

Mitogen-Activated Protein Kinases Partially Regulate Endothelin-1-Induced Contractions through a Myosin Light Chain Phosphorylation-Independent Pathway

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ABSTRACT. Endothelin (ET), derived from the endothelium of blood vessels, is a potent vasoactive peptide. Although it has been reported to be involved in cardiovascular diseases, such as hypertension, the mechanism by which ET evokes vasoconstriction is still unclear. On the other hand, p42/p44 mitogen-activated protein kinase (MAPK) and p38 MAPK are activated by a variety of growth factors and cellular stresses, respectively. However, the role of p42/p44 MAPK and p38 MAPK on the ET-1-induced vasoconstriction is not fully understood. This study was undertaken to determine whether p42/p44 MAPK and p38 MAPK participate in the regulation of vascular smooth muscle contraction by ET-1. The isometric vasoconstriction and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) were simultaneously measured using CAF-100. Phosphorylation of myosin light chain (MLC) and p42/p44 MAPK, p38 MAPK were determined by Western blots. In rat thoracic aorta, ET-1 induced a sustained contraction. In contrast, $[\text{Ca}^{2+}]_i$ was decreased with time. Both PD98059, an inhibitor of p42/p44 MAPK, and SB203580, an inhibitor of p38 MAPK, partially attenuated ET-1-induced contractions in concentration-dependent manners. ET-1 increased phosphorylation of both p42/p44 MAPK and p38 MAPK, and PD98059 and SB203580 completely decreased phosphorylation of p42/p44 MAPK and p38 MAPK in response to ET-1 stimulation, respectively. On the other hand, PD98059 and SB203580 did not affect MLC phosphorylation in response to ET-1 stimulation. These results indicate that p38 MAPK, as well as p42/p44 MAPK, may partially regulate the ET-1-induced contraction through a MLC phosphorylation-independent pathway.

KEY WORDS: endothelin, mitogen-activated protein kinase, vasoconstriction.

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Endothelin (ET) is a potent vasoactive peptide particularly derived from the endothelium of blood vessels [38]. ET is composed of 21-amino acids, and is classified into ET-1, ET-2 and ET-3 according to their amino acid composition [19]. ET-1 is the major ET generated in the endothelium. ET acts via specific plasma membrane receptors. ET_A and ET_B receptors have distinctive characteristics for ET [1, 32]. ET_A and ET_B receptors on smooth muscle induce contraction and stimulate proliferation and cell hypertrophy [6]. Endothelial ET_B receptors stimulate the production of nitric oxide and prostacyclin [33]. ET is known to be involved in cardiovascular diseases, including hypertension, myocardial infarction, congestive heart failure, restenosis and atherosclerosis [24].

Many studies have evaluated ET-induced vasoconstriction. ET-1 evokes a long-lasting contraction, slow in onset, of isolated arteries, veins and in microcirculatory vessels from experimental animals and humans [2, 4, 25, 38]. The mechanism by which contractions are evoked by ET-1 in isolated vascular preparations is complicated. The increase in the sensitivity of the contractile apparatus to intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), as well as the increase of $[\text{Ca}^{2+}]_i$ concentration, may be involved in ET-1-induced vasoconstriction [8, 26, 28, 29]. On the other hand, ET-1 evoked an increase of myofilament Ca^{2+} sensitivity by a mechanism(s) indepen-

dent of tyrosine kinase in the rat pulmonary artery [10]. Moreover, ET-1 increased the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in the canine pulmonary artery [37]. However, the roles of p42/p44 MAPK as well as of p38 MAPK on ET-1-induced vasoconstriction are not fully clarified. In this study, we have addressed the mechanism(s) by which p42/p44 MAPK and p38 MAPK regulate ET-1-induced vascular smooth muscle contraction in the rat thoracic aorta.

MATERIALS AND METHODS

Contractility and $[\text{Ca}^{2+}]_i$ Measurements: Sprague-Dawley rats (250–300 g) were killed by 100% CO_2 inhalation. The thoracic aorta was isolated and cut into strips of 8–10 mm length and placed in physiological salt solution (PSS). These solutions were saturated with 95% O_2 and 5% CO_2 at 37°C to maintain pH at 7.4. The force of contraction was recorded isometrically. Muscle preparations were attached to a holder under a resting force of 5 mN and equilibrated for 60–90 min. $[\text{Ca}^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura-2 [22]. Muscle strips were exposed to the acetoxymethyl ester of fura-2 (fura-2/AM; 5 $\mu\text{mol/L}$) in the presence of 0.02% cremophor EL for 5–6 hr at room temperature. The muscle strips were then transferred to the muscle bath that is part of the fluorimeter (CAF-100; Jasco, Tokyo). 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

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a 500 nm filter.

MLC Phosphorylation: MLC phosphorylation levels were determined on strips of the thoracic aorta as described previously [35]. Briefly, the smooth muscle homogenates prepared by urea buffer (20 mmol/L Tris, 23 mmol/L glycine, 8 mol/L urea, 0.04% bromophenol blue, 10 mmol/L DTT, pH 8.6) were subjected to urea/glycerol gel electrophoresis. Following electrophoresis, the separated proteins were subjected to high-field intensity Western blotting onto nitrocellulose membranes. Visualization of the blotted proteins was performed using an Amersham enhanced chemiluminescence (ECL) kit. Values are reported as mol P_i/mol MLC by integration of the spot corresponding to the phosphorylated MLC to the total of both the phosphorylated and unphosphorylated MLC.

MAPK Phosphorylation: Rat aortic smooth muscle homogenates were centrifuged at $8,000 \times g$ for 10 min at 4°C. Aliquots of 40 μ g protein was subjected to sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membranes (HybondTM-P, Amersham) for 2–3 hr at 200 mA in transfer buffer (25 mmol/L Tris pH 8.3, 192 mmol/L glycine, 20% methanol). The membranes were incubated with 1 μ g/mL of monoclonal anti-phospho MAP kinase (ERK 1/2) antibody (Upstate Biotechnology) or polyclonal phospho-specific p38 MAP kinase antibody (New England Biolab) diluted in freshly prepared phosphate buffered saline (PBS)-milk (MLK) (overnight with agitation at 4°C). After washing the membranes twice with PBS + 0.05% Tween 20, membranes were incubated with secondary antibody (horseradish peroxidase, 1:5,000 dilution) in PBS-MLK for 1.5 hr at room temperature with agitation. After washing the membranes 3–5 times in PBS-0.05% Tween 20, immunoreactive bands were visualized by ECL reagents. Developed films from ECL were scanned and analyzed using NIH Image software.

Statistics: Data are presented as group means \pm SEM. Statistical significance was determined using unpaired, two-tailed Student's *t*-test. A *P* value <0.05 was taken as significant.

Materials: ET-1 was obtained from Sigma (St Louis, MO). PD98059 and SB203580 were obtained from Calbiochem (La Jolla, CA). All other chemicals were of reagent grade purity or better. ET-1 was dissolved into distilled water. PD98059 and SB203580 were dissolved into dimethyl sulfoxide (DMSO).

RESULTS

Contractility and $[Ca^{2+}]_i$ Induced by ET-1: Stimulation of the rat aortic strips with 10 nmol/L ET-1 resulted in a slowly developing contraction, which reached sustained levels at 30 min. The magnitude of the ET-1 induced contraction was similar to that produced in response to KCl-PSS stimulation (Fig. 1A). However, in contrast to contraction, stimulation of the rat aortic strips with 10 nmol/L ET-1 resulted in a rapid increase of $[Ca^{2+}]_i$, which reached peak levels at 3

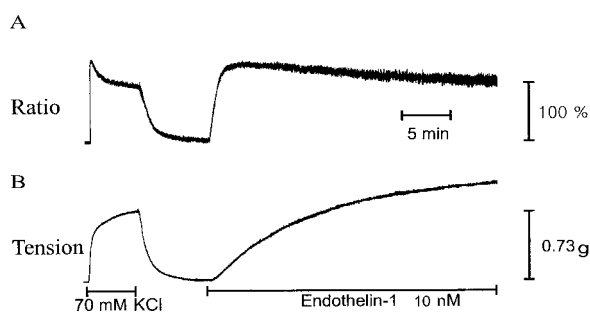


Fig. 1. A) Time course of 10 nmol/L ET-1-induced contraction in rat thoracic aorta. Contraction is expressed as a percent increase in response to high K⁺. Data are expressed as means \pm SEM (n=4). B) Time course of 10 nmol/L ET-1-induced, fura-2 AM-estimated $[Ca^{2+}]_i$ (expressed as % ratio of F340/F380) in response to high K⁺. Data are expressed as means \pm SEM (n=4).

min followed by a gradual decrease of $[Ca^{2+}]_i$ (Fig. 1B).

Effects of PD98059 and SB203580, Inhibitors of MAPK, on ET-1-Induced Contractility and Phosphorylation of MAPK: PD98059 and SB203580 were added during the steady-state force response to investigate the effects of p42/p44 MAPK and p38 MAPK, respectively, on ET-1-induced contraction. The addition of 10–100 μ mol/L PD98059, an inhibitor of p42/p44 MAPK kinase, to strips contracted by 10 nmol/L ET-1 also caused a concentration-dependent relaxation (Fig. 2A). Moreover, the addition of 10–100 μ mol/L SB203580 to strips contracted by 10 nmol/L ET-1 caused a concentration-dependent relaxation (Fig. 2B). On the other hand, 10 nmol/L ET-1 increased phosphorylation of p42/p44 MAPK in the rat thoracic aorta (Figs. 3A, B). By 20 min after ET-1 stimulation, phosphorylation of p42/p44 MAPK was increased to 2.7 ± 0.2 -fold ($P < 0.01$, n=4) compared with controls. By 50 min after ET-1 stimulation, phosphorylation of p42/p44 MAPK was increased to 2.5 ± 0.2 fold ($P < 0.01$, n=4) compared with controls. An aliquot of 100 μ mol/L PD98059, an inhibitor of p42/p44 MAPK kinase, was added 20 min after ET-1 stimulation. During the 30 min application, PD98059 significantly inhibited the phosphorylation of p42/p44 MAPK stimulated by ET-1 to the control level ($P < 0.01$, n=4). Moreover, 10 nmol/L ET-1 increased phosphorylation of p38 MAPK (Figs. 4A, B). By 20 min after ET-1 stimulation, phosphorylation of p38 MAPK was increased to 2.6 ± 0.2 -fold ($P < 0.01$, n=4) compared with control. By 50 min after ET-1 stimulation, phosphorylation of p38 MAPK was increased to 2.7 ± 0.0 -fold ($P < 0.01$, n=4) compared with control. An aliquot of 100 μ mol/L SB203580, an inhibitor of p38 MAPK, was added 20 min after ET-1 stimulation. During 30 min of application, SB203580 significantly inhibited the phosphorylation of p38 MAPK stimulated by ET-1 to the control level ($P < 0.01$, n=4). On the other hand, PD98059 and SB203580 at 100 μ mol/L showed no apparent effect on the 70 mmol/L KCl-PSS-induced contraction (data not shown).

Effects of PD98059 and SB203580 on ET-1-Induced

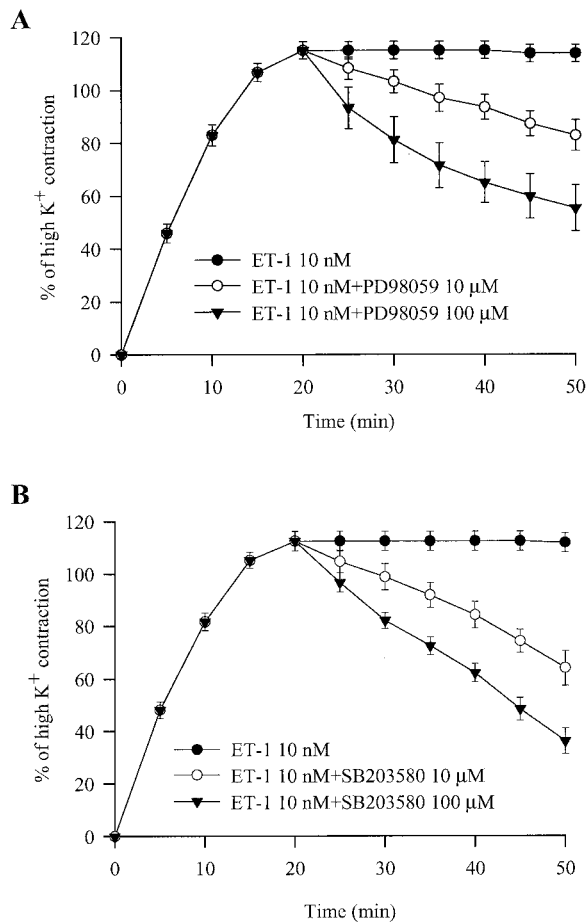


Fig. 2. A) Line graph showing the time course of the inhibition of the 10 nmol/L ET-1-induced contraction by 10–100 μ mol/L PD98059 in the rat thoracic aorta. Vertical bars indicate SEM ($n=4-5$). Addition of PD98059 decreased ET-1-induced contraction concentration-dependently. However, 100 μ mol/L PD98059 did not abolish ET-1-induced contraction completely during the 30 min after application. B) Line graph shows the time course of the inhibition of the 10 nmol/L ET-1-induced contraction by 10–100 μ mol/L SB203580 in the rat thoracic aorta. Vertical bars indicate SEM ($n=4-5$). Addition of SB203580 decreased ET-1-induced contraction concentration-dependently. However, 100 μ mol/L SB203580 did not completely abolish ET-1-induced contraction during the 30 min after application.

Phosphorylation of MLC: The addition of 10 nmol/L ET-1 significantly increased MLC phosphorylation levels from basal values of 0.156 ± 0.022 mol P_i /mol MLC to 0.472 ± 0.013 mol P_i /mol MLC at 20 min stimulation ($P<0.01$, $n=4$) (Figs. 5A, B). MLC phosphorylation levels were measured during 100 μ mol/L PD98059-induced relaxation of ET-1 induced contractions. The addition of 100 μ mol/L PD98059 for 30 min, 20 min after stimulation with ET-1, did not affect MLC phosphorylation levels (0.412 ± 0.023 mol P_i /mol MLC, $n=4$). MLC phosphorylation levels were also

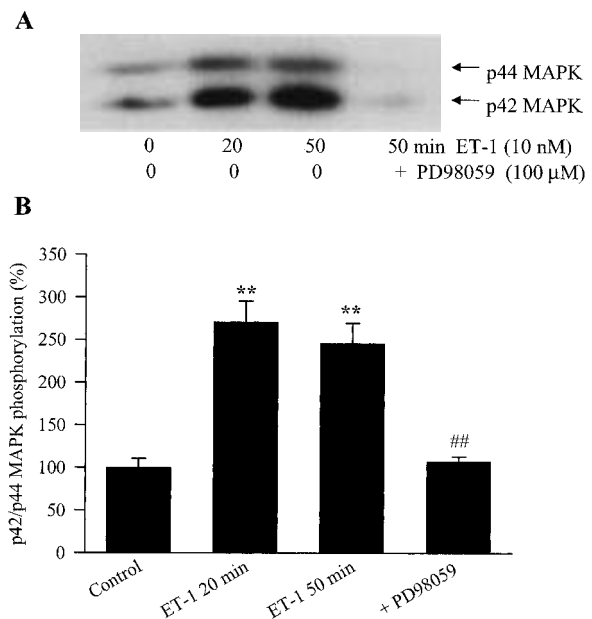


Fig. 3. A) Phosphorylation of p42/p44 MAPK from rat thoracic aorta measured by immunoblotting after SDS gel electrophoresis. Addition of 10 nmol/L ET-1 increased p42/p44 MAPK phosphorylation 20 min after stimulation. This increase was continued until 50 min after ET-1 stimulation. Addition of 100 μ mol/L PD98059, an inhibitor of p42/p44 MAPK kinase, decreased p42/p44 MAPK phosphorylation by ET-1. B) Densitometric quantification of p42/p44 MAPK phosphorylation in rat thoracic aorta. Data are expressed as means \pm SEM ($n=4$). ** Denotes significant difference from control ($P<0.01$). ## Denotes significant difference from ET-1 treated values ($P<0.01$).

measured during 100 μ mol/L SB203580-induced relaxation of ET-1 induced contractions. The addition of 100 μ mol/L SB203580 for 30 min, 20 min after stimulation with ET-1, also did not affect MLC phosphorylation levels (0.454 ± 0.011 mol P_i /mol MLC, $n=4$). To compare the MLC phosphorylation levels induced by PD98059 and SB203580 with those of the time sequence, we also measured MLC phosphorylation levels 50 min after ET-1 stimulation. The addition of 10 nmol/L ET-1 significantly increased MLC phosphorylation levels from basal values of 0.156 ± 0.022 mol P_i /mol MLC to 0.439 ± 0.016 mol P_i /mol MLC at 50 min stimulation ($P<0.01$, $n=4$). These levels were not different from those of MLC phosphorylation following 20 min stimulation with ET-1.

DISCUSSION

The results of our study show that stimulation of rat aortic strips with ET-1 produces an increase in force and in $[Ca^{2+}]_i$. In contrast to the sustained increase in force (Fig. 1A), the $[Ca^{2+}]_i$ decreased with time (Fig. 1B). Although Ca^{2+} - and calmodulin-dependent phosphorylation of the 20 kDa MLC are widely believed to be essential for the initiation of

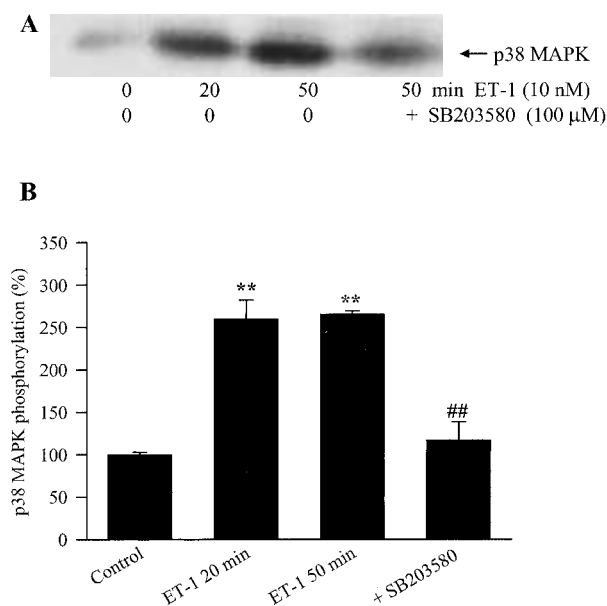


Fig. 4. A) Phosphorylation of p38 MAPK measured by immunoblotting after SDS gel electrophoresis in rat thoracic aorta. Addition of 10 nmol/L ET-1 increased p38 MAPK phosphorylation 20 min after stimulation. This increase was continued until 50 min after ET-1 stimulation. Addition of 100 μ mol/L SB203580, an inhibitor of p38 MAPK, decreased p38 MAPK phosphorylation by ET-1. B) Densitometric quantification of p38 MAPK phosphorylation in rat thoracic aorta. Data are expressed as means \pm SEM (n=4). ** Denotes significant difference from control ($P<0.01$). ## Denotes significant difference from ET-1 treated values ($P<0.01$).

smooth muscle contraction [16], phorbol ester, an activator of protein kinase C, also increases contraction, MLC phosphorylation, and the unloaded shortening velocity induced by low concentrations of $[Ca^{2+}]_i$ in skinned smooth muscle [3, 14, 20]. Therefore, the mechanism(s) responsible for smooth muscle contraction are not solely dependent on $[Ca^{2+}]_i$. Moreover, alterations in Ca^{2+} sensitivity of smooth muscle contraction have been demonstrated in permeabilized fiber preparations. The increase of Ca^{2+} sensitivity of smooth muscle contraction may result from: 1) an alteration of MLC kinase (MLCK)/MLC phosphatases (MLCP) balance, 2) an alteration of certain protein kinase C activity, or 3) an alteration of MAPK activity.

In this study, we focused on the role of MAPK in ET-1-induced contraction of the rat thoracic aorta. The role of MAPK for vasoconstriction is controversial. Agonist and membrane depolarization induced activation of MAPK in the pig carotid artery [21]. However, inhibition of p42/p44 MAPK did not alter smooth muscle contraction in this model [15]. Caldesmon, a protein which is found on actin-based filaments of cells and inhibits the ATPase activity of actomyosin [18], is the substrate for MAPK during vasoconstriction [7, 9, 12]. Moreover, MAPK may be an important regulator for rabbit basilar artery contraction [40]. However, MAPK was not involved in vascular smooth muscle

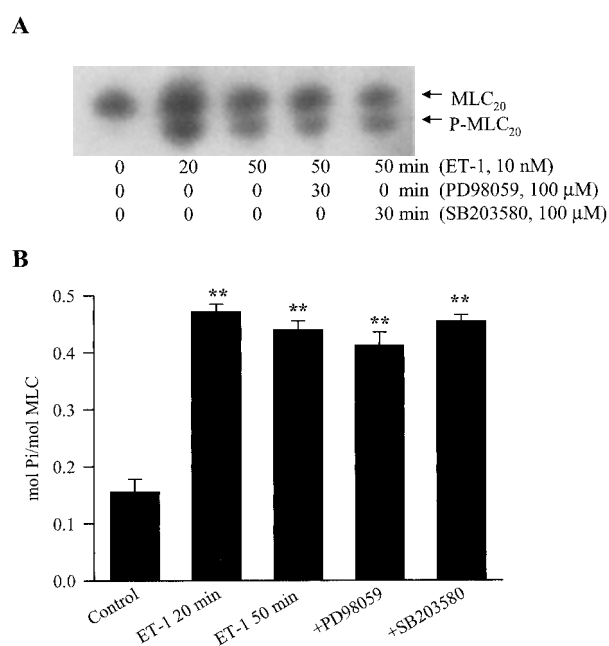


Fig. 5. A) Phosphorylation of MLC measured by immunoblotting after urea-glycerol polyacrylamide gel electrophoresis. 10 nmol/L ET-1 increased the phosphorylation of MLC 20–50 min after stimulation in rat thoracic aorta. At 20 min of ET-1 stimulation, the addition of 100 μ mol/L PD98059 for 30 min did not affect the phosphorylation of MLC by ET-1. Also, at 20 min after ET-1 stimulation, the addition of 100 μ mol/L SB203580 for 30 min did not affect the phosphorylation of MLC by ET-1. B) The bar graph shows means \pm SEM values of phosphorylation of MLC in 10 nmol/L ET-1 treated groups (n=4), demonstrating a significant increase 20–50 min after ET-1 stimulation, a significant increase in PD98059 and SB203580 treated group (n=4), respectively. ** Denotes significant difference from control ($P<0.01$).

contraction nor in myofilament Ca^{2+} sensitivity [27, 34, 36]. Like the SAPK/JNK pathway, p38 MAPK is activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), UV light, and growth factors [13, 17, 23, 30, 31]. Activated p38 MAPK has been shown to phosphorylate and activate MAPKAP kinase-2 [31], and to phosphorylate the transcription factors ATF-2 [30], and Max [39].

In our data, 10–100 μ mol/L PD98059, an inhibitor of p42/p44 MAPK kinase, attenuated ET-1-induced contraction (Fig. 2A). The phosphorylation of p42/p44 MAPK was sustained during 50 min stimulation with ET-1 (Fig. 3). Addition of 100 μ M PD98059 abolished the phosphorylation of p42/p44 MAPK induced by ET-1 (Figs 3A, B). On the other hand, 10–100 μ mol/L SB203580, an inhibitor of p38 MAPK [5], attenuated ET-1-induced contraction (Fig. 2B). Moreover, ET-1 increased p38 MAPK phosphorylation (Fig. 4). The phosphorylation of p38 MAPK was sustained during 50 min stimulation by ET-1. Addition of 100 μ mol/L SB 203580 abolished the phosphorylation of p38 MAPK induced by ET-1 (Figs. 4A, B). In our present stud-

ies, pretreatment with 100 $\mu\text{mol/L}$ of PD98059 or SB203580 did not affect the contraction induced by 70 mmol/L KCl-PSS. These results may show that ET-1 evokes contraction resulting from an activation of p38 MAPK as well as p42/p44 MAPK in the rat thoracic aorta. However, although PD98059 and SB203580 completely inhibited the phosphorylation of p42/p44 MAPK and p38 MAPK by ET-1, respectively, the contractility by ET-1 was partially attenuated. Therefore, p42/p44 MAPK and/or p38 MAPK are not the sole regulatory kinase(s) for rat aortic vascular smooth muscle contraction. On the other hand, SB203580 as well as PD98059 did not affect the phosphorylation of MLC by ET-1 (Figs. 5A, B). Recently, we reported that tyrosine kinase participates in vasoconstriction through a Ca^{2+} - and MLC phosphorylation-independent pathway [11]. Because tyrosine kinase is upstream regulators of MAPK, it is obvious that MLC is not the substrate for the tyrosine kinase-MAPK signaling pathway. Activated p38 MAPK as well as p42/p44 MAPK may modulate another regulatory protein, which may result in an increase in actin-myosin interaction. Recently, the p38 MAPK/heat shock protein (HSP) 27 pathway was suggested to be involved in the generation of maximal force in smooth muscle [36]. They showed that ERK MAP kinases phosphorylate caldesmon *in vivo* but that activation of this pathway is unnecessary for force development. Alternatively, activated p38 MAPK as well as p42/p44 MAPK may directly increase actin-myosin interaction and/or enhance positive co-operativity.

In conclusion, our results suggest that p38 MAPK, as well as p42/p44 MAPK, partially regulate endothelin-1-induced contractions through a MLC phosphorylation-independent pathway.

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