Mechanical Loading Activates Mitogen-activated Protein Kinase and S6 Peptide Kinase in Cultured Rat Cardiac Myocytes*

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The molecular mechanisms by which overloaded cardiac myocytes increase the cell size (hypertrophy) remain unknown. We have previously shown that mechanical loading increased the protein synthesis and the expression of proto-oncogene c-fos mRNA (Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. (1990) J. Biol. Chem. 265, 3585–3598; Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991) J. Biol. Chem. 266, 12865–12868). It has been known that both mitogen-activated protein (MAP) kinase and S6 kinase can be activated by many kinds of growth factors. To clarify whether MAP kinase(s) and S6 kinase(s) are associated with the intracellular signaling of cardiac hypertrophy induced by mechanical loading, we cultured neonatal rat cardiac myocytes in deformable dishes and imposed an in vitro mechanical loading by stretching the adherent myocytes. In this study, we demonstrated that 1) myocyte stretching maximally activated a kinase activity toward myelin basic protein (MBP) at 10 min after stretching, and the kinase activity returned to the control level at 30 min after stretching; 2) kinase assays in MBP-containing gel, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, revealed that stretch-induced MBP kinase activity mainly migrated at 42 kDa in the immunoprecipitated fraction of anti-MAP kinase antibody, suggesting that the stretching mainly increased the 42-kDa MAP kinase activity in cardiac myocytes; 3) phosphorylation of MAP kinase was induced after stretching cardiac myocytes; 4) when protein kinase C was depleted by preincubating myocytes with 100 nm 12-O-tetradecanoylphorbol-13-acetate for 24 h or 2 nm staurosporin for 30 min, stretch-induced MBP kinase activity was decreased by approximately 60–70% as compared with the kinase activity in myocytes without protein kinase C depletion; 5) although the receptor tyrosine kinases were depleted by preincubating myocytes with 50 μM tyrphostin or 20 μM genistein for 30 min, there was no change in the stretch-induced MBP kinase activity; 6) stretch-induced MBP kinase activity was partially dependent on transsarcolemmal influx of Ca2+; 7) myocyte stretching also increased S6 peptide (RRLLSSLRA) kinase activity in the anti-S6 kinase II antibody immunoprecipitates. From these results, we conclude that myocyte stretching increases the activities of MAP kinase and S6 peptide kinase, which may play an important role in the induction of the specific genes and the increase in the protein synthesis.

To increased workload, cardiac myocytes respond by an increase in cell size (hypertrophy), not by an increase in cell number (hyperplasia) (1). Understanding the molecular mechanisms by which cardiac myocytes decipher extracellular signals such as mechanical stress and induce hypertrophy remains to be clarified.

We have previously shown that the stretching increased total RNA content of cultured cardiac myocytes (2). In addition, amino acid incorporation into proteins was also increased after myocyte stretching (3). These results suggest that mechanical loading may directly increase the protein synthesis. Moreover, we have shown that stretching of cardiac myocytes stimulated the expression of fetal-type contractile protein genes as well as the expression of proto-oncogene c-fos mRNA in a protein kinase C-dependent manner (2, 3). However, the black box has been present between the activation of protein kinase C and intranuclear events, and there remains some question whether there is(are) any other signal transduction pathway(s) independent of protein kinase C.

Mitogen-activated protein (MAP)1 kinase (4, 5) is a serine/threonine protein kinase (6) and can be activated by a variety of stimuli such as growth factors (platelet-derived growth factor, epidermal growth factor (EGF), insulin, etc.) and tumor promoters (12-O-tetradecanoylphorbol-13-acetate (TPA), etc.) (7–14). Recently, MAP kinase has been also reported to phosphorylate and activate the product of proto-oncogene c-jun (c-Jun) which is a component of the activator protein 1 (AP-1) transcription factor involved in the mediation of nuclear events elicited by extracellular stimuli (15, 16). Furthermore, it has been known that a variety of growth factors also lead to the activation of S6 kinases (17–22). Therefore, both MAP kinase and S6 kinase may be involved in the molecular mechanisms by which cardiac myocytes induce hypertrophy.

However, more detailed study of the characteristics and regulation of MAP kinase and S6 kinase has been hampered by the lack of specific antibodies and a cDNA clone. Recently,

1 The abbreviations used are: MAP, mitogen-activated protein; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; AP, activator protein; MBP, myelin basic protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; KRP, Krebs-Ringer-phosphate.

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Boulton et al. (11) reported a partial cDNA sequence of the extracellular signal-regulated kinase 1 (ERK1) which has been proposed to encode MAP kinase, and we have prepared the antibody against the synthetic peptide corresponding to the residues 307–327 of the amino acid sequence deduced from the ERK1 cDNA (13). Furthermore, we have also prepared the antibody against the synthetic peptide corresponding to the residues 699–715 of the amino acid sequence (23) deduced from the cDNA of mouse S6 kinase II homologue (rsk<sup>81</sup>) (24). By using these antibodies for immunoprecipitation and immunoblotting, we have established assays to examine both the quantity and the specific activity of MAP kinase and S6 kinase II (13, 23).

In order to elucidate the signal transduction pathways of mechanical loading which induces cardiac hypertrophy, we investigated, in this study, whether the activation of MAP kinase and S6 peptide kinase is involved in the process of cardiac hypertrophy induced by mechanical loading. We here report that myocyte stretching increases MAP kinase and S6 peptide kinase activities in cultured rat cardiac myocytes.

**MATERIALS AND METHODS**

[γ<sup>32</sup>P]ATP (6,600 Ci/mmol) was obtained from Du Point-New England Nuclear. [γ<sup>32</sup>P]Orthophosphate (385 Ci/mg) was from ICN Biomedicals Inc. (Costa Mesa, CA). Myelin basic protein (MBP), protein kinase C inhibitor peptide (rabbit sequence), and TPA were purchased from Sigma. Tyrphostin and genistein (4',5,7-trihydroxyisoflavone) were purchased from Gibco BRL. Nifedipine was a gift from Bayer Yaku hin Ltd. (Tokyo, Japan).

**Cell Culture and Cardiac Myocyte Stretching**—Primary cultures of cardiac myocytes were prepared from the ventricles of 1-day-old Wistar rats essentially according to the method of Simpson et al. (25), and myocyte stretching was conducted as described previously (2, 3). We used silicone rubber culture dishes from Shin-etsu Chemical Co., Ltd., Tokyo, Japan, and cells were plated at a field density of 1 × 10<sup>6</sup> cells/cm<sup>2</sup>. The culture medium was changed 24 h after seeding to a solution consisting of Dulbecco's modified Eagle's medium (DMEM) containing 0.1% fetal bovine serum. We carried out stretch and control experiments simultaneously with the same pool of cells in each experiment. Control cells were treated simultaneously in an identical fashion except for stretching or TPA stimulation so that the manipulation did not make a difference in the other conditions such as the temperature, CO<sub>2</sub> content, or pH of the medium between control, stretched, and TPA-stimulated cells.

**MBP Kinase Assays**—We treated cardiac myocytes with 100 nM TPA for 10 min or stretched them 10% as described previously (2, 3) for 20, 10, or 30 min. The cells were lysed on ice with 0.2 ml of buffer A containing 25 mM Tris-HCl, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM okadaic acid (Wako Pure Chemicals, Japan), 0.5 mM EGTA and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, aliquots of the supernatants of the myocyte extracts were incubated in 40 μl of kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM diithiothreitol (DTT), 40 μM ATP, 2 μCi of [γ<sup>32</sup>P]ATP (2 μM protein kinase inhibitor peptide, 0.5 mM EGTA) and substrates (25 μg MBP). After 10 min at 25 °C, the reaction was stopped by adding 10 μl of stopping solution containing 0.6% HCl, 1 mM ATP, 1% bovine serum albumin. Aliquots of the supernatants (15 μl) were spotted on 1.5 × 1.5-cm squares of P81 paper (Whatman), washed five times for at least 10 min each in 0.5% phosphoric acid, washed in acetic acid, dried, and counted by the Cerenkov technique (13, 26).

**Kinase Assays in MBP-containing Gels after Sodium Dodecyl Sul fate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**—Cardiac myocytes were treated with 100 nM TPA for 10 min or stretched by 10% for 5–30 min. At the termination of stimulation, the cells were lysed. Cell lysates were centrifuged and aliquots of the supernatants were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg/ml MBP (27–29). The gel was removed from the gel by washing the gel with two changes of 100 ml each of 20% 2-propanol in 50 mM Tris-HCl, pH 8.0, for 1 h, and then 250 ml of 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol for 1 h at room temperature. The enzyme was denatured by treating the gel first with two changes of 100 ml of 6 M guanidinium hydrochloride, then renatured with five changes of 250 ml each of 50 mM Tris-HCl, pH 8.0, containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol at 4 °C for 3 h. After renaturation, the gel was renatured at 25 °C for 1 h with 5 ml of 40 mM HEPES, pH 8.0, containing 2 mM DTT, and 10 mM MgCl<sub>2</sub>. Phosphorylation of MBP was carried out by incubating the gel at 25 °C for 1 h with 5 ml of 40 mM HEPES, pH 8.0, 0.5 mM EGTA, 10 mM MgCl<sub>2</sub>, 2 μM protein kinase inhibitor, 40 μM ATP, and 25 μCi of [γ<sup>32</sup>P]ATP. After incubation, the gel was washed with a 7% acetic acid solution until the radioactivity of the solution became negligible. The washed gel was dried and then subjected to autoradiography.

**Kinase Assays of a Y91 Immunoprecipitates in MBP-containing Gels after SDS-PAGE**—We have previously prepared an antibody against the amino acid sequence deduced from the ERK1 cDNA (11), which was used to prepare a polyclonal antibody against a 76 kDa polypeptide whose synthesis was at least a 75% decrease in the presence of 0.1% SDS. The immunoprecipitates were subjected to kinase assays in MBP-containing gels after SDS-PAGE as described above.

**Immunoprecipitation with a Y91 from [γ<sup>32</sup>P]Orthophosphate-labeled Cardiac Myocytes**—Cardiac myocytes were incubated for 3 h with 1 μCi of [γ<sup>32</sup>P]Orthophosphate/culture dish (2 × 4 × 1 cm) in 1 ml of phosphate-free RPMI 1640 medium (Flow Laboratories, United Kingdom); these conditions provide steady-state labeling of endogenous 32<sup>P</sup>-labeled polypeptides (13, 30, 31). Cells were incubated either without or with 10% TPA for 10 min. The cells were then lysed on ice with 0.2 ml of buffer A. Immunoprecipitation with α<sub>Y91</sub> was done in the presence of 0.15% SDS. The immunoprecipitates were washed twice with buffer A containing 250 mM NaCl and twice with buffer A containing 0.025% SDS. The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography.

**Down-regulation of Protein Kinase C and Tyrosine Kinase Activities**—To deplete the protein kinase C or the receptor tyrosine kinase activities, we incubated cardiac myocytes with 100 nM TPA for 24 h, 2 nM staurosporin for 30 min, 50 μM tyrphostin for 30 min, or 20 μM genistein for 30 min before 10% stretching for 10 min. The cells were then lysed and centrifuged, and the supernatants were subjected to MBP kinase assays on the P81 paper described above. It was previously shown that both 100 nM TPA for 24 h and 2 nM staurosporin for 30 min completely inhibited protein kinase C activity in cardiac myocytes (2). Tyrphostin is a membrane-permeable inhibitor of EGF receptor kinase activity and can inhibit signal transduction cascades initiated by the EGF receptor (32–34). Genistein is a nonselective inhibitor of tyrosine kinase activity that competes with ATP for binding to the enzyme (35).

**Inhibition of Transsarcomemmal Influx of Ca<sup>2+</sup>**—To investigate the involvement of transsarcomemmal influx of Ca<sup>2+</sup> in relation to stretch-induced MAP kinase activation, we preincubated cardiac myocytes with Krebs-Ringer-phosphate (KRP) buffer added with 50 μM Ca<sup>2+</sup> for 5 min, and stretched them 10% for 5 min. The cells were subjected to MBP kinase assays on the P81 paper described above. Further, to deplete the influence of either voltage-dependent or -independent Ca<sup>2+</sup> influx, we performed the preincubation with either 10 mM nifedipine (4-(2-nitrophenyl)-2,6-dimethyl-3,5-decarbomethoxy-1,4-dihydropridineline) for 60 min or 1 mM CoCl<sub>2</sub> for 10 min. After the preincubation, myocytes were stretched by 10% for 10 min and subjected to MBP kinase assays on the P81 paper.

**S6 Peptide Kinase Assays**—We have previously prepared an antibodies against the amino acid sequence deduced from the cDNA of mouse S6 kinase II homologue (rsk<sup>81</sup>) (24). This collected antiserum was affinity purified and termed orsk(MC) (23). From the lysates of cardiac myocytes subjected to TPA stimulation or 10% stretching for 10 min, we obtained the orsk(MC) or control preimmune rabbit serum immunoprecipitates, which were incubated with 50 μg of S6 peptide in the presence of 25 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 40 μM ATP, 2 μCi of [γ<sup>32</sup>P]ATP, 2 μM protein kinase inhibitor peptide, 0.5 mM EGTA. After 10 min of incubation at 25 °C, we added 10 μl of stopping solution containing 0.6% HCl, 1 mM ATP, 1% SDS. The kinase activity was calculated by subtracting the radioactivity associated with the immunoprecipitates from the radioactivity of the immunoprecipitates corrected by the Bonferroni method (36). The accepted level of significance was p < 0.05.

**Statistics**—Statistical comparisons of control group with treated groups were carried out using the paired sample t test with p values corrected by the Bonferroni method (36). The accepted level of significance was p < 0.05.
Myocyte Stretching Stimulates Kinase Activity toward MBP—We studied whether cardiac myocyte stretching was able to activate serine/threonine kinase activity. To this end, cardiac myocytes before and after the continuous stretching for 10 min or 100 nM TPA stimulation for 10 min were lysed with buffer A and centrifuged, and the supernatants were subjected to assay for serine/threonine kinase activity using MBP as a substrate. This technique is the defined method which can quantify the MAP kinase activity (13, 26). Myocyte stretching and TPA stimulation activated MBP kinase activity by approximately 1.7- and 2.7-fold, respectively (Fig. 1A). To examine the kinetics of stretch-induced MBP kinase activity, cardiac myocytes before and after stretching for the indicated time periods were subjected to MBP kinase assays. We observed a slight increase in the MBP kinase activity at 5 min after stretching and maximal induction of the activity was observed at 10 min after stretching. The activity returned to the control level at 30 min after stretching (Fig. 1B). Thus, the induction of MBP kinase activity by stretching of cardiac myocytes preceded the accumulation of c-fos mRNA which reached at a maximal level at 30 min (3).

Myocyte Stretching Induces MAP Kinase Activity Migrating at 42 kDa—To determine the proteins responsible for the increased MBP kinase activity, we carried out kinase assays in MBP-containing gels after SDS-PAGE followed by denaturation with 6 M guanidine HCl and renaturation in a buffer containing 0.04% Tween 40. When myocytes were treated with 100 nM TPA for 10 min, we observed MBP kinase activity migrating at 42 and 44 kDa, and the kinase activity of 42-kDa protein was increased by approximately 5.6-fold as compared with the kinase activity in myocytes without TPA stimulation (Fig. 2, A and B). We observed an increased MBP kinase activity migrating at 42 kDa at 10 min after stretching by approximately 2.8-fold, and the kinase activity returned nearly to the control level at 30 min after stretching (Fig. 2, A and C). To identify these molecules, the α91 (one of the anti-MAP kinase antibodies which recognizes both 42- and 44-kDa MAP kinases) (13) immunoprecipitates from cardiac myocytes before and after TPA stimulation or stretching for 10 min were subjected to kinase assays in MBP-containing gels. After 100 nM TPA stimulation for 10 min, we observed an increase in the MBP kinase activity which migrated at 42 and 44 kDa in the α91 immunoprecipitates. After 10 min of stretching, we observed an increase in the MBP kinase activity which migrated at 42 kDa in the α91 immunoprecipitates (Fig. 3). These data confirmed that myocyte stretching indeed increased the kinase activity of 42-kDa MAP kinase.

Next, we examined whether the activated MAP kinase was phosphorylated. When [32P]orthophosphate-labeled myocytes were solubilized and immunoprecipitated with α91, we observed a 42-kDa phosphoprotein. After stretching for 10 min, we observed an increased phosphorylation of this 42-kDa protein (Fig. 4). These data indicate that the MAP kinase which has a molecular mass of 42 kDa is phosphorylated and that this phosphorylation is stimulated by stretching of cardiac myocytes. To identify the phosphoamino acids of the 42-kDa MAP kinase, we further analyzed the [32P]phosphoamino acid content of the phosphorylated MAP kinase. In the basal state, the protein(s) at 42 kDa contained mainly phosphoserine. Myocyte stretching induced de novo threonine and tyrosine phosphorylation of the 42-kDa MAP kinase without a significant change of phosphoserine contents (data not shown). It has been previously shown that MAP kinase is activated when both threonine and tyrosine residues are phosphorylated (13, 37, 38), which supports our present results.

Stretch-induced Increase in MAP Kinase Activity Is via Both Protein Kinase C-independent and -dependent Pathways—We examined whether MAP kinase activation by stretching of cardiac myocytes is via protein kinase C-independent or -dependent pathway. As shown in Fig. 5, TPA stimulation and myocyte stretching activated MBP kinase activity in the cardiac myocytes which were not pretreated with TPA or staurosporin (a-c). However, when myocytes were pretreated with 100 nM TPA for 24 h (d-f), stretch-induced MBP kinase activation was decreased by approximately 60% as compared with the kinase activity in myocytes without the pretreatment (e and f). TPA was not able to activate MBP kinase activity (e), suggesting that protein kinase C was indeed depleted. Prior treatment with staurosporin, a potent and specific protein kinase C inhibitor, also inhibited the stretch-induced MBP kinase activation by approximately 70% (c and i). These data demonstrated that stretch-induced MAP kinase activation was via a partially dependent on the activation of protein kinase C.

Neither Tyrphostin Nor Genistein Inhibits MBP Kinase Activation in Response to Stretching of Cardiac Myocytes—It has been known that a variety of stimuli such as platelet-
Sequential Kinase Activation by Cardiac Myocyte Stretching

Fig. 3. Kinase assays of αY91 immunoprecipitates in MBP-containing gels after SDS-PAGE. Cardiac myocytes were stretched 10% for 10 min (lane b), treated with 100 nM TPA (lane c), or treated without the stimulation (lane a). Next, the cells were lysed in situ and centrifuged, and the supernatants were immunoprecipitated with αY91 in the presence of 0.15% SDS. The immunoprecipitates were electrophoresed on SDS-polyacrylamide gels containing MBP. SDS was removed from the gel, and after denaturation with 6 M guanidine-HCl and renaturation in a buffer containing 0.04% Tween 40, the gel was incubated with [γ-32P]ATP. After washing, the gel was dried and then subjected to autoradiography. The kinase activity at the 42 kDa bands was measured by densitometric scanning of the autoradiogram. Their values were 391.5 (control), 567.2 (10 min), 843.6 (TPA), and 303.3 (background).

Fig. 4. Immunoprecipitation with αY91 from [32P]orthophosphate-labeled cardiac myocytes. [32P]Orthophosphate-labeled cardiac myocytes were treated without (lane a) or with 10% stretching for 10 min (lane b) at 37 °C, lysed, and immunoprecipitated with αY91. The immunoprecipitates were washed and subjected to SDS-PAGE followed by autoradiography.

derived growth factor, EGF, or insulin etc. activate MAP kinase through the activation of the receptor tyrosine kinase. To clarify whether myocyte stretching activates MAP kinase indirectly through another stimuli, we preincubated cardiac myocytes with the receptor tyrosine kinase inhibitors and stretched them 10% for 10 min. After the preincubation with tyrphostin or genistein, myocyte stretching activated MBP kinase activity by approximately 1.7-fold (Fig. 6), and stretch-induced MBP kinase activation remained unchanged (b, d, and f) (p = 0.751, b versus d; p = 0.924, b versus f). After prior treatment with tyrphostin or genistein, stimulation with...
FIG. 5. MBP kinase assays in protein kinase C down-regulated cardiac myocytes after stretching. Cardiac myocytes were pretreated with 100 nM TPA for 24 h (d-f) or 2 nM stauros- porin for 30 min (g-i). They were either stretched by 10% for 10 min (b, e, and h). After lysis and centrifuga-
tion, aliquots of the supernatants were incubated in kinase buffer and sub-
strates (25 μg of MBP). After stopping the reaction, aliquots of the supernatants were spotted on P81 paper, washed, dried, and counted by the Cerenkov tech-
nique. Each histogram represents the average percentage of control (a) from five independent experiments (mean ± S.E.). Statistical analysis was performed using paired sample t test with p values cor-
rected by the Bonferroni method (36) (**p < 0.01, ***p < 0.001 versus control (a), †p < 0.01 versus control (d), ‡p < 0.05 versus control (g)).

FIG. 6. Effect of tyrphostin and genistein on stretch-induced MBP kinase activation. Cardiac myocytes were pretreated with 50 μM tyrphostin (c and d) or 20 μM genistein (e and f) for 30 min. They were stretched by 10% for 10 min (b, d, and f), and MBP kinase assays on P81 paper were performed. Data represent the average percentage of control (a) from five independent experiments (mean ± S.E.) (**p < 0.01, ***p < 0.001 versus control (a)).

EGF was not able to activate MBP kinase activity (data not shown). Therefore, both tyrphostin and genistein failed to inhibit stretch-induced MAP kinase activation.

Stretch-induced Increase in MAP Kinase Activity Is only Partially Involved in Both Voltage-dependent and -independ-
ent Transsarcolemmal Influx of Ca2+—We investigated whether stretch-induced MAP kinase activation is involved in transsarcolemmal influx of Ca2+ because it has been known that the stretching increases the resting intracellular Ca2+ concentration in cardiac myocytes (39). First, to eliminate the influence of transsarcolemmal influx of Ca2+, cardiac myo-
cytes were preincubated with KRP buffer containing only 50 μM Ca2+ for 30 min (Fig. 7A, e and f). In this buffer myocyte stretching activated MBP kinase activity by approximately 1.3-fold (e and f) although in DMEM or KRP buffer containing 1.3 mM Ca2+ myocyte stretching increased MBP kinase
activity by approximately 1.7-fold (a–d). The reduction of the MBP kinase activation was significant (p = 0.022, d versus f). Next, we examined the relationship between stretch-induced MBP kinase activation and voltage-dependent or -independent transsarcolemmal influx of Ca\(^{2+}\) (Fig. 7B). When voltage-dependent or -independent Ca\(^{2+}\) channels were blocked by 10 nM nifedipine (c and d) or 1 mM CoCl\(_2\) (e and f), the activities of MBP kinase were decreased by 33% (d) or 29% (f) compared with the activity in cardiac myocytes without the pretreatment (b), respectively (p = 0.025, b versus d; p = 0.004, b versus f). Thus, we concluded that the stretch-induced MAP kinase activation was only partially dependent on transsarcolemmal Ca\(^{2+}\) influx through both voltage-dependent and -independent Ca\(^{2+}\) channels.

**FIG. 7.** The effects of transsarcolemmal influx of Ca\(^{2+}\) on stretch-induced MBP kinase activation in cardiac myocytes. A, cardiac myocytes were preincubated with DMEM containing 1.3 mM Ca\(^{2+}\) (a and b), KRP buffer containing 1.3 mM Ca\(^{2+}\) (c and d), or KRP buffer containing 50 mM Ca\(^{2+}\) (e and f) for 30 min. MBP kinase assays on P81 paper were performed. Each histogram represents the percentage of control (a) from eight independent experiments (mean ± S.E.) (**p < 0.01 versus control (a), † † † p < 0.01 versus control (c), † † † † p < 0.05 versus control (e)). B, myocytes were preincubated with DMEM added with 10 nM nifedipine for 60 min (c and d) or 1 mM CoCl\(_2\) for 10 min (e and f). They were stretched by 10% for 10 min (b, d, and f). MBP kinase assays on P81 paper were performed. Each histogram represents the average percentage of control (a) from at least 10 independent experiments (mean ± S.E.) (**p < 0.001 versus control (a), † † † p < 0.001 versus control (e), † † † † p < 0.01 versus control (c)).

**Myocyte Stretching Also Stimulates S6 Peptide Kinase Activity**—We measured S6 peptide kinase activity in the immunoprecipitates with control serum (Fig. 8, a–c) or anti-S6 kinase II antibody (ask(m)C) (d–f) from cardiac myocytes. Control serum did not immunoprecipitate TPA- or stretch-induced S6 peptide kinase activity using S6 peptide (RRLSSLRA) as a substrate (a–c). However, both 100 nM TPA stimulation for 10 min and myocyte stretching by 10% for 10 min increased S6 peptide kinase activity by approximately 4- and 3-fold, respectively (d–f).

**DISCUSSION**

The major findings of the present study are that stretching of cardiac myocytes principally activates 42-kDa MAP kinase via both protein kinase C-dependent and -independent pathways and via the receptor tyrosine kinase-independent pathways, and that this activation is partially dependent on transsarcolemmal influx of Ca\(^{2+}\). Cardiac hypertrophy is one of the most important clinical complications of many cardiovascular disorders. It has been reported that cardiac death occurs at a higher incidence in hypertensive patients with cardiac hypertrophy than in those without hypertrophy (40, 41). Hypertrophied heart has been shown to have impaired contractile as well as relaxing functions and the reduced reactive hyperemia following transient ischemia, all of which could lead to the higher mortality of ischemic heart diseases (41–43). Therefore, management of cardiovascular diseases should also be directed to reducing as well as preventing cardiac hypertrophy. In order to achieve this, it is mandatory to understand the cellular and molecular mechanisms by which cardiac hypertrophy is induced and maintained. In particular, cardiac myocytes have developed unique subcellular mechanisms to induce hypertrophy in response to mechanical loading. In this study, we tried to elucidate the intracellular signal transduction system of cardiac myocytes elicited by mechanical stress.
Stretching of Cardiac Myocytes in Vitro Induces the Protein Synthesis and the Gene Expression Characteristic of Hypertrophied Hearts in Vivo—There are many reports that pressure or volume overload not only induces specific gene expression but also accelerates protein synthesis in cardiac hypertrophy (44-47). However, molecular mechanisms of cardiac hypertrophy induced by mechanical stimuli have not been well characterized due to technical difficulties in directly imposing mechanical stimuli on cardiac myocytes. It remains unknown whether mechanical loading directly regulates gene expression and protein synthesis without participation of humoral or neural factors. To overcome this, we have developed original deformable culture dishes made of silicone rubber which can provide the various degree of stretching on the cell. By using this culture system, we have previously shown that myocyte stretching directly increases protein synthesis as well as induces the specific gene expression. Namely, we have reported that the gene expression of skeletal actin, β-myosin heavy chain, and c-fos was increased (2), all of which are known to be expressed in hypertrophied hearts (44-47), and that [3H]phenylalanine incorporation into proteins was also increased by 25% even 2 h after stretching (3). These results indicate that myocyte stretching in vitro can be a good model for studying the intracellular events that occur during the process of stretch-induced cardiac hypertrophy.

MAP Kinase Is Activated by Stretching of Cardiac Myocytes—We have already clarified that mechanical loading in vitro stimulates protein kinase C activity as well as c-fos mRNA expression, which is likely to play key roles in transcription of many genes and an increase in protein synthesis (2, 3). However, it remains unknown what kind of signal transduction systems exist downstream protein kinase C and which enzymes are more specifically involved in this process.

First, we showed that kinase activity toward MBP, the specific substrate of MAP kinase, is increased by stretching as well as by TPA. Second, using in-gel kinase assay, we demonstrated that 42-kDa protein in cardiac myocytes is responsible for the increased MBP kinase activity following stretching. Third, we identified the 42-kDa protein kinase which phosphorylates MBP is one of MAP kinases by using a specific antibody. All of these results clearly indicate that 42-kDa MAP kinase is mainly activated by stretching of cardiac myocytes. We were not able to detect the stretch-induced increase in the kinase activity of 44-kDa MAP kinase, probably because the amount of 44-kDa MAP kinase in cardiac myocytes may have been too low to detect the kinase activity in our assay system. In fact, Western blotting with αY91 revealed that the amount of 44-kDa MAP kinase was lower as compared with that of 42-kDa MAP kinase (data not shown). Recently, cDNA of MAP kinase was cloned (48) and the purified MAP kinase was shown to specifically phosphorylate the regulatory tyrosine and threonine residues of MAP kinase causing its full activation (49, 50). Both TPA and stretch-induced MAP kinase activation are probably via the activation of MAP kinase kinase which is an activator of both ERK1 and ERK2 (51) because the restricted substrate specificity is found for MAP kinase kinase (26, 49). Therefore, we believe that myocyte stretching increases both 42- and 44-kDa MAP kinase activities and that the reason we failed to detect the 44-kDa MAP kinase activation is the limitation of our detection system.

Activation of MAP Kinase Occurs by Both Protein Kinase C-dependent and -independent Pathways and via the Receptor Tyrosine Kinase-independent Pathways—Recently, it has been reported that MAP kinase activation by EGF is protein kinase C-independent, but that nerve growth factor-induced activation of MAP kinase is partially dependent on protein kinase C activation (6). In the present study, stretch-induced MAP kinase activation in myocytes pretreated with TPA was decreased by approximately 60% as compared with the kinase activity in myocytes without pretreatment. Furthermore, prior treatment with staurosporin also inhibited stretch-induced MAP kinase activation by approximately 70%. In this study, we have demonstrated that MAP kinase is activated by myocyte stretching in both protein kinase C-dependent and -independent manner. The presence of multiple signal transduction pathways may be favorable for cardiac myocytes to quickly respond to mechanical stress. On the other hand, neither tyrphostin nor genistein had any effect on the stretch-induced activation of MAP kinase. These results indicate that stretch-induced MAP kinase activation can occur via the receptor tyrosine kinase-independent pathway.

MAP Kinase Activation Is Only Partially Dependent on Both Voltage-dependent and -independent Ca++ Influx—It has been shown that stretching of cardiac myocytes increases intracellular Ca++ concentration in a few seconds (39). Furthermore, Sigurdson et al. (52) indicated that mechanical stimuli on a model system of tissue-cultured heart cells produced a transsarcolemmal influx of Ca++ which led to waves of calcium-induced calcium release and that the response was blocked by removing extracellular Ca++. Therefore, we examined the relationship between MAP kinase activation by stretching and transsarcolemmal influx of Ca++. We showed that stretch-induced MAP kinase activation in cardiac myocytes is decreased by 30% after blocking either voltage-dependent or -independent Ca++ channels and decreased by 50% by blocking both channels. Stimulation with 10-7 M Bay K 8644, voltage-dependent Ca++ channel agonist, also activated MBP kinase activity (data not shown). Thus, we have shown for the first time that stretch-induced MAP kinase activation is only partially dependent on the Ca++ signaling pathway. Consistent with these results, Tsuda et al. (53) reported that angiotensin II-induced MAP kinase activation in vascular smooth muscle cells was inhibited by 25% by prior treatment with EGTA. Moreover, since MAP kinase activity is sensitive to Ca++ in crude cell extracts, but insensitive in the partially purified fraction (27), intracellular Ca++ is thought to indirectly (through other factors) activate MAP kinase.

S6 Kinase Is Also Activated in Cardiac Myocytes by Stretching—Our present study has demonstrated for the first time that stretching activates S6 kinase in cardiac myocytes. S6 kinase was recently identified as one potentially important lamin kinase by Ward et al. (54). S6 kinase may act as a mediator between the signal transduction pathways and the intranuclear events because the laminins are intermediate filament proteins that form a fibrous layer at the periphery of the nucleus. Therefore, stretch-induced S6 kinase activation may also lead to increase in the specific gene expression.

Significance of MAP Kinase Activation in the Specific Gene Expression in Cardiac Hypertrophy—We have previously shown that myocyte stretching induces c-fos expression (2, 3). In this study, we have demonstrated that stretching of cardiac myocytes activates MAP kinase. Recently, Gille et al. (55) reported that MAP kinase phosphorylates the transcription factor p62TCT and enhances the complex formation at c-fos promoter which can lead to the c-fos induction. Therefore, MAP kinase activation should play a critical role in stretch-induced c-fos expression. Furthermore, it has been recently shown that MAP kinase is able to phosphorylate and activate c-Jun (15, 16). Since both c-Fos (the product of c-fos) and c-Jun are components of AP-1 transcription factor, the induction of c-fos caused by stretching and possible phosphorylation...
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and activation of c-Jun by MAP kinase may synergistically activate the function of AP-1 complex (56). In this regard, we have previously presented evidence showing that AP-2 is involved in the altered expression of the human ventricular myosin alkali light chain gene (57). Likewise, altered function of AP-1 induced by MAP kinase may also be involved in the altered expression of a panel of other genes which can lead to an increase in the specific gene expression. However, Nagai et al. (58) indicated that not only ribosomal RNA content but also the efficiency of protein synthesis (synthesis/ribosomal RNA) is significantly increased in the pressure-overloaded rabbit hearts. It remains to be clarified whether myocyte stretching induces the posttranscriptional activation of protein synthesis.

Cardiac hypertrophy has been regarded as a secondary response of the heart to a sustained increase in load (59). However, it is known that excessive hypertrophy predisposes to the cardiac failure and leads to an increased mortality rate (40, 41). Therefore, the development of prevention and treatment of cardiac hypertrophy is an important theme for the cardiologist, which requires elucidation of the mechanisms of cardiac hypertrophy.

In many clinical studies, some kinds of anti-hypertensive agents such as angiotensin converting enzyme inhibitors reduce left ventricular hypertrophy with moderate blood pressure control (60–63), whereas vasodilator hydralazine does not reduce ventricular hypertrophy or even accelerates it even though blood pressure is controlled to normotensive levels (64, 65). These discrepancies between reduction of afterload and regression of hypertrophy suggest that humoral or neural factors as well as pressure overload directly regulate cardiac hypertrophy. These signal transduction pathways should be common at least in part because angiotensin II (one of humoral factors) can activate the function of AP-1 and activation of c-Jun by MAP kinase may also be involved in the altered expression of the human ventricular myosin alkali light chain gene (57). To elucidate intracellular signaling of cardiac hypertrophy may lead to the development of new cardioprotective agents.

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