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Tyrosine kinase participates in vasoconstriction through a Ca^{2+}- and myosin light chain phosphorylation-independent pathway

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Abstract This study was undertaken to determine the role of tyrosine kinase on intracellular Ca^{2+} ($[Ca^{2+}]_i$), myosin light chain (MLC) phosphorylation, and contraction caused by norepinephrine (NE) in rat aorta. NE induced a sustained contraction with an increase of $[Ca^{2+}]_i$. On the other hand, NE increased the phosphorylation of the 20 kDa MLC transiently. Pretreatment with genistein and tyrphostin 25, tyrosine kinase inhibitors, significantly inhibited NE-induced contraction, but did not affect the increase of $[Ca^{2+}]_i$ and MLC phosphorylation. These results suggest that tyrosine kinase may regulate the NE-mediated contraction without altering $[Ca^{2+}]_i$ and MLC phosphorylation in rat aorta.

Key words: Genistein; Tyrphostin 25; Norepinephrine; Ca^{2+}; Myosin light chain; Tyrosine kinase

1. Introduction

Calcium- and calmodulin-dependent phosphorylation of the 20 kDa myosin light chain (MLC) has been clearly shown to be a major regulatory step in the activation of smooth muscle contraction [1]. However, because stimulation-induced MLC phosphorylation levels are increased only transiently while force is maintained, additional regulatory pathways have been proposed to account for the maintenance of muscular force [2–4].

Previous studies using guinea-pig taenia coli, rat aorta, and pulmonary artery suggest that there is communication between the tyrosine kinase pathway and the MLC kinase pathway [5,6], which may influence smooth muscle contraction. Indeed, involvement of tyrosine kinase was suggested recently as a possible mechanism for agonist-induced vascular smooth muscle contraction [7–11]. The proposed mechanism involves the phosphorylation of protein on tyrosine residues by tyrosine kinase, which thereby contributes to signaling processes and leads to muscle contraction.

Tyrosine kinase inhibitors have become widely used in cell biology experiments as probes for tyrosine kinase activity and they are also being considered as potential antiproliferative chemotherapeutic agents [12]. Genistein and other putative tyrosine kinase inhibitors decrease agonist-induced contraction in several types of smooth muscle, but have little effect on intact vessels stimulated by high K^+ or on the Ca^{2+}-induced contractions in β-escin-skinned microvessels [10].

There is evidence that tyrosine kinase participates in the regulation of Ca^{2+} entry associated with agonist-induced contraction in smooth muscle cells [13]. It has been reported that genistein attenuates the agonist-induced increase in cytosolic Ca^{2+} concentration in smooth muscle cells from porcine coronary artery and in A7r5 vascular smooth muscle cells [14,15]. Furthermore, some tyrosine kinase inhibitors are also reported to decrease calcium channel currents [16]. The pathway leading to Ca^{2+} entry probably involves tyrosine kinase and protein kinase C [17]. Thus, phosphorylation of tyrosine is thought to be linked to transplasmalemmal Ca^{2+} influx and to regulate smooth muscle contraction. Genistein also decreased the Ca^{2+} sensitivity of the force generated in agonist-stimulated, α-toxin-skinned smooth muscle [18], as well as in small mesenteric resistance arteries of the rat aorta [17]. However, in the intact, histamine-stimulated, swine carotid artery, the inhibitory action of genistein on force was secondary to its attenuation of increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), without any concomitant change in the $[Ca^{2+}]_i$ sensitivity of myosin phosphorylation or force [19]. In uterine smooth muscle, extracellular Ca^{2+}-independent contraction was inhibited by genistein [20]. Taken together, these results indicate that the vasodilating mechanism of tyrosine kinase inhibitors on smooth muscle contraction is still not clarified.

We used genistein and tyrphostin 25, putative tyrosine kinase inhibitors, to evaluate the role of tyrosine kinase on the contraction, $[Ca^{2+}]_i$, and MLC phosphorylation caused by the α_1-adrenoceptor agonist, norepinephrine (NE), in rat thoracic aorta.

2. Materials and methods

2.1. Materials

The following drugs were used in the present study. The monoclonal antibody (mouse IgM) to myosin (20 kDa light chain) was obtained from Sigma Chemicals (St. Louis, MO, USA). Horseradish peroxidase-conjugated secondary antibody, rainbow protein molecular weight marker, enhanced chemiluminescence (ECL) reagent kit, and ECL X-ray film were purchased from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). Pharmacological reagents

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Abbreviations: NE, norepinephrine; MLC, myosin light chain

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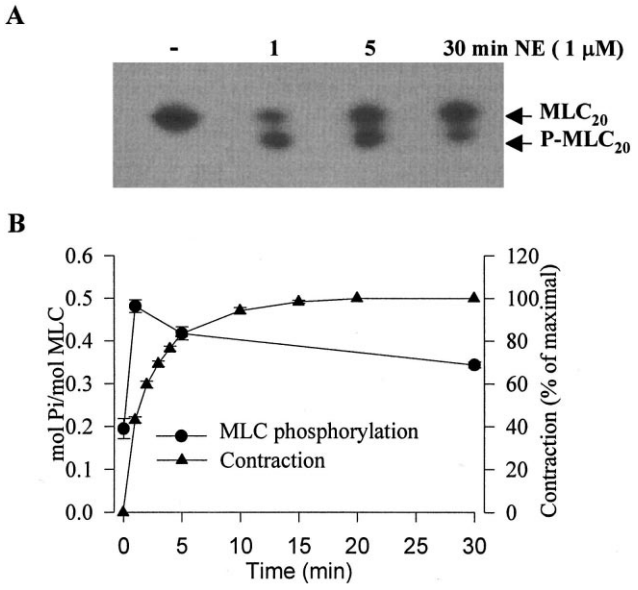

Fig. 1. A: Phosphorylation of MLC measured by immunoblotting after urea–glycerol polyacrylamide gel electrophoresis. B: Time course of 20 kDa MLC phosphorylation ($n=4$), and smooth muscle contraction ($n=5$), induced by 1 μM NE in rat thoracic aorta. Data are expressed as mean ± S.E.M.

such as NE, genistein, tyrphostin 25, and other analytical chemicals were obtained from Sigma Chemicals. The protein assay kit and protein standard (bovine serum albumin) were obtained from Bio-Rad Laboratories (Richmond, CA, USA) and Sigma Chemicals, respectively.

2.2. Tissue preparation and tension measurements

Adult male rats (Sprague–Dawley, 250–350 g) were killed by inhalation of 100% CO_2. Thoracic aortae were isolated carefully and immediately immersed in ice-cold physiological salt solution (PSS) of the following composition (in mM): NaCl 136.9, KCl 5.4, $NaHCO_3$ 23.8, glucose 5.5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, and ethylenediaminetetraacetic acid 0.01. Fat and connective tissues were cleaned off the preparation. Endothelium was removed by gently rubbing the intima with a cotton swab to avoid the endothelium-dependent relaxation effect of acetylcholine. Helical strips of thoracic aorta (2 mm in width and 10–12 mm in length) were dissected.

Aortic strips were placed in Magnus chambers containing 7 ml of PSS (pH 7.4), which was aerated continuously with 95% O_2/5% CO_2. A passive tension of 1.0 g was applied initially to each aortic strip, which was then allowed to equilibrate at 37°C for 60 min. Following equilibration, strip contraction was initiated by the addition of KCl-PSS (65.4 mM KCl; made by substituting 60 mM NaCl in normal PSS with equimolar KCl) in order to provide a reference force for standardizing the experimental values [21]. Changes in muscle tension were recorded isometrically using force displacement transducers connected to a Gould polygraph (Cleveland, OH, USA).

2.3. MLC phosphorylation detection

Muscle strips were frozen rapidly in a slurry of dry ice with 10% (w/v) trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) in acetone. After 1 h, strips were removed to air-dry at room temperature. Dried strips were homogenized in a solution of 10% (w/v) TCA and 10 mM DTT/H_2O, and then the muscle homogenates were centrifuged at 10 000 rpm for 1 min. Supernatant was removed and water-saturated ethyl ether was added and mixed, before further centrifugation. Supernatant was again removed to wash out TCA. Proteins were extracted from centrifuged pellets in urea buffer (20 mM Tris, 23 mM glycine, 8 M urea, 0.04% bromophenol blue, 10 mM DTT; pH 8.6 at 20°C). Protein concentration was determined by the Bradford method (Bio-Rad protein assay kit), employing bovine serum albumin (Sigma) as a standard and subjecting 10 μg protein to urea/glycerol gel electrophoresis. The unphosphorylated and phosphorylated forms of 20 kDa MLC extracted from the frozen muscle strips were separated by urea/glycerol gel electrophoresis according to the protocol of Walsh et al. [22]. Immunoblot analysis was performed using monoclonal anti-myosin (light chain 20K) antibody (1:500). The immunoreactive bands were visualized by ECL reagent, and the images were analyzed by densitometry using Scion imaging software (NIH, Bethesda, MD, USA). All values are reported as mol P_i/mol MLC, corresponding to phosphorylated MLC as a percentage of the total of both the phosphorylated and unphosphorylated MLC.

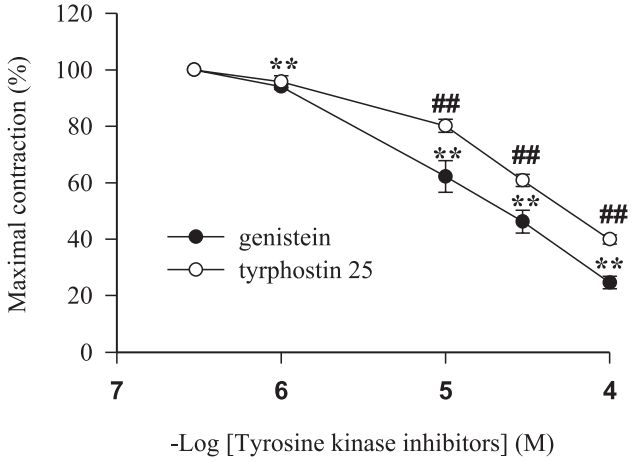

Fig. 2. Effect of tyrosine kinase inhibitors on NE (1 μM)-induced contraction in rat thoracic aorta. Rat thoracic aortic strips were preincubated with 10 μM genistein or 30 μM tyrphostin 25 for 30 min prior to the addition of NE. The contractile responses were evoked by adding NE for 30 min with or without tyrosine kinase inhibitor. Data are expressed as mean ± S.E.M. ($n=5$); ** or ## denotes significant differences from control ($P<0.01$).

2.4. Measurement of $[Ca^{2+}]_i$ in rat thoracic aorta

$[Ca^{2+}]_i$ was measured according to the method described by Kwon et al. [23], using the fluorescent calcium indicator fura-2. Muscle strips were exposed to the acetoxymethyl ester of fura-2 (fura-2/AM; 5 μM) in the presence of 0.02% cremophor EL for 5–6 h at room temperature. The muscle strips were then transferred to the muscle bath that is part of the fluorimeter (CAF-100; Jasco, Tokyo, Japan). The muscle strips were illuminated alternately (48 Hz) with 340 nm and 380 nm light. The light emitted from the muscle strips was collected by a photomultiplier through a 500 nm filter.

2.5. Statistical analysis

All data are expressed as the mean ± S.E.M. The unpaired Student's *t*-test was used to test for significant differences between means, with $P<0.05$ considered to be statistically significant.

3. Results

NE (1 μM) induced sustained contraction of rat thoracic aorta (Fig. 1B). On the other hand, NE (1 μM) increased the phosphorylation of 20 kDa MLC only transiently. After 1 min stimulation with NE (1 μM), the phosphorylation of 20 kDa MLC was increased maximally from 0.195 ± 0.024 to 0.481 ± 0.151 mol P_i/mol MLC ($n=4$). After 5 min stimulation, phosphorylation was 0.417 ± 0.015 mol P_i/mol MLC ($n=4$), and after 30 min stimulation, it was 0.344 ± 0.071 mol P_i/mol MLC ($n=4$).

Therefore, NE induced a transient increase in the phosphorylation of 20 kDa MLC of rat thoracic aorta (Fig. 1A,B). The maximal phosphorylation was observed at 1 min after NE application and it gradually decreased with time. Pretreatment with genistein and tyrphostin 25 (10^{-6}–10^{-4} M) for 30 min reduced the NE-induced contraction in a concentration-dependent manner (Fig. 2). The IC_{50} values of genistein and

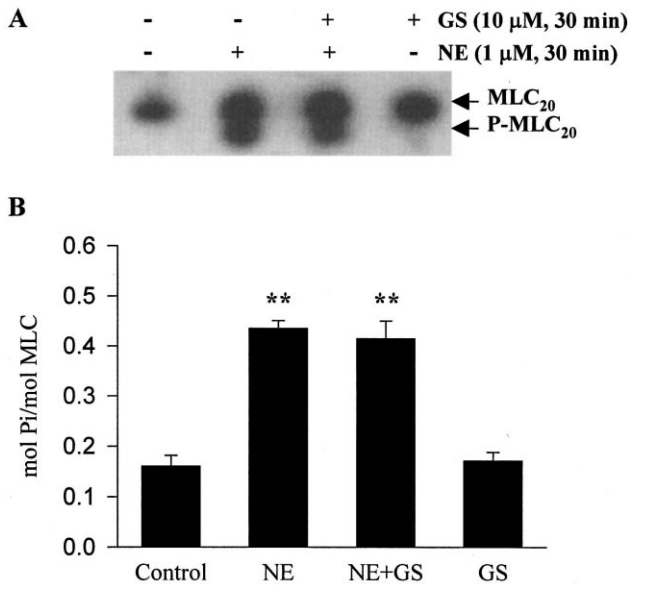

Fig. 3. A: Phosphorylation of MLC measured by immunoblotting after urea–glycerol polyacrylamide gel electrophoresis. B: Effect of 10 μM genistein (GS) on NE (1 μM)-induced MLC phosphorylation in rat thoracic aorta. The rat thoracic aorta was pretreated with 10 μM genistein for 30 min prior to the addition of NE. MLC phosphorylation was measured 30 min after the addition of NE with or without genistein. Data are expressed as mean ± S.E.M. ($n = 4$); ** denotes significant differences from control ($P < 0.01$).

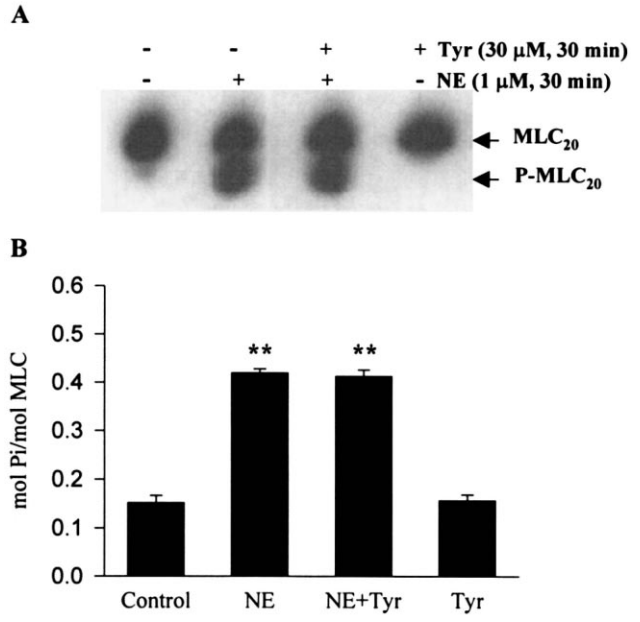

Fig. 4. A: Phosphorylation of MLC measured by immunoblotting after urea–glycerol polyacrylamide gel electrophoresis. B: Effect of 30 μM tyrphostin 25 (Tyr) on NE (1 μM)-induced MLC phosphorylation in rat thoracic aorta. The rat thoracic aorta was pretreated with 30 μM tyrphostin 25 for 30 min prior to the addition of NE. MLC phosphorylation was measured 30 min after the addition of NE with or without tyrphostin 25. Data are expressed as mean ± S.E.M. ($n = 4$); ** denotes significant differences from control ($P < 0.01$).

tyrphostin 25 for NE-induced contraction were 15.05 μM ($n = 5$) and 54.95 μM ($n = 5$), respectively.

On the other hand, to evaluate the effect of tyrosine kinase inhibitor on the 20 kDa MLC phosphorylation induced by NE, strips were preincubated in genistein (10 μM) or tyrphostin 25 (30 μM) for 30 min prior to the addition of NE. It was found that 10 μM genistein did not inhibit the 20 kDa MLC phosphorylation induced by NE (Fig. 3A,B). 30 μM tyrphostin 25 also did not inhibit the 20 kDa MLC phosphorylation induced by NE (Fig. 4A,B).

Fig. 5 shows the changes in $[Ca^{2+}]_i$ with respect to the contraction amplitude of the aortic strips. Pretreatment of the strips with 10 μM genistein (58.83 ± 2.36% of high K^+-induced Ca^{2+}, $n = 6$) for 30 min did not affect the increase of $[Ca^{2+}]_i$ induced by 1 μM NE (57.33 ± 4.16% of high K^+-induced Ca^{2+}, $n = 6$). Pretreatment of the strips with 30 μM tyrphostin 25 (57.80 ± 2.58% of high K^+-induced Ca^{2+}, $n = 5$) for 30 min also did not affect the increase of $[Ca^{2+}]_i$ induced by 1 μM NE. However, 10 μM genistein significantly diminished the amplitude of the sustained contraction evoked by 1 μM NE from 106.83 ± 7.33% of high K^+-induced contraction ($n = 6$) to 61.83 ± 3.27% of high K^+-induced contraction ($P < 0.01$, $n = 6$). 30 μM tyrphostin 25 also significantly diminished the amplitude of the sustained contraction evoked by 1 μM NE to 61.00 ± 3.39% of high K^+-induced contraction ($P < 0.01$, $n = 5$).

4. Discussion

α-Adrenoceptors have been shown to stimulate phosphoinositide hydrolysis, leading to $[Ca^{2+}]_i$ release and protein kinase C activation [24]. Yet in other tissues, contraction occurs in the absence of an increase of $[Ca^{2+}]_i$ [25], implying an alternative mechanism. The contractile response to NE has been shown to involve two components: (i) a phasic response, which can only be elicited once after removal of extracellular Ca^{2+}, and (ii) a sustained response, which can be generated repeatedly in the absence of extracellular Ca^{2+} [26]. Two different contractile mechanisms may, therefore, contribute to the overall response.

It is well known that the phosphorylation of the 20 kDa MLC is not the sole determinant for smooth muscle contraction. In most cases, during stimulation, MLC phosphorylation decreases to suprabasal levels although force is sustained. Thus, force may be maintained by slowly cycling unphosphorylated myosin–actin cross-bridges [27]. In our data, the sustained contraction was developed by NE, even though the phosphorylation of MLC increased only transiently (Fig. 1A,B). Therefore, secondary mechanism(s) for smooth muscle contraction may be involved in the development and/or maintenance of contractile tension.

On the other hand, it has been reported that the tyrosine kinase inhibitors, genistein and tyrphostin, decreased agonist-induced contraction in various tissues of different species, thereby suggesting that tyrosine phosphorylation of some proteins may play a role in agonist-induced contraction [10,18,28–30].

Our data show that, when the two structurally different tyrosine kinase inhibitors, genistein (10 μM) and tyrphostin 25 (30 μM), significantly reduce the amplitude of the contraction evoked by NE (Fig. 2), the same concentrations do not produce any inhibitory effect on NE-induced 20 kDa MLC phosphorylation (Figs. 3A,B and 4A,B), and even on $[Ca^{2+}]_i$ in rat aortic vascular smooth muscle (Fig. 5A–C).

In a recent study by Jin et al. [6], tyrosine kinase activation

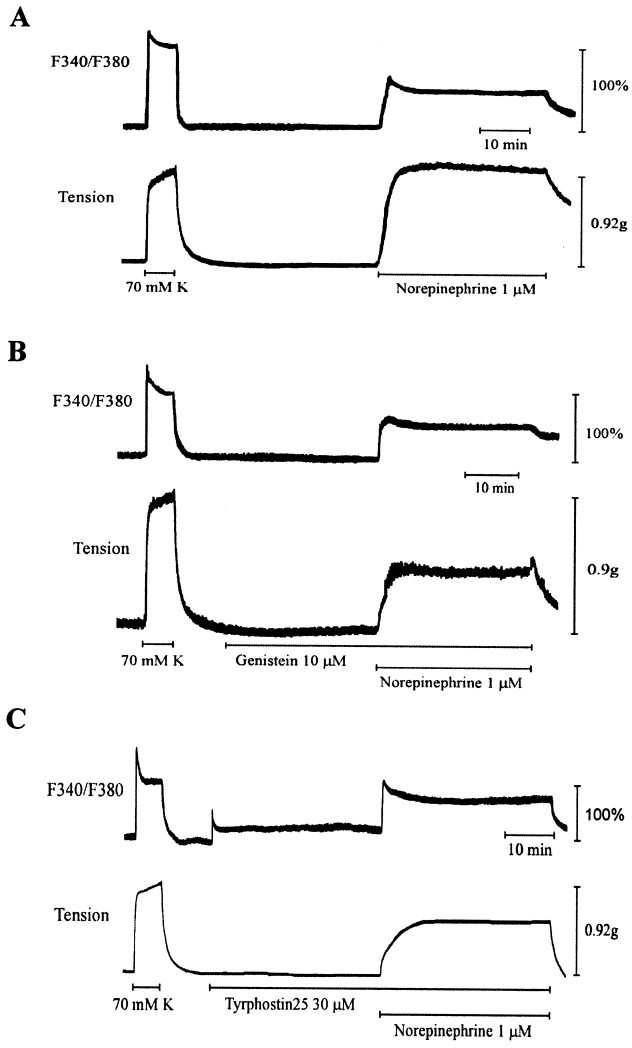

Fig. 5. Effect of tyrosine kinase inhibitors on increase of $[Ca^{2+}]_i$ induced by NE (1 μM) and smooth muscle contraction in rat thoracic aorta. Representative recordings showing the changes in myoplasmic $[Ca^{2+}]$, estimated by fura-2/AM (expressed as a percentage of the F_{340}/F_{380} ratio), and force (expressed in g) after stimulation with 1 μM NE in rat thoracic aorta, without pretreatment (A), or with pretreatment by 10 μM genistein (B) and 30 μM tyrphostin 25 (C) for 30 min.

has been shown to increase phosphorylation of MLC stimulated with fetal calf serum in vascular smooth muscle. Tyrosine phosphorylation is involved in smooth muscle contraction by regulating the Ca^{2+} sensitivity of the contractile apparatus [18,31]. In the present study, the tyrosine kinase-dependent pathway has also been clearly demonstrated not to be involved in transmembranous $[Ca^{2+}]_i$ entry in rat thoracic aorta.

The α_1-adrenoceptor subtypes (α_{1A} and α_{1B}) increase the Ca^{2+} sensitivity of contractile elements, and the Ca^{2+} sensitization produced by the α_{1A} subtype receptor plays an important role in contraction of rabbit thoracic aorta smooth muscle [32]. It has also been reported that the α_{1A}-adrenoceptor subtype activates mainly MLC kinase-independent pathways of the contractile mechanisms in rabbit vascular smooth muscle [33]. Therefore, the sustained contraction induced by NE could be due to an increase in $[Ca^{2+}]_i$, an increase in Ca^{2+} sensitivity of contractile elements, or a Ca^{2+}-independent mechanism [34]. Taken together, these data suggest that tyrosine kinase may regulate the contraction by NE without modification of $[Ca^{2+}]_i$ and MLC phosphorylation. It should also be noted that, at the concentration used, genistein, a putative tyrosine kinase inhibitor, did not inhibit other kinases linked to the signal transduction that results in vascular smooth muscle contraction, such as MLC kinase [10].

In conclusion, tyrosine kinase participates in NE-induced contraction of rat aortic smooth muscle. Although this may involve one or more steps in the signal transduction pathway, the enzyme appears to have a role in mediating sustained contraction and plays no role in certain other processes, including those involving $[Ca^{2+}]_i$ and MLC phosphorylation.

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