Evidence for modulation of smooth muscle force by the p38 MAP kinase/HSP27 pathway

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Evidence for modulation of smooth muscle force by the p38 MAP kinase/HSP27 pathway. Am J Physiol Heart Circ Physiol 278: H1899–H1907, 2000. — Mitogen-activated protein (MAP) kinases signal to proteins that could modify smooth muscle contraction. Caldesmon is a substrate for extracellular signal-related kinases (ERK) and p38 MAP kinases in vitro and has been suggested to modulate actin-myosin interaction and contraction. Heat shock protein 27 (HSP27) is downstream of p38 MAP kinases presumably participating in the sustained phase of muscle contraction. We tested the role of caldesmon and HSP27 phosphorylation in the contractile response of vascular smooth muscle by using inhibitors of both MAP kinase pathways. In intact smooth muscle, PD-098059 abolished endothelin-1 (ET-1)-stimulated phosphorylation of ERK MAP kinases and caldesmon, but p38 MAP kinase activation and contractile response remained unaffected. SB-203580 reduced muscle contraction and inhibited p38 MAP kinase and HSP27 phosphorylation but had no effect on ERK MAP kinase and caldesmon phosphorylation. In permeabilized muscle fibers, SB-203580 and a polyclonal anti-HSP27 antibody attenuated ET-1-dependent contraction, whereas PD-098059 had no effect. These results suggest that ERK MAP kinases phosphorylate caldesmon in vivo but that activation of this pathway is unnecessary for force development. The generation of maximal force may be modulated by the p38 MAP kinase/HSP27 pathway.

Endothelin-1; mitogen-activated protein kinases; caldesmon; skinned muscle fibers; heat shock protein 27

SMOOTH MUSCLE CONTRACTION is initiated by Ca2+-dependent mechanisms activating actin-myosin coupling and cross-bridge cycling, but additional proteins have been proposed to participate in the sustained contractile phase. In smooth muscle cell, the thin filament-binding protein caldesmon has been localized in close proximity to actin, myosin, and tropomyosin, suggesting that caldesmon may be involved in contractile functions performed by this protein complex (22). This is supported by observations that caldesmon blocks myosin binding to actin and inhibits actin-dependent myosin ATPase activity, and these inhibitory effects are reversed on caldesmon phosphorylation (19, 30). Caldesmon phosphorylation in differentiated smooth muscle is regulated by mitogen-activated protein (MAP) kinases, and therefore MAP kinases are considered likely modulators of smooth muscle contraction (1, 8, 16, 21). The physiological relevance of MAP kinase coupling to caldesmon, however, has been challenged by evidence that activated extracellular signal-regulated kinase (ERK) MAP kinase does not lead to disinhibition of actin-tropomyosin-activated ATPase activity in vitro (17) and does not enhance muscle force in chemically skinned fibers (20). In intact vascular smooth muscle, inhibition of ERK MAP kinase activation by PD-098059 does not reduce histamine or phorbol 12,13-dibutyrate-elicited contractile response (13). Thus whether caldesmon exerts inhibitory effects on active cross-bridge cycling (9) and, subsequently, on smooth muscle contraction is unclear, and so is the question of whether this effect is reversed when caldesmon is phosphorylated by MAP kinases.

Phosphorylation of the small heat shock protein 27 (HSP27) is another MAP kinase-mediated mechanism proposed to modulate the sustained phase of smooth muscle contraction (3, 5). Elevated phosphorylation of HSP27 has been observed after stimulation with a variety of contractile agonists such as carbachol (18), thrombin (6), C2 ceramide and endothelin-1 (ET-1) (26), and cyclosporin A (3). Inhibition of HSP27 phosphorylation (3, 5) or treatments interfering with intracellular distribution of HSP27 (26) have been shown to reduce muscle contraction. HSP27 is involved in the regulation of actin assembly in vivo (14, 24), and hence it is possible that it plays a role in the sustained smooth muscle contraction.

In the present study we tested the role of ERK and p38 MAP kinases and their putative downstream effectors proteins caldesmon and HSP27 in the regulation of contractile responses of canine pulmonary artery smooth muscle. We provide evidence that ERK MAP kinases, but not p38 MAP kinases, are the physiologically relevant caldesmon kinases in vivo. The ERK MAP kinase-caldesmon pathway is activated by contractile neurotransmitters, but this activation appears unnecessary for development of muscle force. We show that inhibition of the p38 MAP kinase/HSP27 pathway reduces the initial rate and maximal contractile force, indicating a possibility for modulation of smooth muscle contraction.

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METHODS

Materials. Wortmannin, GTP (sodium salt), ATP (disodium salt), creatine phosphate (CrP; disodium salt), rabbit muscle creatine phosphokinase (CPK), carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), BSA, and ET-1 were purchased from Sigma (St. Louis, MO). PD-098059 and SB-203580 were from Calbiochem (La Jolla, CA). Recombinant active GST-ERK2 and Xenopus p38 (Mpk2) MAP kinases were from Upstate Biotechnology (Lake Placid, NY). Alkaline phosphatase was purchased from Boehringer-Mannheim (Mannheim, Germany); phosphospecific anti-ERK MAP kinase (catalog no. 9101S) and anti-p38 MAP kinase (catalog no. 9211S) antibodies were purchased from New England Biolabs (Beverly, MA). Anti-rabbit IgG alkaline phosphatase-conjugated antibody was purchased from Promega (Madison, WI). Polyclonal anti-HSP27 antibody was affinity purified with a rabbit polyclonal phosphospecific antibody recognizing HSP27 (HSP27-Sepharose CL4B column). A sequence-specific polyclonal anti-caldesmon antibody (3/90 antibody) was raised by injecting rabbits with full-length pig stomach h-caldesmon. Polyclonal phosphospecific anti-caldesmon antibodies were raised against peptide sequences surrounding the phosphorylated MAP kinase consensus domains of mammalian smooth muscle caldesmon, e.g., Ser-759 (B1 antibody) and Ser-789 (B3 antibody). These anti-caldesmon antibodies were affinity purified as previously described (10). Their immunoreactivity and the linear range of detection of canine h-caldesmon were determined by Western immunoblotting with the use of dilution series of total canine vascular smooth muscle tissue homogenates.

Phosphorylation of caldesmon by recombinant active ERK and p38 MAP kinases in vitro. Purified porcine caldesmon was first dephosphorylated by incubation with alkaline phosphatase in vitro, following the manufacturer’s guidelines (Boehringer Mannheim). The progress of dephosphorylation of the MAP kinase consensus sites was monitored by the use of site-specific antibodies B1 (Ser-759) and B3 (Ser-789) until no detectable immunoreactivity was present. The dephosphorylated caldesmon was then subjected to phosphorylation by activated recombinant GST-ERK2 MAP kinase and Mpk2 MAP kinase in vitro. Phosphorylation reactions were carried out at 30°C in a reaction volume of 80 μl containing 25 mM Tris·HCl (pH 7.0), 0.1 mM EGTA, 0.1 mM Na2VO4, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), 2 μg of purified porcine stomach caldesmon, 250 μM ATP/10 μCi [γ-32P]ATP, and enzyme. Aliquots (15 μl) were withdrawn at 0, 30, and 60 min, and the reaction was stopped by the addition of 5 μl of 4X SDS sample buffer to produce final concentrations of 0.06 M Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mM DTT, and 0.03% bromophenol blue. After samples were boiled for 5 min, protein was resolved by SDS-PAGE (8% acrylamide) on three parallel, equally loaded gels. All gels were transferred to nitrocellulose membranes, which were exposed to a screen to assay the radioactivity incorporated into caldesmon during the phosphorylation reaction (model 525 Molecular Imager, Bio-Rad, Hercules, CA). One of the nitrocellulose membranes was then probed with a general rabbit polyclonal anti-caldesmon antibody to verify uniform loading (3/90 antibody, dilution 1:10,000), a second membrane was probed with a rabbit polyclonal phosphospecific antibody recognizing phosphorylated Ser-759 (B1 antibody, dilution 1:1000), and a third membrane was probed with a rabbit polyclonal phosphospecific antibody recognizing phosphorylated Ser-789 (B3 antibody, dilution 1:1,000). Immunoreactive bands were visualized by a color reaction with the use of alkaline phosphatase conjugates and then quantified by densitometry with the use of a UMAX Powerlook flat-bed scanner. The bands were analyzed using Molecular Analyst software (Bio-Rad) as previously described (28).

Smooth muscle stimulation and homogenization. Second-order branches of pulmonary artery were dissected from adult mongrel dogs of either sex that were euthanized with pentobarbital sodium (62 mg/kg iv). Muscle rings (1–2 mm wide) were excised, mounted on stainless steel hooks, and equilibrated at 37°C while bathed with oxygenated physiological salt solution (PSS) composed of 2 mM MOPS (pH 7.4), 140 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM Na2HPO4, 0.02 mM EDTA, and 5.6 mM d-glucose. Tissues were stimulated three times for 5 min with 70 mM potassium depolarizing solution to obtain reproducible contractile responses. The arterial rings were then incubated for 1 h with 0.1% DMSO (vehicle control), 50 μM PD-098059, or 25 μM SB-203580 and stimulated with 100 nM ET-1 for 10 min. In preliminary experiments we established that these concentrations of PD-098059 and SB-203580 inhibited completely and specifically the catalytic activity of ERK and p38 MAP kinases, respectively. Tissues were frozen in liquid nitrogen and homogenized in extraction buffer (20 μl/mg tissue wet weight) composed of 20 mM HEPES (pH 7.5), 6 mM EDTA, 190 mM NaCl, 0.25 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1 μM leupeptin, and 1 mM Na3VO4. Extracts were clarified by centrifugation at 10,000 g for 10 min, and protein concentration in the clear supernatants was assayed using the bicinchoninic acid method.

Assay of MAP kinase and caldesmon phosphorylation in vivo. Tissue homogenates containing equal amounts of total protein (15 μg) were loaded in each lane, separated by SDS-PAGE, and transferred to nitrocellulose in 25 mM Tris·HCl, 192 mM glycine, and 10% methanol for 2 h at 24 V and 4°C (Genie blotter, Idea Scientific, Minneapolis, MN). The nitrocellulose membranes were blocked for 4 h in 0.5% gelatin in TNT buffer [100 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20]. Phosphorylated ERK and p38 MAP kinases were immunodetected by phosphospecific anti-MAP kinase antibodies (New England Biolabs, Beverly, MA) diluted 1:1,000 in 0.1% gelatin-TNT. Caldesmon phosphorylation was assayed using the phosphospecific antibodies B1 and B3 apt peptides. The immunoreactive bands were visualized by goat anti-rabbit alkaline phosphatase conjugate diluted to 1:10,000 with 0.1% gelatin-TNT using color detection. Blots were then scanned and quantified as previously described (28). ERK and p38 MAP kinase threonine/tyrosine phosphorylation was expressed relative to the phosphorylation of control muscle strips.

MAP kinase-activated protein kinase-2 activity assay. MAP kinase-activated protein (MAPKAP) kinase-2 activity was assayed in an in vitro reaction with the use of bacterially expressed canine rHSP27, as previously described (18). The kinase reaction (20 μl) contained 25 mM Tris·HCl (pH 7.0), 0.1 mM EGTA, 0.2 mM Na2VO4, 10 mM magnesium acetate, 1 mM DTT, 0.5 μg of rHSP27, 250 μM ATP/2 μCi [γ-32P]ATP, and clarified tissue homogenate. After incubation at 30°C for 1 h, the reaction was stopped by adding 6.7 μl of 4X SDS sample buffer and boiling for 5 min. Final concentrations of SDS sample buffer components were 0.06 M Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mM DTT, and 0.03% bromophenol blue. Protein was resolved by SDS-PAGE (12% acrylamide), and the gels were stained with Coomassie brilliant blue and exposed on a radiographic screen. The phosphorylation of rHSP27 was detected with a model 525 Molecular Imager (Bio-Rad). Background signal due to the phosphorylation of endogenous HSP27 comprised <5% of the total kinase activity. The obtained MAPKAP kinase-2 catalytic activity was
were used to evaluate the roles of ERK and p38 MAP kinases in the contractile curve without and with PD-098059 or SB-203580 to determine the initial force rate, maximum force, and area under the force-time trace. Tissues were stretched to 100 nM ET-1 for 10 min with 0.1% DMSO (vehicle control) or 10 µM of either PD-098059 or SB-203580, and fibers were stimulated with 0.1% DMSO (vehicle control), 25 µM SB-203580, or 50 µM PD-098059. Force traces obtained before and after 1-h preincubation with 0.1% DMSO (vehicle control), 25 µM SB-203580, or 50 µM PD-098059. Force traces obtained before and after incubation with the inhibitors were digitized to determine initial force rate, maximum force, and area under the force-time trace. The relative changes in the area under the contractile curve without and with PD-098059 or SB-203580 were used to evaluate the roles of ERK and p38 MAP kinases in smooth muscle contraction.

Skinned fiber experiments. Small pulmonary artery rings (−1 mg tissue wet wt) were excised from second-order branches, cleaned of connective tissue, and cut open. The fibers were mounted on stainless steel clips, and after equilibration in PSS at room temperature, they were stretched to adjust the resting tension. Tissue viability was verified by the contractile response to 1 µM phenylephrine (PE) to obtain a control contractile response, and the same stimulation was repeated after 1-h preincubation with 0.1% DMSO (vehicle control), 25 µM SB-203580, or 50 µM PD-098059. Force traces obtained before and after incubation with the inhibitors were digitized to determine initial force rate, maximum force, and area under the force-time trace. The relative changes in the area under the contractile curve without and with PD-098059 or SB-203580 were used to evaluate the roles of ERK and p38 MAP kinases in smooth muscle contraction.

RESULTS

Effect of PD-098059 and SB-203580 on force development of phenylephrine-stimulated intact smooth muscle. Pulmonary artery smooth muscle rings were mounted on stainless steel hooks and equilibrated in oxygenated PSS at 37°C in a tissue bath. Tissues were treated with 70 mM potassium-depolarizing solution (3 times for 5 min each) to obtain reproducible contractions. Muscle rings were then stimulated with 1 µM phenylephrine (PE) to obtain a control contractile response, and the same stimulation was repeated after 1-h preincubation with 0.1% DMSO (vehicle control), 25 µM SB-203580, or 50 µM PD-098059. Force traces obtained before and after incubation with the inhibitors were digitized to determine initial force rate, maximum force, and area under the force-time trace. The relative changes in the area under the contractile curve without and with PD-098059 or SB-203580 were used to evaluate the roles of ERK and p38 MAP kinases in smooth muscle contraction.

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inhibit p38 MAP kinase activity. ET-1 significantly increased the phosphorylation of both ERK1 and p38 MAP kinases to $1.73 \pm 0.15$ and $4.03 \pm 1.60$ times basal activity at 10 min, respectively (Fig. 2, A and B). The phosphorylation of caldesmon at Ser-759 was marginal at basal conditions and remained unchanged 10 min after ET-1 stimulation (not shown). In contrast, the low Ser-789 phosphorylation of caldesmon at rest was substantially increased to $1.49 \pm 0.06$ times basal at 10 min (Fig. 2C). These results again imply that there may be a preferred MAP kinase phosphorylation site in canine smooth muscle caldesmon.

Both ERK and p38 MAP kinases are proline-directed kinases; therefore, the phosphorylation of the adjacent Ser-789 could reflect catalytic activity of either MAP kinase or could be the result of a joint catalytic modification. To distinguish between these possibilities in separate experiments, we inhibited the two MAP kinase pathways and then assayed ET-1-stimulated caldesmon phosphorylation. After 1-h preincubation of muscle strips with 50 µM PD-098059, the ET-1-stimulated ERK1 MAP kinase phosphorylation was completely abolished (Fig. 2A), and so was the phosphorylation of caldesmon (Fig. 2C), whereas the phosphorylation of p38 MAP kinase and activation of MAPKAP kinase-2 remained unaffected.

SB-203580 has been shown to interact with the ATP-binding site of p38 MAP kinase, and this binding does not interfere with phosphorylation of the threonine/glu./tyrosine sequence and activation of p38 MAP kinase by upstream regulators (29). Accordingly, preincubation with SB-203580 was not expected to decrease ET-1-stimulated phosphorylation of p38 MAP kinase. Surprisingly, we detected a substantial reduction of p38 MAP kinase phosphorylation by SB-203580 (Fig. 2B). Because threonine/tyrosine phosphorylation does not reflect catalytic activity, we tested whether SB-203580 inhibited p38 MAP kinase pathway by assaying the activity of MAPKAP kinase-2, an enzyme downstream of p38 MAP kinases. In control muscle strips, ET-1-stimulated activation of MAPKAP kinase-2 to $2.18 \pm 0.15$ times basal, and this was eliminated in strips preincubated with SB-203580 (Fig. 2D). The inhibition of MAPKAP kinase-2 would be expected to result in a net decrease of HSP27 phosphorylation. To test this hypothesis, we assayed the charge isoform

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Fig. 1. Recombinant activated GST-extracellular signal-regulated kinase-2 (ERK2) and p38 (Mpk2) mitogen-activated protein (MAP) kinases phosphorylate the same consensus sequences of caldesmon in vitro. Phosphorylation of purified porcine stomach caldesmon was carried out at 30°C with 250 µM/10 µCi [$γ^{-32}$P]ATP. Aliquots were withdrawn at 0, 30, and 60 min, and reaction was stopped by addition of SDS sample buffer and boiling. Protein was resolved by SDS-PAGE (8% acrylamide) on parallel, equally loaded gels, which were then transferred to nitrocellulose and exposed to develop radioactive bands (panels A). Bands were quantified by scanning densitometry and plotted to illustrate time course of caldesmon phosphorylation (panels C). Membranes were then probed with a general rabbit polyclonal anti-caldesmon antibody (3/90 Ab) to verify uniform loading (panels B), with a phosphospecific antibody recognizing phosphorylated Ser-789 (panels D) or with a phosphospecific antibody recognizing phosphorylated Ser-759 (panels E). Immunoreactive bands were visualized by a color reaction using alkaline phosphatase conjugates, quantified by densitometry, and plotted (panels F) to differentially represent phosphorylation of each caldesmon phosphorylation site. Results are based on a single phosphorylation experiment with both MAP kinases.
Fig. 2. Effects of PD-098059 and SB-203580 on endothelin-1 (ET-1)-stimulated phosphorylation of ERK and p38 MAP kinases, MAP kinase-activated protein (MAPKAP) kinase-2, and caldesmon in intact smooth muscle. Canine pulmonary artery strips were incubated with 0.1% DMSO (vehicle control), 50 µM PD-098059, or 25 µM SB-203580 for 1 h before a 10-min stimulation with 100 nM ET-1. Strips were frozen and homogenized, and protein phosphorylation was assayed by Western immunoblotting. Top: immunoblots were prepared by probing total protein with phosphospecific antibodies raised against threonine/tyrosine-phosphorylated ERK (A) and p38 MAP kinases (B) and Ser-789-phosphorylated caldesmon (C). MAPKAP kinase-2 activity was assayed by in vitro phosphorylation of recombinant canine heat shock protein 27 (rHSP27). Protein was resolved by SDS-PAGE, gels were stained with Coomassie brilliant blue (CBB; D, top blot), destained, and exposed on screen to develop radioactive images (D, bottom blot). Bottom: phosphorylation was calculated by densitometry of immunoreactive or radioactive bands, as appropriate, and expressed relative to basal activity. *P < 0.05, compared with basal conditions (Student’s t-test).

shift of HSP27 after a one-dimensional IEF separation and Western immunoblotting of equal amounts of total protein in each IEF tube gel. Four bands, corresponding to nonphosphorylated (Fig. 3, spot a), monophosphorylated (Fig. 3, spot b), diphosphorylated (Fig. 3, spot c), and triphosphorylated (Fig. 3, spot d) HSP27 were detected with this technique. At rest, the HSP27 pool was mainly composed of nonphosphorylated and monophosphorylated isofoms (Fig. 3A, spots a and b), whereas only a faint immunoreactive signal of the other isoforms was detected (Fig. 3A, spots c and d). This pattern underwent a substantial change toward the more phosphorylated, acidic HSP27 isoforms in the ET-1-stimulated tissues (Fig. 3B, spots c and d). Although SB-203580 preincubation did not completely eliminate the rightward HSP27 isoform shift, it was reversed toward more basal isoform distribution (Fig. 3C). This SB-203580-elicited inhibition of the p38 MAP kinase pathway, however, resulted in no apparent change of ERK MAP kinases or caldesmon phosphorylation (Fig. 2C). Therefore, although both ERK and p38 MAP kinase pathways are activated by ET-1, their activation does not appear to converge on caldesmon or HSP27. Only ERK MAP kinases regulate caldesmon phosphorylation in vivo, preferentially on Ser-789, and only p38 MAP kinases appear to regulate phosphorylation of HSP27.

Pulmonary artery smooth muscle contraction is not affected by PD-098059 but is reduced by SB-203580. In addition to being a potent contractile agonist, ET-1 induces sustained tachyphylaxis, thus limiting the number of consecutive muscle stimulations with ET-1 in one experiment. Therefore, PE, a sympathomimetic with a readily reversible contractile effect, was used to evaluate the role of MAP kinases in the development of muscle force. At a concentration of 1 µM, PE elicited a tonic contraction consisting of a steep initial rise of force, followed by a slower phase that leveled off after 6 min after the onset of stimulation (Fig. 4). Preincubation of smooth muscle strips with DMSO or PD-098059 did not change the time course of PE-stimulated contraction (Fig. 4A). SB-203580 significantly reduced the fast initial rate of contraction (from 0.9439 to 0.5191 min⁻¹) and the maximum force (from 1.77 to 1.59 g), and hence reduced the total area under the force-time curves by approximately 12% (Fig. 4B). These experiments demonstrate a lack of effect of the ERK MAP kinase pathway and a possible modulation of vascular smooth muscle contraction by p38 MAP kinases.

SB-203580 inhibits smooth muscle contraction by a p38 MAP kinase-dependent but Ca²⁺-independent mechanism. SB-203580 is regarded as a specific inhibitor of p38 MAP kinase, lacking an inhibitory effect for a number of other kinases and phosphatases (7). Inhibition of Ca²⁺-dependent smooth muscle contraction by SB-203580 has not been previously shown. It is possible, therefore, that the reduction of muscle contraction in our experiments (Fig. 4) is not only due to inhibition of p38 MAP kinase pathway but also to some unrecognized effects on Ca²⁺-dependent mechanisms.

To test this hypothesis, we used Staphylococcus α-toxin-skinning fibers as a reliable technique for distinguishing between Ca²⁺-dependent and -independent contraction (25). A Ca²⁺ concentration-force curve was first generated with the use of activating solutions with Ca²⁺ concentrations ranging from 10 nM to 10 µM at room temperature. This approach allowed us to select a submaximal Ca²⁺ concentration of 0.3 µM, which was used in our routine contraction experiments. The contractile response at this concentration was approximately 30% of the maximum response to 10 µM Ca²⁺. ET-1 alone (100 nM) did not evoke muscle contraction, but after preincubation with ET-1 for 10 min, the contractile responses of permeabilized muscle fibers to 0.3 µM Ca²⁺ were enhanced two to three times over the control contraction (Fig. 5). This ET-1-dependent contraction has been observed by other investigators in skinned muscle fiber preparations and is believed to reflect enhanced Ca²⁺ sensitivity of smooth muscle myofilaments, which is caused by activation of intracellular mechanisms down-
stream of ET-1 receptors (23). Here we hypothesized that this ET-1-dependent contraction is mediated in part by MAP kinase-dependent mechanisms. To test this hypothesis, we preincubated skinned fibers with 0.1% DMSO (vehicle), 10 µM PD-098059, or 10 µM SB-203580 for 10 min. This protocol did not affect the Ca\(^{2+}\)-stimulated force generation, suggesting that the major Ca\(^{2+}\)-dependent contractile mechanisms are sensitive to neither DMSO nor MAP kinase inhibitors. Also, DMSO and PD-098059 did not produce any significant effect on the ET-1-dependent contraction (Fig. 5, A and C). In contrast, SB-203580 significantly reduced the ET-1-dependent contraction by 30% (Fig. 5, B and C). These results suggest that p38 MAP kinase, rather than ERK MAP kinase pathway modulates smooth muscle contraction.

p38 MAP kinases regulate smooth muscle contraction via an HSP27-dependent mechanism. As depicted in Fig. 2, when p38 MAP kinase was blocked by SB-203580, caldesmon phosphorylation in intact muscle

Fig. 3. ET-1 stimulation increases HSP27 phosphorylation in intact vascular smooth muscle, and this effect is reversed by SB-203580. HSP27 phosphorylation was assayed at rest (basal; A), after ET-1 stimulation (B), and after ET-1 stimulation following preincubation with SB-203580 (SB; C) by 1-dimensional isoelectric focusing (IEF; arrow under blots shows direction of protein resolution) followed by Western immunoblotting. Top: immunoreactive bands of nonphosphorylated (spots a), monophosphorylated (spots b), diphosphorylated (spots c), and triphosphorylated HSP27 (spots d). Bottom: immunoreactivity was quantified by densitometry and expressed as a percentage of total HSP27 in the loaded sample. Results are averages of 3 parallel experiments. *P < 0.05, compared with basal conditions (Student’s t-test).

Fig. 4. PD-098059 (A) has no effect on, whereas SB-203580 (B) partially inhibits, phenylephrine (PE)-stimulated contraction of intact vascular smooth muscle. Canine pulmonary artery strips were incubated with 0.1% DMSO (vehicle control), 50 µM PD-098059 (PD), or 25 µM SB-203580 (SB) for 1 h before a 10-min stimulation with 1 µM PE. Top: force traces were digitized to calculate initial rate of contraction, maximum contraction force, and area under force-time curves. Bottom: areas are presented relative to area under force-time traces of control strips. *P < 0.05, compared with basal conditions (Student’s t-test).
remained unchanged. This is consistent with the interpretation that p38 MAP kinases regulate smooth muscle contraction via a caldesmon-independent mechanism. Because we showed that activation of p38 MAP kinase increases the activity of MAPKAP kinase-2 and phosphorylation of HSP27 (Figs. 2 and 3), here we evaluated the possibility that this pathway is a factor in the development of muscle force. Saponin-permeabilized muscle fibers were incubated with ET-1 and a preimmune rabbit IgG (control), or with ET-1 and a polyclonal rabbit anti-HSP27 antibody, before contraction stimulation with 0.3 µM Ca\(^{2+}\). The effect of preimmune IgG on ET-1-dependent contraction was trivial (Fig. 6, A and C), but the anti-HSP27 antibody produced a significant 20% inhibition of muscle force (Fig. 6, B and C). These observations are consistent with the hypothesis that HSP27 is involved in mechanisms modulating force generation of vascular smooth muscle.

**DISCUSSION**

The role of caldesmon in smooth muscle contraction has been extensively studied in the last decade in an attempt to explain the dissociation between maintained force and rapidly decreasing intracellular Ca\(^{2+}\) concentration and phosphorylation of myosin light chain. A functional coupling between ERK MAP kinases and caldesmon has been proposed and is supported by findings that ERK MAP kinases are physiologically relevant caldesmon kinases in vivo (2). It has also been shown that ERK MAP kinase activity and caldesmon phosphorylation follow a time course similar to that of smooth muscle contraction elicited by a variety of contractile agonists (10, 11, 12, 16). Likewise,
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calmodulin-dependent kinase II, or casein kinase II were proposed to regulate caldesmon functions (17). Thus whether the ERK MAP kinase-caldesmon pathway plays a regulatory role in muscle contraction is still to be determined.

More recent data from our laboratory have shown that not only ERK MAP kinases but also p38 MAP kinases can phosphorylate caldesmon in vitro (15), thus extending the possibilities for multiple regulation of caldesmon phosphorylation. For the first time, in the present study we report that in vitro p38 MAP kinase phosphorylates the same consensus sequences of caldesmon that are phosphorylated by ERK MAP kinases, e.g., Ser-759 and Ser-789. We further demonstrate that even though SB-203580 inhibited ET-1-induced phosphorylation of p38 MAP kinase, MAPKAP kinase-2, and HSP27 in vivo, SB-203580 failed to block caldesmon phosphorylation at these sites. Our results indicate, therefore, that in vivo caldesmon is mainly phosphorylated by ERK MAP kinases, rather than by p38 MAP kinases. This may reflect different spatial intracellular distribution of activated MAP kinases and the possibility that only ERK MAP kinases reside in close proximity with caldesmon, whereas p38 MAP kinases are localized in different cell compartments.

The p38 MAP kinase pathway has been implicated in the regulation of actin assembly via activation of MAPKAP kinase-2 and HSP27 (14). It has also been proposed that HSP27 mediates the development of smooth muscle contraction stimulated by bombesin or PKC (5, 27). We showed in previous experiments with intact canine smooth muscle (15, 18), and we show in the present study, that contractile agonists increase phosphorylation of HSP27 in a SB-203580-sensitive manner. Not only did SB-203580 reduce the initial rate and steady-state muscle force (Fig. 4) but also SB-203580 and the anti-HSP27 antibody significantly decreased endothelin-dependent contraction in skinned fibers (Figs. 5 and 6). Therefore, our results suggest that the p38 MAP kinase/HSP27 pathway is involved in the maintenance of the contractile response of vascular smooth muscle. HSP27 regulates actin polymerization (4, 14), focal adhesions, and cellular interactions with the extracellular environment (24) in a phosphorylation-dependent manner. It could be expected, therefore, that inhibition of HSP27 phosphorylation might decrease contractile responses of vascular smooth muscle. In this respect our skinned-fiber experiments support the idea that reduction of muscle contraction by the anti-HSP27 antibody is solely dependent on HSP27, rather than on other p38 MAP kinase-dependent mechanisms. SB-203580 leads to complete inhibition of ET-1-stimulated MAPKAP kinase-2 activation (Fig. 2D), whereas both SB-203580 and the anti-HSP27 antibody only partially reduce muscle contraction (Figs. 4–6). A likely interpretation is that the HSP27-dependent effects on muscle contraction do not immediately interfere with, but are complementary to, the major contractile mechanism of actin-myosin coupling.

In summary, in this study we tested the role of ERK and p38 MAP kinases and their downstream targets...
caldesmon and HSP27 in the regulation of vascular smooth muscle contraction. Our results suggest that 1) ERK, but not p38 MAP kinases are the major caldesmon kinases in vivo; 2) ET-1-stimulated phosphorylation of ERK MAP kinases and caldesmon occurs simultaneously with smooth muscle contraction but has little effect on muscle force; and 3) ET-1-stimulated activation of p38 MAP kinase pathway modulates smooth muscle contraction via phosphorylation of HSP27.

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