion of neostigmine (without neostigmine, $EC_{50}$: WKY, 1.02 ± 0.55 μM; SHR, 0.89 ± 0.22 μM; with 10 μM neostigmine, $EC_{50}$: WKY:

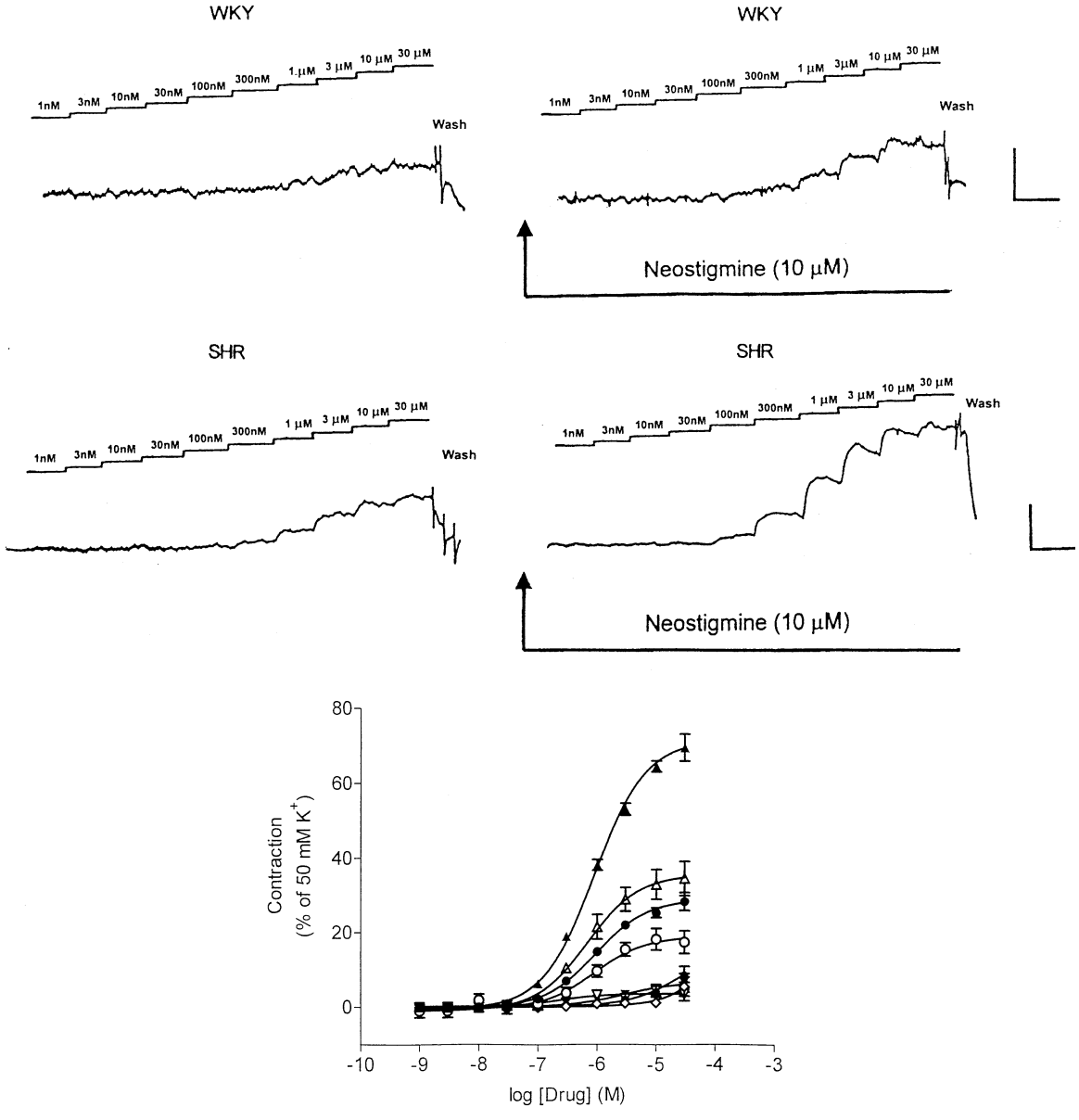


Fig. 1. Top: Representative traces illustrating the effect of neostigmine (10 μM, an anti-cholinesterase) on tension development in response to acetylcholine challenge of the pulmonary artery (endothelium-denuded, under resting tension) of the normotensive Wistar–Kyoto (WKY) and Spontaneously hypertensive rats (SHR). Calibration bars: 4 mN and 5 min. Bottom: Cumulative concentration–response curves of acetylcholine on the pulmonary artery (endothelium-denuded, under resting tension) of the normotensive WKY (○, ●) and SHR (△, ▲) with (closed symbols) and without (open symbols) the presence of 10 μM neostigmine. For comparison, effect of McN-A-343 (◇, ◆) and oxotremorine (▽, ▼) were also examined in the normotensive WKY (open symbols) and SHR (closed symbols) (without neostigmine). Results are expressed as mean ± S.E.M. ($n = 5–9$).

1.33 ± 0.82 μM; SHR: 1.09 ± 0.91 μM) ($n$ = 6–9) ($P$> 0.05). These results demonstrate the significance of the inclusion of an anti-cholinesterase in acetylcholine experiments and therefore 10 μM neostigmine was present at all time in the subsequent experiments. Moreover, Hill coefficient of 1.13 and 0.98 were estimated in SHR and the WKY rats, respectively ($P$>0.05).

### 3.2. Muscarinic receptor characterisation

Muscarinic receptor characterisation experiments were performed as previously described using a range of pharmacological tools (Choy et al., 2002). Atropine (a non-selective muscarinic receptor antagonist) (1, 10, 30 and 100 nM) caused a parallel rightward shift of the concentration–response curve with no apparent change in the magnitude of maximum contraction of acetylcholine. p$A_2$ values of 9.48 and 9.52 in the WKY and SHR ($n$ = 6 for each concentration of atropine), respectively, were estimated with no significant difference between these values ($P$>0.05) (Table 1). With pirenzepine (a muscarinic $M_1$ receptor antagonist) (0.1, 0.3, 1, 3 and 10 μM) ($n$ = 6 for each concentration of pirenzepine), a parallel rightward shift with no apparent change in the maximum response of acetylcholine-induced pulmonary artery contraction was recorded in both strains of rat. p$A_2$ values of 7.06 and 7.13 were estimated in the WKY and SHR ($P$>0.05), respectively (Table 1). Unlike pirenzepine, Green Mamba toxin-7 (MT-7) (a highly selective muscarinic $M_1$ receptor antagonist) (0.3, 1, 3, 10 and 30 nM) ($n$ = 4 for each concentration) did not modify acetylcholine-induced pulmonary artery contraction of the WKY and SHR. In contrast to acetylcholine, 4-hydroxy-2-butynyl-trimethylammonium-*m*-chlorocarbanilate (McN-A-343, a muscarinic $M_1$ receptor agonist) (1 nM to 10 μM) had a minimal effect on the pulmonary artery of the WKY and SHR and only a small contraction was recorded at 30 μM (WKY: ~ 5%; SHR: ~ 8%) ($n$ = 5) (Fig. 1).

The presence of *p*-fluoro-hexahydro-sila-difenidol (*p*-FHHSiD, a selective muscarinic $M_3$ receptor antagonist) (0.03, 0.1, 0.3 and 1 μM) (p$A_2$: WKY, 8.11; SHR, 8.15) ($P$>0.05) ($n$ = 6 for each concentration of *p*-FHHSiD), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP, a selective muscarinic $M_3$ receptor antagonist) (0.001, 0.003, 0.01, 0.03 and 0.1 μM) (p$A_2$: WKY, 8.91; SHR, 9.02) ($P$>0.05) ($n$ = 5 for each concentration of 4-DAMP) and tropicamide (a muscarinic $M_4$ receptor antagonist) (0.1, 0.3, 1, 3 and 10 μM) (p$A_2$: WKY, 7.15; SHR, 7.14) ($P$>0.05) ($n$ = 6 for each concentration of tropicamide) caused a parallel rightward shift with no change in the maximum response of acetylcholine-elicited pulmonary artery contraction (Table 1). Unlike tropicamide, Green Mamba toxin-3 (MT-3, a highly selective muscarinic $M_4$ receptor antagonist) (0.3, 1, 3, 10 and 30 nM) ($n$ = 4 for each concentration of MT-3) did not modulate the contractile response of acetylcholine in the WKY and SHR. With all conventional muscarinic receptor antagonists examined, only methoctramine (a muscarinic $M_2$ receptor antagonist) (0.001, 0.01, 0.1, 1, 3, 30 and 100 μM) ($n$ = 5 for each concentration of methoctramine) failed to affect acetylcholine-mediated pulmonary artery contraction of the WKY and SHR. Similar to McN-A-343, oxotremorine (a muscarinic $M_2$ receptor agonist) (1 nM to 10 μM) only had a minimal effect ($n$ = 5) (Fig. 1).

Table 1
Summary of the estimated p$A_2$ values of different muscarinic receptor antagonists against acetylcholine-mediated pulmonary artery (endothelium-denuded) contraction (with of 10 μM neostigmine) of the Wistar–Kyoto (WKY) and Spontaneously hypertensive rats (SHR)

| Antagonist | WKY | | | SHR | | |
|---|---|---|---|---|---|---|
| | p$A_2$ | $r^2$ | Slope of Schild plot | p$A_2$ | $r^2$ | Slope of Schild plot |
| Atropine | 9.48 | 0.98 | 0.94 | 9.52 | 0.96 | 1.10 |
| Pirenzepine | 7.06 | 0.91 | 0.98 | 7.13 | 0.93 | 0.93 |
| Methoctramine | >4 | – | – | >4 | – | – |
| *p*-FHHSiD | 8.11 | 0.96 | 1.10 | 8.15 | 0.98 | 1.06 |
| 4-DAMP | 8.91 | 0.95 | 0.97 | 9.02 | 0.97 | 1.02 |
| Tropicamide | 7.15 | 0.98 | 0.95 | 7.14 | 0.96 | 0.96 |
| MT-3 | >7.5 | – | – | >7.5 | – | – |
| MT-7 | >7.5 | – | – | >7.5 | – | – |

### 3.3. Role(s) of $Ca^{2+}$, $Na^+$ and the ion exchangers ($Na^+/K^+$ ATPase, $Na^+/H^+$ exchanger and $Na^+/Ca^{2+}$ exchanger)

Administration of 5-*N*-ethyl-*N*-isopropyl-amiloride (EIPA) (1 μM, a selective sodium–hydrogen exchanger isoform 1 (NHE-1) inhibitor) had no effect on acetylcholine-elicited pulmonary artery contraction of the WKY and SHR (WKY: 31.71 ± 3.04% vs. 30.82 ± 3.95%; SHR: 70.33 ± 5.11% vs. 73.27 ± 3.25%). Interestingly, EIPA 10 μM only suppressed acetylcholine-induced contraction in SHR ($n$ = 6) (Fig. 2), whereas no apparent effect was recorded in the WKY rats (30 μM acetylcholine, WKY: 31.31 ± 4.13% vs. 28.44 ± 5.02%) ($n$ = 5). A higher concentration of EIPA (30 μM) caused no further inhibition of acetylcholine-mediated contraction in SHR ($n$ = 5) (data not shown). A similar degree of inhibition, compared to EIPA, was observed with amiloride (500 μM, a general NHE blocker) in SHR and no apparent effect was recorded in the WKY rats ($n$ = 5–6). Neither EIPA (1 and 10 μM) nor amiloride (500 μM) altered the basal tension of the pulmonary artery (data not shown).

In order to demonstrate the importance of $[Na^+]_o$, $[Na^+]_o$ present in Krebs' solution was partially substituted with an equal molar concentration of choline (10, 30 and 60 mM). Interestingly, choline with concentrations of ≤ 30 mM only attenuated acetylcholine-induced response observed in SHR ($n$ = 6 for each concentration of choline) and no apparent effect was recorded in the WKY rats ($n$ = 5) (Fig. 3). A similar suppressive effect by *N*-methyl-D-glucamine substitution (10 and 30 mM) on acetylcholine-induced contraction in SHR was observed ($n$ = 6) (data not shown). However, a further substitution of $[Na^+]_o$ with choline (60 mM) sup-

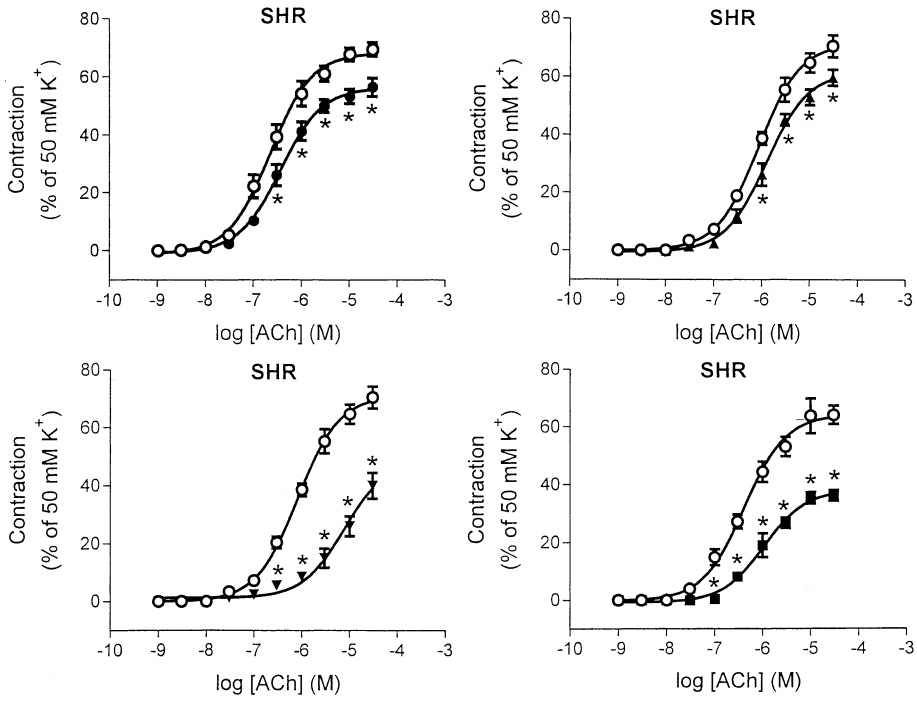


Fig. 2. Cumulative concentration–response curves of acetylcholine (control, ○) (with neostigmine 10 μM) on the pulmonary artery (endothelium-denuded, under resting tension) of Spontaneously hypertensive rats (SHR) in the presence of EIPA (10 μM, ●), SK and F 96365 (1 μM, ▲), a combination of EIPA (10 μM) plus SK and F 96365 (1 μM) (▼), and KB-R 7943 (5 μM, ■). Results are expressed as mean ± S.E.M. ($n = 5–6$), *$P < 0.05$ compared to control.

pressed acetylcholine-mediated response in both the WKY and SHR ($n = 7$) (Fig. 3). Ouabain (0.1, 1 and 10 μM) ($n = 5–6$) and tetrodotoxin (100 nM) ($n = 5$) were without effect on acetylcholine-mediated pulmonary artery contraction (data not shown).

The presence of 1-[β-[3-(4-Methoxyphenyl)-propoxyl]-4-methoxyphenethyl]-1H-imidazole (SK&F 96365) (1 μM, a putative store-operated channel blocker) significantly reduced acetylcholine-mediated pulmonary artery contraction in SHR but not the WKY rats (30 μM acetylcholine, WKY: 31.31 ± 4.13% vs. 28.72 ± 6.02%; SHR: 73.17 ± 4.04% vs. 56.13 ± 5.21%) ($n = 6$). SK&F 96365 10 μM attenuated acetylcholine-elicited contraction with a greater inhibition in SHR, compared to the WKY rats (30 μM acetylcholine, WKY: 20.33 ± 5.11%; SHR: 33.22 ± 3.69%) ($n = 5$). SK&F 96365 (1 and 10 μM) did not modulate the basal tension of the pulmonary artery.

In SHR, a combination of SK&F 96365 (1 μM) plus EIPA (10 μM) inhibited acetylcholine response ($n = 5–6$) (Fig. 2). The degree of inhibition was slightly greater, but non-significantly different, compared to the summation of the degree of suppression recorded when these drugs were applied alone (Fig. 2).

Administration of 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea (KB-R 7943, 5 μM) (a selective inhibitor of the reverse mode of the $Na^+/Ca^{2+}$ exchanger (NCX)) ($n = 6$) (Fig. 2) and 2,4-dichlorobenzamil (10 μM, a NCX blocker) ($n = 5$) markedly inhibited acetylcholine-elicited

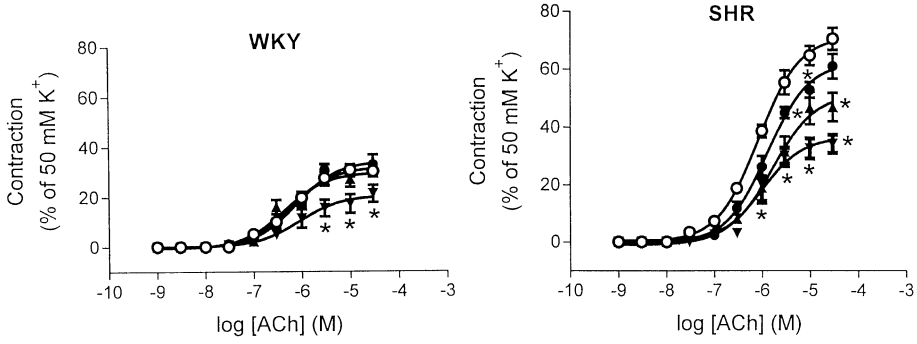


Fig. 3. Cumulative concentration–response curves of acetylcholine (control, ○) (with neostigmine 10 μM) on the pulmonary artery (endothelium-denuded, under resting tension) of the normotensive Wistar–Kyoto (WKY) and Spontaneously hypertensive rats (SHR), with an equal molar substitution of $[Na^+]_o$ with $[choline^+]_o$ in Krebs' solution (10 mM, ●; 30 mM, ▲; 60 mM, ▼). Results are expressed as mean ± S.E.M. ($n = 5–6$), *$P < 0.05$ compared to control.

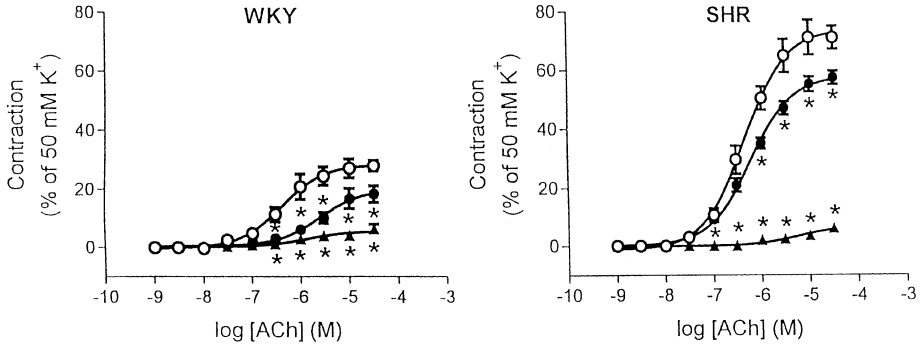


Fig. 4. Cumulative concentration–response curves of acetylcholine (control, ○) (with neostigmine 10 μM) on the pulmonary artery (endothelium-denuded, under resting tension) of the normotensive Wistar–Kyoto (WKY) and Spontaneously hypertensive rats (SHR) after caffeine treatment (10 mM, ● with 2.5 mM $[Ca^{2+}]_o$ Krebs' solution), and in $[Ca^{2+}]_o$-free, EGTA (0.5 mM)-containing Krebs' solution (▲). Results are expressed as mean ± S.E.M. ($n = 5–6$), *$P < 0.05$ compared to control.

contraction in SHR only. No apparent effect was observed in the WKY rat ($n = 5$) (data not shown). The estimated degree of inhibition produced by KB-R 7943 was fairly similar to that observed when a combination of SK&F 96365 (1 μM) plus EIPA (10 μM) were used (Fig. 2). Neither KB-R 7943 (5 μM) nor 2,4-dichlorobenzamil (10 μM) altered the basal tension of the pulmonary artery of both strains of rat (data not shown).

Caffeine (10 mM) caused a transient increase in tension (magnitude of contraction, WKY: 16.42 ± 7.15%; SHR: 25.22 ± 8.09%) ($n = 5–6$) which returned to baseline level after ~ 10 min. Subsequent application of acetylcholine resulted in an attenuated contraction in both strains of rat (Fig. 4). In nominally $[Ca^{2+}]_o$-free, EGTA (0.5 mM)-containing solution, acetylcholine failed to elicit contraction except at the highest concentration (30 μM) ($n = 6$) (Fig. 4).

The role of protein kinase C activation in acetylcholine-mediated pulmonary artery contraction was determined using bisindolylmaleimide I (a highly specific protein kinase C inhibitor). Application of bisindolylmaleimide I (200 nM) only depressed acetylcholine-mediated pulmonary artery contraction in SHR, whereas no apparent effect was recorded in the WKY rat (30 μM acetylcholine, WKY: 30.23 ± 5.11% vs. 32.17 ± 6.02%; SHR: 74.77 ± 3.45% vs. 53.29 ± 7.04%) ($n = 5–6$). However, a higher concentration of bisindolylmaleimide I (500 nM) suppressed the contraction observed in both strains of rat (WKY: 18.66 ± 3.22%; SHR: 38.53 ± 5.51%) ($n = 5$). On its own, bisindolylmaleimide I (200 and 500 nM) had no effect on the resting tension of the pulmonary artery of the WKY and SHR (data not shown).

## 4. Discussion

In contrast to the huge number of studies in examining acetylcholine-mediated endothelium-dependent relaxation, the effort spent in evaluating the contractile effect of acetylcholine is relatively sparse. It may be because, in most cases, the magnitude of acetylcholine-evoked contraction recorded in different vascular beds was relative small (left panel, Fig. 1) and regarded as physiologically non-significant. This assumption may be incorrect as it has been shown that acetylcholinesterase (a highly efficient enzyme responsible for the catabolism of endogenous acetylcholine) is present in different vasculatures including pulmonary circulation (Bradley et al., 1970; Altiere et al., 1994). Hence, studies using acetylcholine as a muscarinic receptor agonist, without the inclusion of anti-cholinesterase, will obviously result in a significantly reduced contractile response (Fig. 1), as the concentration of acetylcholine at the receptor site is lower than anticipated. In this study, the presence of neostigmine (10 μM, an anti-cholinesterase) (Wilson, 1966) enhanced acetylcholine-elicited pulmonary artery (endothelium-denuded, under resting tension) contraction of the normotensive Wistar–Kyoto (WKY) and Spontaneously hypertensive rats (SHR). A marked upward (an increase in maximum contraction) shift of the concentration–response curve of acetylcholine, as observed in human isolated pulmonary artery (Walch et al., 1997), was recorded in both strains of rat.

Using a range of muscarinic receptor antagonists (in the presence of neostigmine), similar $pA_2$ values were estimated with individual receptor antagonist irrespective of the strain of rat used. The highest $pA_2$ values were observed with atropine (a non-selective muscarinic receptor antagonist, ~ 9.5), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP, a selective muscarinic $M_3$ receptor antagonist, ~ 9) and *p*-fluoro-hexahydro-sila-difenidol (*p*-FHHSiD, a selective muscarinic $M_3$ receptor antagonist, ~ 8.1) that are similar to the reported $pA_2$ values of individual antagonist interacting with muscarinic $M_3$ receptor (Buckley et al., 1989). Our findings are consistent with observation reported by Phillips et al. (1997) in which muscarinic m3 and m5 receptors mRNA are found, whereas muscarinic m2, m4 and m1 receptor mRNA expression is only weakly detected/not detected in the pulmonary artery of the WKY rats. In our pharmacological study using 4-DAMP and *p*-FHHSiD, the affinities calculated at least for the moderately muscarinic $M_3/M_5$ recep-

tor discriminating antagonists, 4-DAMP and *p*-FHHSiD, in pulmonary artery taken from the WKY and SHR ($pA_2 \approx$ 9.0 and 8.1, respectively) better fit to values at muscarinic $M_3$ receptors ($pK_i$ of 9.1 and 7.7, respectively), similar to human pulmonary artery (Norel et al., 1996), than at muscarinic $M_5$ receptors ($pK_i$ of 8.3 and 6.9, respectively) (Eglen et al., 2001). However, a lack of selective receptor agonists and antagonists for muscarinic m5 receptors has obviously obstructed the evaluation of the possible role of this receptor type on acetylcholine-induced pulmonary artery contraction of the WKY and SHR even though m5 receptor mRNA is expressed in the pulmonary artery (Phillips et al., 1997).

The magnitude of acetylcholine-mediated maximum contraction (with neostigmine) was greater ( ~ 2 folds) in SHR compared to the WKY rats. An enhanced contraction in response to certain contractile agents, e.g. 5-hydroxytryptamine and noradrenaline has also been reported in different preparations obtained from SHR (Fronhoffs et al., 1999). Perhaps, in SHR, there is an increased expression of muscarinic $M_3$ receptors or, unlike the WKY rats, the receptors are coupled to multiple signal transduction pathways, as suggested in the blood cells of hypertensive patients (Rosskopf et al., 1993). It has been reported (De Michele et al., 1991) that a higher density of the muscarinic receptor was found in the pulmonary artery of the WKY rat (age: 16 weeks old which is the borderline of the stable stage of hypertension) than in SHR. However, the stoichiometry of muscarinic $M_3$ receptors and acetylcholine (with neostigmine) estimated in the WKY and SHR suggest that there was no change in the number of acetylcholine per muscarinic $M_3$ receptor as revealed by a similar Hill coefficient of 0.98 and 1.13 was estimated in the WKY and SHR, respectively.

Changes in the activity of the ubiquitous $Na^+/H^+$ exchanger (NHE) have been documented in different cell types from patients with essential hypertension and animal models (Orlov et al., 2000). Pulmonary artery smooth muscle cells possess a NHE (the NHE-1 isoform) and NHE-1 plays a significant role in the regulation of intracellular $Na^+$ ($[Na^+]_i$) homeostasis (Quinn et al., 1991; Silverman et al., 1995). Activation of NHE resulted in $1Na^+$ influx in exchange for $1H^+$ efflux across the cell membrane and influx of $[Na^+]_o$ causes membrane depolarisation. In this study, pre-treatment with amiloride (500 μM, a non-selective NHE inhibitor) or 5-*N*-ethyl-*N*-isopropyl-amiloride (EIPA) (10 μM, a selective NHE-1 blocker) blunted but not abolished acetylcholine-mediated pulmonary artery contraction observed in SHR and no apparent change was recorded in the WKY rats. These novel results suggest, for the first time, the importance of NHE-1 activation (Berk et al., 1989) in mediating the exaggerated acetylcholine-elicited contraction of the pulmonary artery of SHR. However, our results were in contrast to previous reports (McMurty et al., 1979; Janssens et al., 1994; Silverman et al., 1995) in which the NHE activation played no important role in the haemodynamic response of the pulmonary artery in SHR (age: ≤ 14 weeks old) (Silverman et al., 1995). The reason(s) for this discrepancy may be related to the age of the animals (Janssens et al., 1994; Aharinejad et al., 1996).

Recently, Arnon et al. (2000) provided convincing evidence that the vasoconstrictor-induced elevation of $[Na^+]_i$ in rat mesenteric arterial myocytes is due to an influx of extracellular $Na^+$ ($[Na^+]_o$) through the store-operated channels (SOC) located in the plasmalemma after the unloading of the intracellular $Ca^{2+}$ stores. Similar to EIPA and amiloride, the inhibitory effect of 1-[β-[3-(4-Methoxyphenyl)-propoxyl]-4-methoxyphenethyl]-1H-imidazole (SK&F 96365) (1 μM, a putative SOC blocker) (Fasolato et al., 1990) in SHR may also suggest that the entry of $[Na^+]_o$ through the SOC (Arnon et al., 2000) also participated in the development of the exaggerated contractile response of acetylcholine. It is interesting to note that the NHE-1 and the SOC seem to contribute independently to influx of $[Na^+]_o$ as a combination of EIPA plus SK&F 96365 resulted in a "fairly similar" degree of inhibition, compared to the summation of the effects of EIPA and SK&F 96365 when applied alone. In addition, a partial replacement of $[Na^+]_o$ ( ≤ 30 mM) in Krebs' solution with an equal molar concentration of either choline or *N*-methyl-D-glucamine resulted in an attenuation of acetylcholine response in SHR but not the WKY rats. Besides, the ouabain-sensitive $Na^+/K^+$ ATPase (Janssens et al., 1993) and $Na^+$ channels played no apparent role in the exaggerated contraction of acetylcholine observed in SHR as ouabain and tetrodotoxin failed to modify acetylcholine-induced contraction. Taken together, these results strongly suggest the importance of $[Na^+]_o$ and its entry, upon the muscarinic $M_3$ receptor activation, probably through the SOC and NHE-1 for the development of the exaggerated acetylcholine-elicited contraction in SHR. The coupling between muscarinic $M_3$ receptor and NHE-1 warrants further investigation.

In addition to NHE, other ion exchanger such as $Na^+/Ca^{2+}$ exchanger (NCX) also exists in the plasma membrane of heart (reviewed by Philipson and Nicoll, 1993) and pulmonary artery smooth muscle cells (Sprague–Dawley rat) (Wang et al., 2000). Under steady-state condition, NCX exchanges $3Na^+$ (influx) for $1Ca^{2+}$ (efflux) (the forward mode) in order to maintain the cytosolic $[Ca^{2+}]_i$ at low level. Under certain diseased conditions that there is a cytosolic $[Na^+]_i$ overload, the NCX can operate in a reverse mode manner, i.e. $3Na^+$ efflux for $1Ca^{2+}$ influx in order to prevent $[Na^+]_i$ accumulation. The entry of $[Ca^{2+}]_o$ can trigger the release of $Ca^{2+}$ from intracellular organelle such as sarco/endoplasmic reticulum.

In this study, 2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]-isothiourea (KB-R 7953) (5 μM, a selective inhibitor of the reverse mode of the NCX) (Iwamoto et al., 1996) and 2,4-dichlorobenzamil (a relatively selective inhibitor of the NCX) (Lipp and Pott, 1988) suppressed acetylcholine-

induced pulmonary artery contraction in SHR but not the WKY rat. These results suggest that the NCX (operates in the reverse mode fashion) is largely contributed to the exaggerated contraction by acetylcholine in SHR. Besides, the estimated degree of inhibition by KB-R 7953 was "fairly similar" to that recorded with a combination of EIPA and SK&F 96365. It is tempting to speculate that the process of $[Na^+]_o$ influx (through NHE-1 and SOC) is coupled to the influx of $[Ca^{2+}]_o$ (through the reverse mode of the NCX, due to the elevated $[Na^+]_i$) in SHR.

It is important to point out that a full activation of the reverse mode of the NCX occurred when $[Na^+]_o$ was totally replaced with other ion substitutes (reviewed by Philipson and Nicoll, 1993). The increased in $[Ca^{2+}]_i$ leads to an enhanced responsiveness of tissues to agonists. In rat aortic smooth muscle cell line A7r5 (Gillespie et al., 1992), it has been suggested that a complete removal of $[Na^+]_o$ resulted in the generation of a small reverse driving force for the NCX which is too small to elicit an appreciable influx of $[Ca^{2+}]_o$. In this study, we have only performed a partial substitution of $[Na^+]_o$ with choline and *N*-methyl-D-glucamine, and a suppression of acetylcholine-induced contraction was recorded in SHR. Hence, a minimal contribution of the enhanced influx of $[Ca^{2+}]_o$ due to the decrease in $[Na^+]_o$ is expected. On the other hand, $[Na^+]_o$ influx through the NHE-1 and SOC seems to provide the required amount of $[Na^+]_i$ for the operation of the NCX in a reverse mode manner.

It has been proposed (Reuter et al., 1973) and recently demonstrated (Moore et al., 1993) that there is a clustering of the NCX to regions closely apposed to portions of the underlying junctional elements of the sarco/endoplasmic reticulum specialised for $Ca^{2+}$ storage/release, suggesting important functional implications of the NCX on $[Ca^{2+}]_i$ homeostasis in vascular smooth muscle. Hence, the activities of the NCX can, in turn, regulate the $[Ca^{2+}]$ content of the sarco/endoplasmic reticulum and therefore determine the magnitude of contraction upon receptor activation. However, a possible contribution to the enhanced contraction of an enhanced mobilisation of $Ca^{2+}$ from the sarco/endoplasmic reticulum of various blood vessels of SHR is controversial (Kanagy et al., 1994; Toyoda et al., 1995; Tostes et al., 1996; Arii et al., 1999).

In our study, caffeine (10 mM) caused a similar magnitude of the transient increase in tension in the WKY and SHR. Perhaps, the exaggerated acetylcholine-mediated pulmonary artery contraction in SHR may not be due to an altered mobilisation of $Ca^{2+}$ released from the caffeine-sensitive store. Subsequent administration of acetylcholine resulted in attenuated contraction in both strains of rat. In nominally $[Ca^{2+}]_o$-free, EGTA-containing solution, acetylcholine failed to produce a significant effect, in both strains of rat, implying that $[Ca^{2+}]_o$ is essential for contraction of the pulmonary artery.

To examine the participation of protein kinase C, effect of bisindolylmaleimide I (a selective protein kinase C inhibitor) (Toullec et al., 1991) was evaluated. Interestingly, bisindolylmaleimide I (200 nM) only attenuated acetylcholine-mediated pulmonary artery contraction in SHR and no apparent effect was recorded in the WKY rats. A higher concentration of bisindolylmaleimide I (500 nM), however, markedly suppressed the acetylcholine response recorded in both strains of rat. We are therefore tempting to speculate that at low concentration, bisindolylmaleimide I only influences the pathway in the pulmonary artery of SHR such as phosphorylation of NHE-1 (Rosskopf et al., 1993; Vallega et al., 1988). The suppressive effect of 500 nM bisindolylmaleimide I observed in both strains of rat may simply represent the essential function of protein kinase C activation in mediating smooth muscle contraction such as phosphorylation of ion channels (He et al., 2000; Kim et al., 2000).

In conclusion, we have demonstrated that the endogenous neurotransmitter acetylcholine elicited an excitatory response in endothelium-denuded pulmonary artery (under resting tension) of the WKY and SHR through the activation of muscarinic $M_3$ (probably muscarininc $M_5$ receptor as well) receptors. Our novel results provided evidence that in SHR, activation of the NHE-1 and the SOC leads to an influx of $[Na^+]_o$ and resulted in an elevation of cytosolic $[Na^+]_i$. To restore the altered $[Na^+]_i$ homeostasis, the NCX operates in a reverse mode manner ($Na^+$ efflux with $Ca^{2+}$ influx) and subsequently leads to an influx of $[Ca^{2+}]_o$. Hence, an increased $Ca^{2+}$ is available for the exaggerated contraction of acetylcholine observed in the pulmonary artery of SHR.

### 4.1. Limitations

We admitted that we have not measured the pulmonary arterial blood pressure and the degree of cardiac hypertrophy of both strains of rat in this study. However, it has been reported that hypertension started to develop in the pulmonary artery of SHR at the age $\geq 14$ weeks and reached a maintained elevation of blood pressure (age $\geq 22$ weeks) (Janssens et al., 1994; Aharinejad et al., 1996). Besides, it has been demonstrated that ventricular hypertrophy developed in 4-month-old SHR (Camilión de Hurtado et al., 2002). On the other hand, it is well known that none of the currently available conventional muscarinic receptor antagonists is specific for a particular subtype of muscarinic receptor. Nonetheless, it is important to point that the $pA_2$ value of various conventional muscarinic receptor antagonists estimated in our pharmacological study (acetylcholine with neostigmine) was "fairly similar" to the reported $pA_2$ value of individual receptor antagonists obtained from radioligand binding studies (Dörje et al., 1991). However, the underlying reason(s) responsible for the ineffectiveness of methoctramine (up to 100 μM tested) (the reported $pK_i$>6.0) in antagonising acetylcholine-evoked pulmonary artery responses (contraction, this study; relaxation, our previous study; Choy et al., 2002) is not known at present.

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