Molecular Analysis of Cardiac Hypertrophy by Mechanical Stress: Involvement of Stretch-Induced Protein Kinase Cascade

機械的刺激により生じる心筋肥大の分子生物学的解析
ー蛋白リン酸化酵素カスケードの役割ー

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Summary

The molecular mechanism(s) by which overloaded cardiac myocytes increase the cell size (hypertrophy) remain unknown. Previously, it has been shown that mechanical stress increases protein synthesis as well as the expression of proto-oncogene *c-fos* mRNA in a protein kinase C-dependent manner. It has been known that protein synthesis is increased by the activation of S6 kinase, which is phosphorylated and activated by mitogen-activated protein (MAP) kinase. In addition, it has been recently reported that MAP kinase kinase which is a MAP kinase activator lies downstream of Raf-1 kinase. Furthermore, both MAP kinase and Raf-1 kinase induce the transcriptional activation of specific target genes. Thus, to clarify the role of phosphorylation cascade as the intracellular signaling of mechanical stress-induced cardiac hypertrophy, I examined the activation of these four kinases (Raf-1 kinase, MAP kinase kinase, MAP kinase and S6 kinase) by stretch of cardiac myocytes in deformable dishes. In this study, I demonstrated that (1) MAP kinase was activated by stretch and the activity reached maximum at 10 min after stretch, (2) kinase assays in substrate-containing gel revealed that stretch activated 42 kDa-MAP kinase, (3) threonine and tyrosine residues of MAP kinase were phosphorylated *de novo* by stretch, (4) stretch-induced MAP kinase activation was partially dependent on transsarcolemmal influx of Ca^{2+} but independent of the receptor tyrosine kinase activation, (5) depletion of protein kinase C partially suppressed stretch-induced MAP kinase activation, (6) myocyte-stretch also activated S6 kinase, and (7) sequential activation of Raf-1 kinase and MAP kinase kinase was induced by stretch. From these results, I conclude that phosphorylation cascade involved in protein synthesis as well as gene expression is activated in cardiac myocytes by mechanical stress. The results also suggest the presence of multiple pathways in the activation of phosphorylation cascade, which may facilitate the formation of cardiac hypertrophy in response to mechanical loading.

Footnotes

Abbreviations used in this paper are:

PKC, protein kinase C;

MAP, mitogen-activated protein;

AP-1, activator protein 1;

MBP, myelin basic protein;

TPA, 12-*O*-tetradecanoylphorbol-13-acetate;

TPCK, tosylphenylalanine-chloromethyl-ketone;

DMEM, Dulbecco's modified Eagle's medium;

ERK1, extracellular signal-regulated kinase 1;

EGTA, ethylene glycol-bis(b-aminoethyl ether) N,N,N',N',-tetraacetic acid;

SDS, sodium dodecyl sulfate;

PAGE, polyacrylamide gel electrophoresis;

DTT, dithiothreitol;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

2-D, two dimension or two-dimensional;

KRP, Krebs-Ringer-phosphate;

rMAPK, recombinant MAP kinase;

S.E., standard error;

EGF, epidermal growth factor;

PLC, phospholipase C.

Introduction

Cardiac hypertrophy which is an adaptation to a sustained increase in overload is one of the most important clinical complications of cardiovascular disorders. It is known that hypertrophy is associated with cardiac failure and an increased mortality rate [1, 2]. Understanding the molecular mechanism(s) involved in cardiac hypertrophy is extremely important because the development of new treatments for heart diseases and their evaluation will become possible on these basis.

It has been shown that stretch of cardiac myocytes increased not only total RNA content in the cell [3] but also amino acid incorporation into proteins [4], suggesting that mechanical loading directly increases protein synthesis, and that stretch stimulates the expression of fetal-type contractile protein genes. Moreover, the expression of proto-oncogene *c-fos*, an important transcription factor, was activated by stretch in a protein kinase C (PKC)-dependent manner. However, the intracellular signal transduction in cardiac myocytes which mediates between mechanical stress and the acceleration of protein synthesis or specific gene expression has not been well characterized.

Increased protein synthesis is generally associated with an increase in phosphorylation of S6 protein in 40 S ribosome [5]. It has been suggested that phosphorylation of S6 protein is increased by the activation of S6 kinase. Recent reports have suggested that one of S6 kinases is phosphorylated and activated by mitogen-activated protein (MAP) kinase [6, 7]. More recently, Raf-1 kinase which is the product of *raf-1* proto-oncogene has been shown to activate MAP kinase kinase [8-10]. Therefore, these four kinases (Raf-1 kinase, MAP kinase kinase, MAP kinase and S6 kinase) can be the candidates mainly involved in the acceleration of protein synthesis in hypertrophied heart (translational activation).

Furthermore, MAP kinase has also been shown to phosphorylate and activate the proto-oncogene *c-jun* product (c-Jun) which is a component of the activator protein 1 (AP-1) transcription factor [11, 12]. In addition, Bruder *et al.* have indicated that Raf-1 kinase can trans-activate genes containing the AP-1-binding site [13]. These data suggest that MAP kinase and Raf-1 kinase are the mediators of specific gene expression in cardiac hypertrophy (transcriptional activation).

However, more detailed analyses on the characteristics and the regulation of these four kinases have been hampered by lack of specific antibody. Recently, antibodies against MAP kinase [14], S6

kinase [15], Raf-1 kinase [16] and MAP kinase kinase [17] have been produced. By immunoprecipitation and immunoblotting using these four antibodies, assay systems to measure both the quantity and the specific activities of MAP kinase, S6 kinase, Raf-1 kinase and MAP kinase kinase have been established [14-18].

In order to elucidate the involvement of protein kinase cascade in cardiac hypertrophy, I investigated whether the sequential activation of Raf-1 kinase, MAP kinase kinase, MAP kinase and S6 kinase can be induced by myocyte-stretch. I here report that mechanical stress induces phosphorylation of these protein kinases followed by the activation of their enzymatic activities.

Materials and Methods

[γ-^{32}P]ATP (6,000 Ci/mmole) was obtained from Du Point-New England Nuclear (Boston, MA). [^{32}P]orthophosphate (285 Ci/mg) was from ICN Biomedicals (Irvine, CA). Myelin basic protein (MBP), protein kinase inhibitor peptide (rabbit sequence) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma, and tosylphenylalanine-chloromethyl-ketone (TPCK)/trypsin from Worthington.

Cell Culture and Cardiac Myocyte-Stretch

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.* [19], and myocyte-stretch was conducted as described previously [3, 4]. I used silicone rubber culture dishes from Shin-Etsu Chemical Co., Ltd., Tokyo, Japan, and cells were plated at a field density of 1 x 10^5 cells/cm^2. The culture medium was changed 24 h after seeding to a serum-free solution consisting of Dulbecco's modified Eagle's medium (DMEM). Stretch and control experiments were carried out simultaneously with the same pool of cells in each experiment. Control cells were treated simultaneously in an identical fashion except for stretch or TPA stimulation so that the experiments could be performed under the same conditions concerning temperature, CO_2 content or pH of the medium between control, stretched and TPA-stimulated cells. It has been previously reported that a 10 % change in the length of the dish resulted in a 9.3 ± 0.9 % (n=100) change in the length of the cell along a single axis [4], and after pretreatment with TPA, the same result was obtained (9.4 ± 1.2 % (n=10)).

Immunoblotting with anti-MAP kinase antibody, anti-Raf-1 kinase antibody or anti-MAP kinase kinase antibody

Antibodies against MAP kinase (αY91), Raf-1 kinase, MAP kinase kinase and S6 peptide kinase were provided from Dr. Kadowaki's group [14, 15, 16, 17]. Cardiac myocytes were stretched or treated with 100 nM TPA, and lysed on ice with buffer A containing 25 mM Tris-HCl, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 nM okadic acid (Wako Pure Chemicals, Japan), 0.5 mM ethylene glycol-bis (b-aminoethyl ether) N,N,N',N',-

tetraacetic acid (EGTA) and 1 mM phenyl-methyl sulfonyl fluoride. After centrifugation, aliquots of the supernatants of myocyte-extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto Immobilon-P membrane (Millipore) using a Milli Blot-SDS system. The unoccupied protein binding sites on the membrane were blocked by 3 % bovine serum albumin in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1 % Triton X-100 for 1 h at room temperature. The membrane was incubated with 10 μg/ml of αY91, anti-Raf-1 kinase antibody or anti-MAP kinase kinase antibody for 10 h at 4 °C. After washing, it was incubated 1 h with alkaline phosphatase-coupled second antibody (Promega Biotec), and developed using the ProtoBlot immunoblotting system (Promega Biotec) according to the manufacturer's instructions.

MBP Kinase Assays

Cardiac myocytes were treated with 100 nM TPA for 10 min, or stretched by 10 % as described previously [3, 4] for 5, 10, 20 or 30 min. The cells were lysed on ice with 0.2 ml of buffer A. After centrifugation, aliquots of the supernatants of myocyte-extracts were incubated in 40 μl of kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 40 μM ATP, 2 μCi [γ-^{32}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 x 1.5 cm squares of P81 paper (Whatman), washed five times for at least 10 min each in 0.5 % phosphoric acid, washed in acetone, dried and counted by Cerenkov [14].

Kinase Assays in MBP-Containing Gels after SDS-PAGE

Cardiac myocytes were treated with 100 nM TPA for 10 min, or stretched by 10 % for 5 to 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 [20-22]. SDS 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 guanidine/HCl at room temperature for 1 h and 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 preincubated at 25 °C for 1 h with 5 ml of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0) containing 2 mM DTT, and 10 mM $MgCl_2$. 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 $MgCl_2$, 2 μM protein kinase inhibitor, 40 μM ATP, and 25 μCi [γ-^{32}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 αY91 Immunoprecipitates in MBP-Containing Gels after SDS-PAGE

Cardiac myocytes were treated with 100 nM TPA for 10 min, or stretched by 10 % for 10 min. Cell lysates were centrifuged and the supernatants were immunoprecipitated with αY91 in the presence of 0.15 % of SDS. The immunoprecipitates were subjected to kinase assays in MBP-containing gels after SDS-PAGE as described above.

Immunoprecipitation with αY91 from [^{32}P]orthophosphate-labeled cardiac myocytes

Cardiac myocytes were incubated for 3 h with 1 mCi of [^{32}P]orthophosphate per culture dish (2 x 4 x 1 cm) in 1 ml phosphate-free RPMI 1640 medium (Flow Laboratories, U.K.). These conditions provide steady-state labeling of endogenous ^{32}P-labeled polypeptides [23, 24]. Cells were incubated either with or without 10 % stretching for 10 min at 37 °C. The cells were then lysed on ice with 0.2 ml of buffer A. Immunoprecipitation with αY91 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.

Phosphoamino Acid Analysis

The phosphoprotein bands were excised from the dried gel. The gel pieces were then washed twice with 25 % isopropyl alcohol for 30 min, twice with 10 % ethanol for 30 min, and dried [25].

The gel pieces were resuspended in 1 ml of 50 mM NH_4HCO_3 at pH 8.3 and 100 µg of TPCK-trypsin was added. After incubation at 37 °C for 6 h, additional 100 µg of TPCK-trypsin was added and digestion was continued for additional 12 h. The digests were dried, resuspended in H_2O, and dried again twice and redissolved in a small volume of 30 % formic acid. The phosphopeptides were separated in two dimensions (2-D) as described previously [25]. Two-dimensional (2-D) phosphoamino acid analysis was performed by hydrolyzing the TPCK/trypsin-treated phosphopeptides, or phosphopeptides recovered from the cellulose plates, in 6 N HCl at 110 °C for 60 min, followed by electrophoresis (first dimension; formic acid/acetic acid/water (11:39:450), pH 1.9, second dimension, pyridine/acetic acid/water (1:10:189), pH 3.5 [26].

Inhibition of Transsarcolemmal Influx of Ca^{2+}

To investigate the involvement of transsarcolemmal influx of Ca^{2+} in relation to stretch-induced MAP kinase activation, we preincubated cardiac myocytes with Krebs-Ringer-phosphate (KRP) buffer added with 50 µM Ca^{2+} for 30 min, and stretched them by 10 % for 10 min. The cells were subjected to MBP kinase assays on P81 paper described above. Further, to deplete the influence of either voltage-dependent or -independent Ca^{2+} influx, I performed the preincubation of cardiac myocytes with either 10 nM nifedipine (4-(2'-nitrophenyl) -2, 6-dimethyl -3, 5-decarbomethoxy-1, 4-dihydropyridine) for 60 min or 1 mM $CoCl_2$ for 10 min. After the preincubation, myocytes were stretched by 10 % for 10 min and subjected to MBP kinase assays on P81 paper.

Down-Regulation of the Receptor Tyrosine Kinase and PKC Activities

To deplete the receptor tyrosine kinase or PKC activities, I incubated cardiac myocytes with 50 µM tyrphostin for 30 min, 20 µM genistein for 30 min, 100 nM TPA for 24 h or 2 nM staurosporine for 30 min before 10 % stretch for 10 min. The cells were lysed and centrifuged, and the supernatants were subjected to MBP kinase assays on P81 paper described above. Tyrphostin is a membrane-permeable inhibitor of EGF receptor kinase activity and can inhibit signal transduction cascades initiated by the epidermal growth factor (EGF) receptor [27-29]. Genistein is a nonselective inhibitor of tyrosine kinase activity that competes with ATP for binding to the enzyme [30]. It has been shown that both tyrphostin and genistein are potent inhibitors of the receptor tyrosine kinase

with little significant effect on serine/threonine kinases such as PKC [31]. Moreover, it has been previously shown that both 100 nM TPA for 24 h and 2 nM staurosporine for 30 min completely inhibited PKC activity in cardiac myocytes [3].

S6 Peptide Kinase Assays

Antibody against S6 peptide kinase was provided from Dr. Kadowaki's group [15]. From the lysates of cardiac myocytes subjected to TPA stimulation or stretch for 10 min, we obtained the anti-S6 peptide kinase antibody or control preimmune rabbit serum immunoprecipitates, which were incubated with 50 μg of S6 peptide (RRLSSLRA) in the presence of 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, 2 μCi [γ-^{32}P]ATP, 2 μM protein kinase inhibitor peptide, 0.5 mM EGTA. After 10 min incubation at 25 °C, I added 10 μl of stopping solution containing 0.6 % HCl, 1 mM ATP, 1 % bovine serum albumin. After centrifugation, aliquots of the supernatants (15 μl) were spotted on 1.5 x 1.5 cm squares of P81 paper, washed five times for at least 10 min each in 0.5 % phosphoric acid, washed in acetone, dried and counted by Cerenkov.

Assay of MAP kinase kinase activity

MAP kinase kinase activity was assayed using recombinant MAP kinase fused to maltose binding protein (rMAPK). To chromatographically separate MAP kinase kinase from endogenous MAP kinase, cell lysates were applied to Q-sepharose column and flow through fractions [32, 33] were immunopreciptated with anti-MAP kinase kinase antibody. The immunoprecipitates were incubated with buffer B containing 25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, 2 μCi [γ-^{32}P]ATP, 2 μM protein kinase inhibitor peptide and 0.5 mM EGTA and substrates (100 μg rMAPK) for 30 min at 25 °C. rMAPK was collected using amylose resin and was electrophoresed on 10 % polyacrylamide gel.

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 [34]. The accepted level of significance was $P<0.05$.

Results

42 kDa-MAP Kinase is Predominant in Cardiac Myocytes.

The anti-MAP kinase antibody, αY91 recognizes both 42 kDa- and 44 kDa-MAP kinase in the presence of 0.15 % SDS [14]. By using this antibody, I first examined the relative abundance of 42 kDa- and 44 kDa-MAP kinase in cultured neonatal rat cardiac myocytes. As shown in Fig. 1, 42 kDa-MAP kinase was predominant in rat cardiac myocytes. MAP kinase stimulated by myocyte-stretch was also recognized by αY91.

Fig. 1. 42 kDa-MAP kinase is predominant in both stretched and unstimulated cardiac myocytes.

Cardiac myocytes stimulated without (*a*) or with (*b*) myocyte-stretch for 10 min were lysed and equalized for protein content before electrophoretic separation on 12% SDS-PAGE and transferred to nitrocellulose filters. Protein blots were probed with anti-MAP kinase antibody (αY91) and immunological reactions were identified by using an alkaline phosphatase detection system.

Stretch of Cardiac Myocytes Stimulates Kinase Activity toward MBP.

I studied whether stretch of cardiac myocytes was able to activate MAP kinase activity. To this end, cardiac myocytes before and after the continuous stretch 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 MAP kinase activity using MBP as a substrate. Myocyte-stretch and TPA stimulation activated MBP kinase activity by approximately 1.7-fold and 2.7-fold, respectively (Fig. 2A). Next, to examine the kinetics of stretch-induced MBP kinase activity, cardiac myocytes before and after stretch for the indicated periods of time were subjected to MBP kinase assay. I observed a slight increase in the MBP kinase activity at 5 min after stretch and maximal induction of the activity was observed at 10 min after stretch. The activity returned to the control level at 30 min after stretch (Fig. 2B). Thus, the induction of MBP kinase activity by stretch preceded the accumulation of *c-fos* mRNA by stretching which reached at a maximal level at 30 min [4].

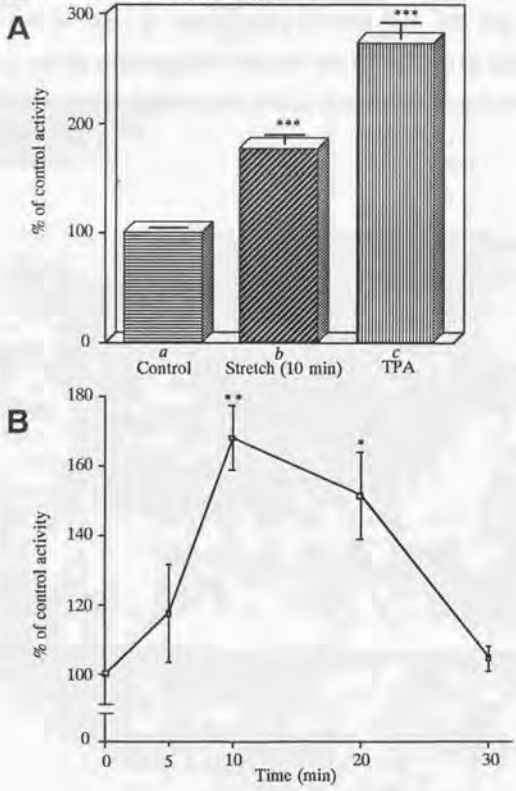
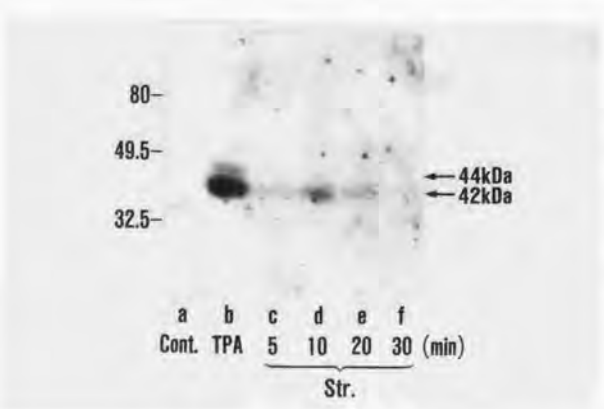


Fig. 2. Cardiac myocyte-stretch activates MBP phosphorylation activity.

Cardiac myocytes were stretched by 10 % for the indicated periods of time or treated with 100 nM TPA for 10 min. Aliquots of the supernatants of cardiac myocyte-extracts were incubated in buffer A and MBP. After stopping the reaction, aliquots of the supernatants were spotted on P81 paper (Whatman), washed, dried and counted by Cerenkov. Data represent the average percentage of control from eight (A) or five (B) independent experiments (mean ± standard error (S.E.)) (*P<0.05 , **P<0.01 and ***P<0.001 versus control).

Stretch of Cardiac Myocytes Induces 42 kDa-MAP Kinase Activity.

To determine the proteins responsible for the increased MBP kinase activity, I 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, I 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 (Fig. 3A, 3B). We observed an increased MBP kinase activity migrating at 42 kDa at 10 min after stretch by approximately 2.8-fold, and the kinase activity returned nearly to the control level at 30 min after stretch (Fig. 3A, 3C).



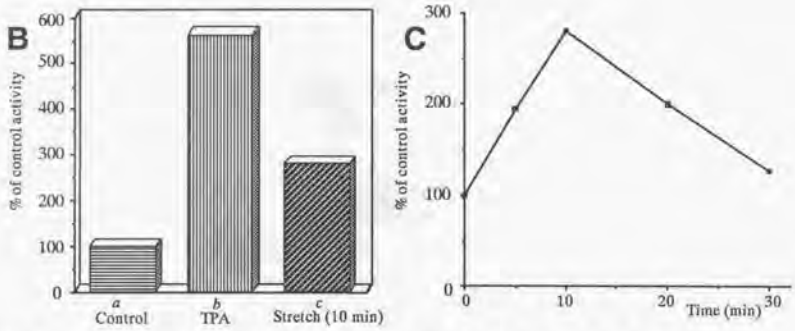


Fig. 3. Stretch-induced MBP phosphorylation activity migrates at 42 kDa.

A, cardiac myocytes were treated with 100 nM TPA (lane *b*), or stretched by 10 % for 5, 10, 20 or 30 min (lane *c, d, e, f*). The cells were lysed *in situ* and the aliquots of the supernatants of the lysates were electrophoresed on SDS-polyacrylamide gels containing MBP. The gel was incubated with [γ-^{32}P]ATP and subjected to autoradiography. The aliquots of the supernatants of the cell lysates were also subjected to MBP phosphorylation study using P81 paper, and each MBP kinase activity counted by Cerenkov was 2909 (control), 8790 (TPA), 4045 (5 min), 5011 (10 min), 4223 (20 min) and 3031 cpm (30 min). B and C, the kinase activity at the 42 kDa bands was measured by densitometric scanning of the autoradiogram. Their values were 299.8 (control), 995.8 (TPA), 443.2 (5 min), 574.6 (10 min), 452.0 (20 min), 341.5 (30 min) and 148.3 (background). After the background subtraction, the values became 151.5 (control), 847.5 (TPA), 294.9 (5 min), 426.3 (10 min), 303.7 (20 min) and 193.2 (30 min). Each datum represents the percentage of control.

To identify these molecules, the anti-MAP kinase antibody (αY91)-immunoprecipitates from cardiac myocytes before and after TPA stimulation or stretch for 10 min were subjected to kinase assays in MBP-containing gels. After 100 nM TPA stimulation for 10 min, I observed an increase in the MBP kinase activity which migrated at 42 and 44 kDa in αY91-immunoprecipitates. After stretch for 10 min, I observed an increase in MBP kinase activity which migrated at 42 kDa in αY91-immunoprecipitates (Fig. 4). These data confirmed that myocyte-stretch indeed increased the kinase activity of 42 kDa-MAP kinase. I was not able to detect stretch-induced increase in the kinase activity of 44 kDa-MAP kinase, because the amount of 44 kDa-MAP kinase in cardiac myocytes may have been too low to detect the kinase activity of 44 kDa-MAP kinase in my assay system. In fact, Western blotting with αY91 revealed that the amount of 44 kDa-MAP kinase was low as compared with that of 42 kDa-MAP kinase (Fig. 1).

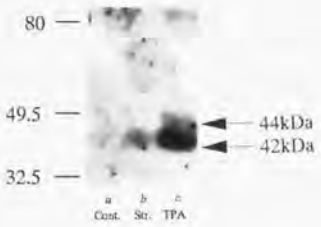


Fig. 4. Forty-two kDa-MAP kinase is principally activated by both TPA and Stretch.

Cardiac myocytes were treated with 100 nM TPA (lane *b*), or stretched by 10 % for 10 min (lane *c*). The supernatants of the lysates were immunoprecipitated with anti-MAP kinase antibody (αY91) in the presence of 0.15 % SDS. The immunoprecipitates were electrophoresed on SDS-polyacrylamide gels containing MBP. The gel was incubated with [γ-^{32}P]ATP and subjected to autoradiography.

I examined whether the activated MAP kinase was phosphorylated. When [^{32}P]orthophosphate-labeled stretched myocytes were solubilized and immunoprecipitated with anti-MAP kinase antibody (αY91), I observed a 42 kDa-phosphoprotein. After stretch for 10 min, phosphorylation of this 42 kDa-protein was further observed (Fig. 5). These data indicate that MAP kinase which has a molecular weight of 42 kDa is phosphorylated in cardiac myocytes, and this phosphorylation is increased by stretch. To identify the phosphoamino acids of 42 kDa-MAP kinase, I further analyzed the ^{32}P-phosphoamino acid content of phosphorylated MAP kinase. In the basal state, the protein(s) at Mr 42 kDa contained mainly phosphoserine. Myocyte-stretch induced *de novo* threonine and tyrosine phosphorylation of 42 kDa-MAP kinase (Fig. 6). It has been previously shown that MAP kinase was activated when both threonine and tyrosine were phosphorylated [14, 35, 36], which support the present results.

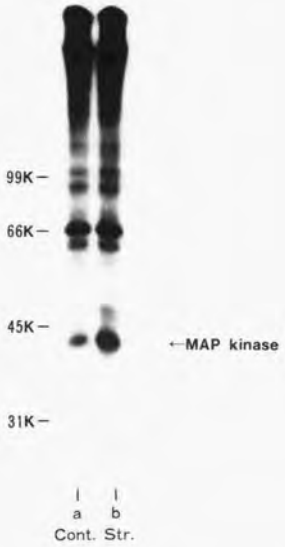


Fig. 5. Cardiac MAP kinase is phosphorylated by stretch.

[^{32}P]orthophosphate-labeled cardiac myocytes were treated without (Cont.) or with 10 % stretch for 10 min (Str.) at 37 °C, lysed and immunoprecipitated with anti-MAP kinase antibody (αY91). The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography.

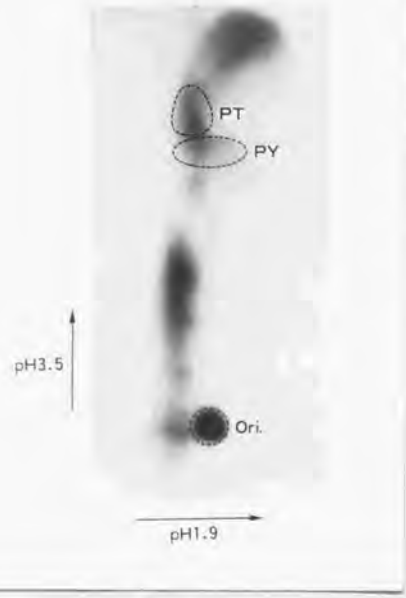


Fig. 6. Threonine and tyrosine residues of MAP kinase are phosphorylated *de novo* by stretch.

After SDS-PAGE, the bands at Mr 42 kDa were excised from the gel, rehydrated and digested with TPCK/trypsin. The digests were subjected to partial acid hydrolysis, followed by 2-D thin-layer electrophoresis. The positions of the phosphorylated amino acid standards were visualized by ninhydrin staining. (PT, phosphothreonine; PY, phosphotyrosine; Ori, origin.)

Stretch-induced Increase in MAP Kinase Activity is Partially Involved in both Voltage-Dependent and -Independent Transsarcolemmal Influx of Ca^{2+}.

I investigated whether stretch-induced MAP kinase activation is involved in transsarcolemmal influx of Ca^{2+} because it has been known that stretch of cardiac myocytes increases the resting intracellular Ca^{2+} concentration [37]. First, to eliminate the influence of transsarcolemmal influx of Ca^{2+}, cardiac myocytes were preincubated with KRP buffer containing only 50 μM Ca^{2+} for 30 min

(Fig. 7A, *e, f*). In this buffer myocyte-stretch activated MAP kinase activity by approximately 1.3-fold although in DMEM or KRP buffer containing 1.3 mM Ca^{2+} myocyte-stretch increased MAP kinase activity by approximately 1.7-fold (*a, b, c, d*). The reduction of the MAP kinase activation was significant (P=0.022 *d* versus *f*). Next, I examined the relationship between stretch-induced MAP 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, d*) or 1 mM $CoCl_2$ (*e, f*), the activities of MAP kinase were decreased by 33 % (*d*) or 29 % (*f*) compared with the activity in cardiac myocytes without pretreatment (*b*), respectively (P=0.025 *b* versus *d*, P=0.004 *b* versus *f*). Thus, I concluded that stretch-induced MAP kinase activation was partially dependent on transsarcolemmal Ca^{2+} influx through both voltage-dependent and -independent Ca^{2+} channels.

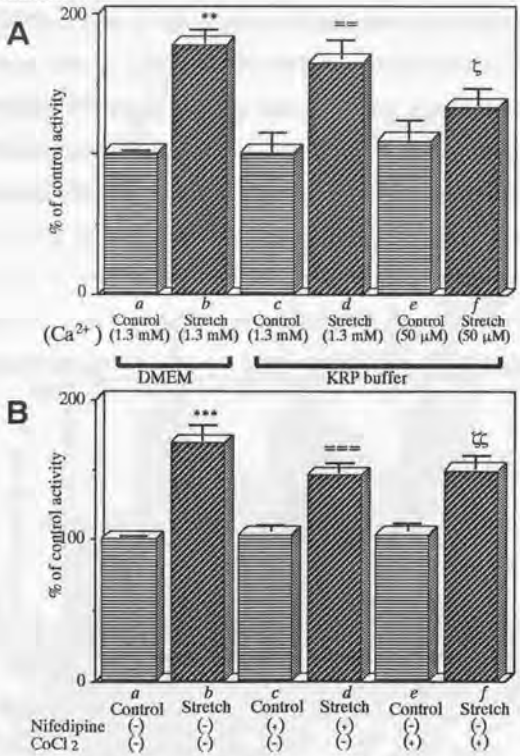


Fig. 7. Stretch-induced MAP kinase activation is partially dependent on transsarcolemmal influx of Ca^{2+}.

A, cardiac myocytes were preincubated with DMEM containing 1.3 mM Ca^{2+} (*a, b*), KRP buffer containing 1.3 mM Ca^{2+} (*c, d*) or KRP buffer containing 50 μM Ca^{2+} (*e, f*) for 30 min. MBP kinase assays on P81 paper were performed. Each histogram represents the average percentage of control (*a*) from eight independent experiments (mean ± S.E.) (**P<0.01 versus control (*a*), ==P<0.01 versus control (*c*), ξP<0.05 versus control (*e*)). B, myocytes were preincubated with DMEM added with 10 nM nifedipine for 60 min (*c, d*) or 1 mM $CoCl_2$ for 10 min (*e, f*). They were stretched by 10 % for 10 min (*b, d, f*). MBP kinase assays on P81 paper were performed. Each histogram represents the average percentage of control (*a*) from at least ten independent experiments (mean ± S.E.) (***P<0.001 versus control (*a*), ===P<0.001 versus control (*c*), ξξ P<0.01 versus control (*e*)).

Neither Tyrphostin nor Genistein Inhibits MAP Kinase Activation in Response to Stretch of Cardiac Myocytes.

It has been known that a variety of stimuli such as platelet derived growth factor, EGF or insulin *etc.* activate MAP kinase through activation of the receptor tyrosine kinases. To clarify whether myocyte-stretch activates MAP kinase indirectly through another stimuli, I preincubated cardiac myocytes with the receptor tyrosine kinase inhibitors and stretched them by 10 % for 10 min. After the preincubation with tyrphostin or genistein, myocyte-stretch activated MAP kinase activity by approximately 1.7-fold (Fig. 8), and stretch-induced MAP kinase activation remained unchanged (*b, d, f*) (P=0.751 *b* versus *d*, P=0.924 *b* versus *f*). After the prior treatment with tyrphostin or genistein, stimulation with EGF was not able to activate MAP kinase activity (data not shown). Therefore, both tyrphostin and genistein failed to inhibit stretch-induced MAP kinase activation.

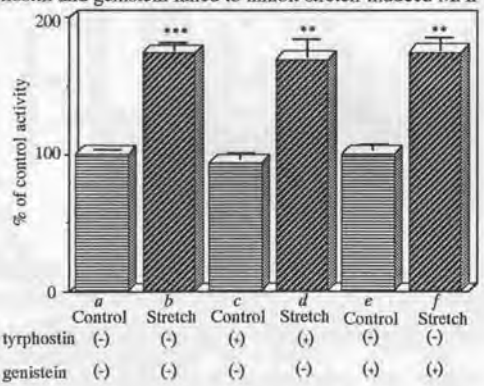


Fig. 8. Stretch-induced MAP kinase activation is independent of activation of the receptor tyrosine kinases.

Cardiac myocytes were pretreated with 50 μM tyrphostin (*c, d*) or 20 μM genistein (*e, f*) for 30 min. They were stretched by 10 % for 10 min (*b, d, 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*)).

Stretch-induced Increase in MAP Kinase Activity is via PKC-Dependent and Independent Pathways.

I examined whether MAP kinase activation by stretch of cardiac myocytes is via PKC-dependent or -independent pathway. As shown in Fig. 9, both TPA stimulation and myocyte-stretch activated MAP kinase activity in cardiac myocytes which were not pretreated with TPA or staurosporine (*a, b, c*). However, when myocytes were pretreated with 100 nM TPA for 24 h (*d, e, f*), stretch-induced MAP kinase activity was decreased by approximately 60 % as compared with the kinase activity in myocytes without pretreatment (*c, f*). TPA was not able to activate MAP kinase activity (*e*), suggesting that PKC was indeed depleted. Pretreatment with staurosporine, a potent and specific PKC inhibitor, also inhibited stretch-induced MAP kinase activation by approximately 70 % (*c, i*). These data demonstrated that stretch-induced MAP kinase activation was via partially dependent on the activation of PKC.

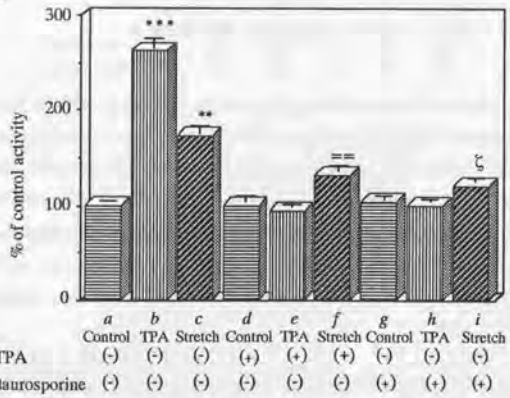


Fig. 9. Stretch-induced MAP kinase activation is partially dependent on the activation of PKC.

Cardiac myocytes were pretreated with 100 nM TPA for 24 h (*d, e, f*) or 2 nM staurosporine for 30 min (*g, h, i*). They were either stretched by 10 % for 10 min (*c, f, i*) or stimulated by 100 nM TPA for 10 min (*b, e, h*). MBP kinase assays on P81 paper were performed. Each histogram represents the average percentage of control (*a*) from five independent experiments (mean ± S.E.) (**P<0.01, ***P<0.001 versus control (*a*), ==P<0.01 versus control (*d*), ζ P<0.05 versus control (*g*)).

Myocyte-Stretch also Stimulates S6 Peptide Kinase Activity.

I measured S6 peptide kinase activity in the immunoprecipitates with control serum (Fig. 10, *a, b, c*) or anti-S6 kinase antibody (*d, e, 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, b, c*). However, both 100 nM TPA stimulation for 10 min and 10 % stretch for 10 min increased S6 peptide kinase activity by approximately 4- and 3-fold, respectively (*d, e, f*).

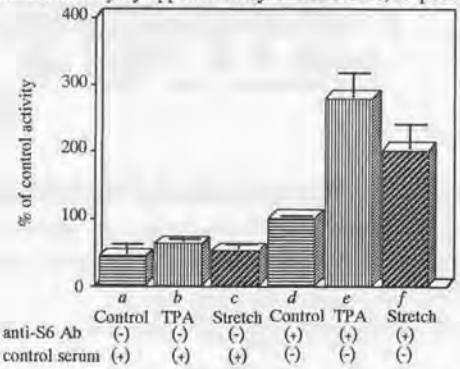


Fig. 10. Stretch of cardiac myocytes activates S6 peptide kinase activity.

Cardiac myocytes were subjected to 10 % stretch for 10 min (*c, f*) or 100 nM TPA stimulation for 10 min (*b, e*). They were lysed and centrifuged, and the supernatants were immunoprecipitated with anti-S6 kinase antibody (*d, e, f*) or control preimmune rabbit serum (*a, b, c*). The immunoprecipitates were incubated in kinase buffer and substrates (50 μg of S6 peptide). After stopping the reaction, aliquots of the supernatants were spotted on P81 paper (Whatman), washed, dried and counted by Cerenkov. Each histogram represents the average percentage of control (*d*) from three independent experiments (mean ± S.E.). The mean values were 45 (*a*), 62 (*b*), 51 (*c*), 100 (*d*), 278 (*e*) and 201 % (*f*).

Both Raf-1 Kinase and MAP kinase kinase are Activated by Myocyte-Stretch.

I examined whether stretch stimulates Raf-1 kinase activity in cardiac myocytes. Since the activation of Raf-1 kinase is correlated with an apparent increase in molecular weight as assayed by a

decrease in mobility during SDS-PAGE [38], immunoblot analysis with anti-Raf-1 kinase antibody was performed. Raf-1 kinase molecules migrated more slowly in 10 %-stretched cells for 5 to 30 min (Fig. 11, *d, e, f*) than the Raf-1 kinase molecules in the unstimulated cells (*a*). The mobility of Raf-1 kinase returned nearly to the control level at 60 min after stretch (*g*).

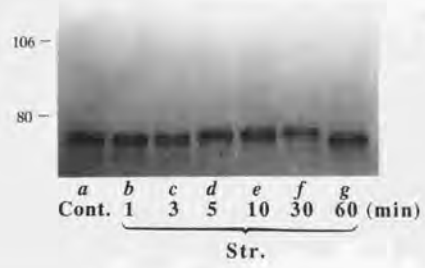


Fig. 11. Myocyte-stretch induces Raf-1 kinase activity.

Cardiac myocytes were stretched by 10 % for the indicated periods of time. Cell lysates were prepared and equalized for protein content before electrophoretic separation on 7% SDS-PAGE and transferred to nitrocellulose filters. Protein blots were probed with anti-Raf-1 kinase antibody and immunological reactions were identified by using an alkaline phosphatase detection system. Molecular mass markers (in kDa) are shown to the left.

Next, I studied whether the activation of MAP kinase kinase can be induced by stretch of cardiac myocytes. As shown in Fig. 12, myocyte-stretch for 10 min induced MAP kinase kinase activity as reflected by a shift in electrophoretic mobility.

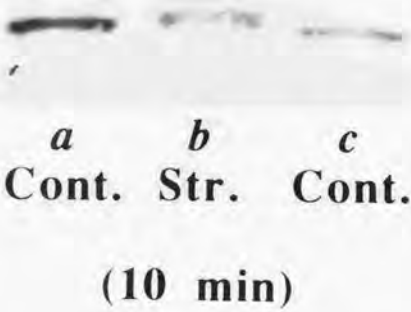


Fig. 12. Myocyte-stretch also activates MAP kinase kinase activity.

Cardiac myocytes were stretched by 10 % for 10 min. Cell lysates were electrophoresed on 12 % polyacrylamide gel, blotted onto nitrocellulose filter and incubated with anti-MAP kinase kinase antibody. Detection of the immune complexes is described in the legend of Fig. 11. Molecular mass markers (in kDa) are shown to the left.

Finally, I examined the time course of the activity of MAP kinase kinase after myocyte-stretch. I observed a slight increase in MAP kinase kinase activity at 1 min after stretch. MAP kinase kinase activity maximally increased 5 min after stretch and gradually decreased thereafter (Fig. 13). The kinase activity returned nearly to the control level at 30 min after stretch (data not shown).

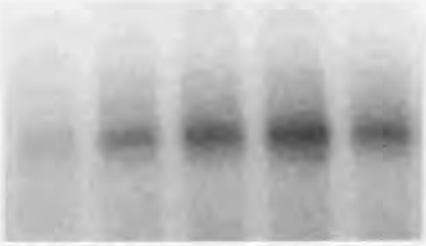


Fig. 13. Time course of the kinase activity of MAP kinase kinase

MAP kinase kinase activity was assayed using rMAPK. To chromatographically separate MAP kinase kinase from endogenous MAP kinase, cell lysates were applied to Q-sepharose column and flow through fractions were immunopreciptated with anti-MAP kinase kinase antibody. The immunoprecipitates were incubated with buffer B for 30 min at 25 ^{o}C. rMAPK was collected using amylose resin and was electrophoresed on 10 % polyacrylamide gel.

Discussion

The major findings of the present study are that stretch of cardiac myocytes activates MAP kinase which is the principal mediator of specific gene expression, and that myocyte-stretch activates S6 kinase which should lead to an increase in the efficiency of protein synthesis in ribosome. Furthermore, I have demonstrated that Raf-1 kinase and MAP kinase kinase are sequentially activated by stretch of cardiac myocytes.

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 [1, 2]. 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 [2, 39, 40]. Therefore, management of cardiovascular diseases should also be directed to preventing as well as reducing 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. Especially, cardiac myocytes have developed unique subcellular mechanisms to induce hypertrophy in response to mechanical loading. In this study, I tried to elucidate the intracellular signal transduction system of cardiac myocytes elicited by mechanical stress.

Stretch of cardiac myocytes in vitro induces protein synthesis and 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 [41-44]. However, molecular mechanisms of cardiac hypertrophy induced by mechanical stimuli have not been well characterized due to technical difficulties in directly imposing mechanical loading on cardiac myocytes. It remains unknown whether mechanical stress directly regulates gene expression and protein synthesis without participation of humoral or neural factors. To overcome this, original deformable culture dishes made of silicone rubber which can provide the various degree of stretch on the cell have been developed. By using this culture system, it has been already clarified that myocyte-stretch directly induces

specific gene expression and increases protein synthesis. Namely, it has been already reported that the gene expression of skeletal actin, β-myosin heavy chain and *c-fos* was increased, all of which are known to be expressed in hypertrophied hearts. Furthermore, ^{14}C-phenylalanine incorporation into proteins was increased by 25 % even 2 h after stretch. These results indicate that myocyte-stretch *in vitro* can be a good model for studying the intracellular events occurring during the process of stretch-induced cardiac hypertrophy.

MAP kinase is activated by stretch of cardiac myocytes.

It has been already clarified that mechanical loading *in vitro* stimulates PKC 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 [3, 4]. However, it remains unknown what kind of signal transduction systems exist downstream PKC and which enzymes are more specifically involved in this process.

First, I showed that kinase activity toward MBP, the specific substrate of MAP kinase is increased by stretch as well as by TPA (Fig. 2). Second, using in-gel kinase assay, I demonstrated that 42 kDa-protein in cardiac myocytes is responsible for the increased MBP kinase activity following stretch (Fig. 3). Third, I identified 42 kDa-protein kinase which phosphorylates MBP is one of MAP kinases by using a specific antibody (Fig. 4). All of these results clearly indicate that 42 kDa-MAP kinase is mainly activated by stretch of cardiac myocytes. Consistent with these results, Sadoshima *et al.* also showed in the recent report that MAP kinase was activated after stretch for 10 min [45]. I was not able to detect 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 (one of anti-MAP kinase antibody) revealed that the amount of 44 kDa-MAP kinase was lower as compared with that of 42 kDa-MAP kinase (Fig. 1). Recently, cDNA of MAP kinase kinase was cloned [46] and the purified MAP kinase kinase was shown to specifically phosphorylate the regulatory tyrosine and threonine residues of MAP kinase causing its full activation [17, 47]. 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 [48], because the restricted substrate specificity is found for MAP kinase kinase [47, 49]. Therefore, I believe that myocyte-stretch increases both 42 kDa and 44 kDa-

MAP kinase activities, and that the reason why I failed to detect the activation of 44 kDa-MAP kinase is the limitation of my detection system.

MAP kinase activation is partially dependent on both voltage-dependent and -independent Ca^{2+} influx.

It has been shown that stretch of cardiac myocytes increases intracellular Ca^{2+} concentration in a few seconds [37]. Furthermore, Sigurdson *et al.* indicated that mechanical stimuli on a model system of tissue-cultured heart cells produced a transsarcolemmal influx of Ca^{2+} which led to waves of calcium-induced calcium release and that the response was blocked by removing extracellular Ca^{2+} [50]. Therefore, I examined the relationship between stretch-induced MAP kinase activation and transsarcolemmal influx of Ca^{2+}. I showed that MAP kinase activation by stretch of cardiac myocytes is decreased by 30 % after blocking either voltage-dependent or -independent Ca^{2+} channels and decreased by 50 % by blocking both channels. Stimulation with 10^{-6} M Bay K 8644, voltage-dependent Ca^{2+} 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 partially dependent on Ca^{2+} signaling pathway. Consistent with these results, Tsuda *et al.* reported that angiotensin II-induced MAP kinase activation in vascular smooth muscle cells was inhibited by 25 % by pretreatment with EGTA [51]. Moreover, since MAP kinase activity is sensitive to Ca^{2+} in crude cell extracts, but insensitive in the partially purified fraction [20], intracellular Ca^{2+} is thought to indirectly (through other factors) activate MAP kinase.

Activation of MAP kinase occurs via both PKC-dependent and -independent pathways and via the receptor tyrosine kinase-independent pathways.

Recently, it has been reported that MAP kinase activation by EGF is PKC-independent, but that nerve growth factor-induced activation of MAP kinase is partially dependent on PKC activation [52]. In the present study, stretch-induced MAP kinase activation in cardiac myocytes pretreated with TPA was decreased by approximately 60 % as compared with the kinase activity in myocytes without pretreatment. Furthermore, prior treatment with staurosporine also inhibited stretch-induced MAP kinase activation by approximately 70 % (Fig. 9). In this study, I have demonstrated that MAP

kinase is activated by myocyte-stretch in both PKC-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 stretch-induced activation of MAP kinase (Fig. 8). These results indicate that MAP kinase activation by stretch of cardiac myocytes can occur via the receptor tyrosine kinase-independent pathway.

S6 kinase is also activated by stretch in cardiac myocytes.

Recent reports have revealed that MAP kinase may play some early intermediate regulatory role in a protein kinase cascade which regulates protein synthesis via activation of S6 kinase in 40 S ribosome [5-7, 53, 54]. A sequential activation of MAP kinases and S6 kinase II in the liver injected with insulin has been recently reported [15]. The present study has demonstrated that stretch activates S6 kinase in cardiac myocytes (Fig. 10), which in turn increases S6 phosphorylation, possibly resulting in an increased efficiency of protein synthesis in 40 S ribosome.

Myocyte-stretch activates Raf-1 kinase and MAP kinase kinase in sequence.

I have clarified in this study that myocyte-stretch activates both Raf-1 kinase and MAP kinase kinase activity in cardiac myocytes (Fig. 11, 12 and 13). I think that Raf-1 kinase and MAP kinase kinase are sequentially activated by myocyte-stretch, because MAP kinase kinase is a kinase activated by threonine phosphorylation [17] and Raf-1 kinase has a serine/threonine-specific protein kinase activity [55-58]. Consistent with this idea, it was recently reported that Raf-1 kinase can activate MAP kinase kinase [32, 33].

I have also indicated that Raf-1 kinase can be activated by 100 nM TPA stimulation for 5 min (data not shown). It has been known that serine/threonine phosphorylation is responsible for the activation of Raf-1 kinase [18] and that PKC is a serine/threonine-specific protein kinase. Further, it has been previously shown that myocyte-stretch induces PKC activation [3, 4]. Therefore, I assume that PKC activated by myocyte-stretch induces Raf-1 kinase activity. However, it remains to be answered whether Raf-1 kinase can also be activated via a PKC-independent pathway.

Activated serine/threonine protein kinase cascade induces cardiac hypertrophy.

In this study, I have demonstrated that stretch-induced Raf-1 kinase activation is possibly induced by activated PKC and that myocyte-stretch induces sequential activation of MAP kinase and S6 kinase. Moreover, it has been clarified that MAP kinase is substrate-specifically activated by MAP kinase kinase [32, 33]. Thus, I propose the presence of stretch-induced protein phosphorylation cascade in cardiac myocytes. PKC activation by stretch phosphorylates and activates Raf-1 kinase, which phosphorylates and activates MAP kinase kinase, which in turn phosphorylates and activates MAP kinase, which finally phosphorylates and activates S6 kinase. Kyriakis *et al.* also assume that insulin or other growth factors sequentially activate in the order of Raf-1 kinase, MAP kinase kinase, MAP kinase and S6 kinase. However, Lee *et al.* insist that MAP kinase is the activator of Raf-1 kinase kinases. Therefore, more detailed analyses are necessary to clarify what kind of phosphorylation cascade is involved and in what order these kinases are activated.

Significance of phosphorylation cascade in the specific gene expression in cardiac hypertrophy.

It has been previously shown that myocyte-stretch induces *c-fos* expression in a PKC dependent manner [3, 4], and in this study, I have demonstrated that stretch activates MAP kinase. Interestingly, it has been recently shown that MAP kinase is able to phosphorylate and activate c-Jun [11, 12]. 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 PKC and possible phosphorylation and activation of c-Jun by MAP kinase may synergistically activate the function of AP-1 complex in cardiac myocytes. Furthermore, I have indicated that myocyte-stretch induces Raf-1 kinase activation. It has recently been shown that the activated form of Raf-1 kinase translocates to the perinuclear area and the nucleus and trans-activates AP-1-binding site [13], suggesting Raf-1 kinase can activate the transcription from AP-1 binding site. In this regard, evidence showing that AP-2 is involved in the altered expression of the human ventricular myosin alkali light chain gene has been previously presented [59]. Likewise, altered function of AP-1 induced by PKC, MAP kinase and Raf-1 kinase may also be involved in the altered expression of a panel of other genes which can lead to an increase in specific gene expression.

Significance of S6 kinase activation in an increase in the acceleration of protein synthesis in cardiac hypertrophy

It has been reported 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 [60], which suggests that pressure overload in the heart has a multitude of effects on protein synthesis at different levels. It has been shown in the present study that S6 kinase activity in cardiac myocytes was increased by approximately 3-fold after stretch for 10 min. Since S6 kinase is known to phosphorylate and activate ribosomal S6 protein, leading to an increase in protein synthesis in ribosome. Therefore, phosphorylation of S6 kinase as observed in this study can be the molecular mechanism of the higher efficiency of protein synthesis in cardiac hypertrophy.

Mechanical stress-induced intracellular signaling proposed by the data presented in this manuscript is illustrated in Fig. 14.

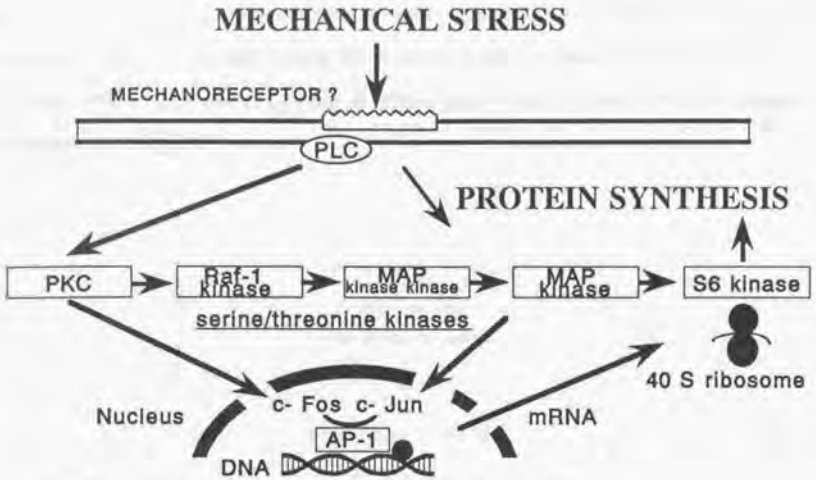


Fig. 14. Hypothetical molecular mechanisms of cardiac hypertrophy: involvement of protein kinase cascade

Mechanical stress increases not only PKC activity via phospholipase C activation (3, 4) but also MAP kinase activity, which is partially dependent on PKC. Mechanical stress also activates Raf-1 kinase and MAP kinase kinase activity. Activated forms of PKC, Raf-1 kinase and MAP kinase activate the promoters, which end up in increasing the efficiency of specific gene expression (transcriptional activation). Furthermore, activated PKC induces Raf-1 kinase activation, which induces the phosphorylation and activation of MAP kinase kinase, which induces the phosphorylation and activation of MAP kinase, which in turn activates S6 kinase in 40 S ribosome. The phosphorylation of S6 protein induced by S6 kinase finally increases the efficiency of protein synthesis (translational activation). (PLC, phospholipase C)

Cardiac hypertrophy has been regarded as a secondary response of the heart to a sustained increase in overload [61]. However, it is known that excessive hypertrophy predisposes to the cardiac failure and leads to an increased mortality rate [1, 2]. Therefore, the development of prevention and treatment of cardiac hypertrophy is an important theme for the cardiologist, which requires elucidation of the mechanism(s) 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 [62-65], whereas vasodilator hydralazine does not reduce ventricular hypertrophy or even accelerates it even though blood pressure is controlled to normotensive levels [66, 67]. 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 MAP kinase [51]. To elucidate the intracellular signaling of cardiac hypertrophy may lead to the development of new cardioprotective agents.

References

1. Devereux, R. B. (1987) *Med. Clin. North. Am.* **71,** 813-826

2. Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., and Castelli, W. P. (1990) *N. Engl. J. Med.* **322,** 1561-1566

3. Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991) *J. Biol. Chem.* **266,** 1265-1268

4. Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. (1990) *J. Biol. Chem.* **265,** 3595-3598

5. Sturgill, T. W. (1991) *Biochem. Biophys. Acta.* **1092,** 350-357

6. Sturgill, T. W., Ray, L. B., Erickson, E., and Maller, J. L. (1988) *Nature* **334,** 715-718

7. Ahn, N. G., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 11495-11501

8. Kovacina, K. S., Yonezawa, K., Brautigan, D. L., Tonks, N. K., Rapp, U. R., and Roth, R. A. (1990) *J. Biol. Chem.* **265,** 12115-12118

9. Blachshear, P. J., Haupt, D. M., App, H., and Rapp, U. R. (1990) *J. Biol. Chem.* **265,** 12131-12134

10. Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M., and Williams, L. T. (1989) *Cell* **58,** 649-657

11. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) *Nature* **353,** 670-674

12. Alvarez, E., Northwood, I. C., Gonzalez, F. A., Latour, D. A., Seth, A., Abate, C., Curran, T., and Davis, R. J. (1991) *J. Biol. Chem.* **266,** 15277-15285

13. Bruder, J. T., Heidecker, G., and Rapp, U. R. (1992) *Genes and Development* **6,** 545-556

14. Tobe, K., Kadowaki, T., Tamemoto, H., Ueki, K., Hara, K., Koshio, O., Momomura, K., Gotoh, Y., Nishida, E., Akanuma, Y., Yazaki, Y., and Kasuga, M. (1991) *J. Biol. Chem.* **266,** 24793-24803

15. Tobe, K., Kadowaki, T., Hara, K., Gotoh, Y., Kosako, H., Matsuda, S., Tamemoto, H., Ueki, K., Akanuma, Y., Nishida, E., and Yazaki, Y. (1992) *J. Biol. Chem.* **267,** 21089-21097

16. Izumi, T., Saeki, Y., Akanuma, Y., Takaku, F., and Kasuga, M. (1988) *J. Biol. Chem.* **263,** 10386-10393

17. Kosako, H., Gotoh, Y., Matsuda, S., Ishikawa, M., and Nishida, E. (1992) *EMBO J.* **11,** 2903-2908

18. Izumi, T., Tamemoto, H., Nagao, M., Kadowaki, T., Takaku, F., and Kasuga, M. (1991) *J. Biol. Chem.* **266,** 7933-7939

19. Simpson, P., and Savion, S. (1982) *Circ. Res.* **50,** 101-116

20. Gotoh, Y., Nishida, E., and Sakai, H. (1990) *Eur. J. Biochem.* **193,** 671-674

21. Gotoh, Y., Nishida, E., Matsuda, S., Shiina, N., Kasako, H., Shiokawa, K., Akiyama, T., Ohta, K., and Sakai, H. (1991) *Nature* **349,** 251-254

22. Kameshita, I., and Fujisawa, H. (1989) *Anal. Biochem.* **183,** 139-143

23. Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, K., Takaku, F., Sakai, H., Yahara, I., and Kasuga, M. (1987) *J. Biol. Chem.* **262,** 7342-7350

24. Yamamoto-Honda, R., Koshino, O., Tobe, K., Shibazaki, Y., Momomura, K., Odawara, M., Kadowaki, T., Takaku, F., Akanuma, Y., and Kasuga, M. (1990) *J. Biol. Chem.* **265,** 14777-14783

25. Koshio, O., Akanuma, Y., and Kasuga, M. (1989) *FEBS Lett.* **254,** 22-24

26. Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) *Methods. Enzymol.* **99,** 387-402

27. Yaish, P., Gazit, A., Gilon, C., and Levitzki, A. (1988) *Science* **242,** 933-935

28. Gazit, A., Yaish, P., Gilon, C., and Levitzki, A. (1989) *J. Med. Chem.* **32,** 2344-2352

29. Lyall, R. M., Zilberstein, A., Gazit, A., Gilon, C., Levitzki, A., and Schlessinger, J. (1989) *J. Biol. Chem.* **264,** 14503-14509

30. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987) *J. Biol. Chem.* **262,** 5592-5595

31. Enright, W. J., and Booth, P. (1991) *Focus* **13,** 23-27

32. Dent, P., Haser, W., Haystead, T. A. J., Vincent, L. A., Roberts, T. M., and Sturgill, T. W. (1992) *Science* **257,** 1404-1406

33. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992) *Nature* **358,** 417-421

34. Wallenstein, S., Zucker, C. L., and Fless, J. L. (1980) *Circ. Res.* **47,** 1-9

35. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) *Nature* **343,** 651-653

36. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *EMBO J.* **10,** 885-892

37. Guennec, J.-Y. L., White, E., Gannier, F., Argibay, J. A., and Garnier, D. (1991) *Quat. J. Exp. Physiol.* **76,** 975-978

38. Morrison, D. K., Kaplan, D. R., Rapp, U., and Roberts, T. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8855-8895

39. Anderson, K. M., Odell, P. M., Wilson, P. W. F., and Kannel, W. B. (1990) *Am. Heart J.* **121,** 293-298

40. Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., and Castelli, W. P. (1989) *Ann. Int. Med.* **110,** 101-107

41. Izumo, S., Lompre, A.-M., Matsuoka, R., Koren, G., Schwartz, K., Nadal-Ginard, B., and Mahdavi, V. (1987) *J. Clin. Invest.* **79,** 971-978

42. Izumo, S., Nadal-Ginard, B., and Mahdavi, V. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 339-343

43. Komuro, I., Kurabayashi, M., Takaku, F., and Yazaki, Y. (1988) *Circ. Res.* **62,** 1075-1079

44. Schwartz, K., Bastie, D. d. l., bouveret, P., Oliveiro, P., Alonso, S., and Budkingham, M. (1986) *Circ. Res.* **59,** 551-555

45. Sadoshima, J., and Izumo, S. (1993) *EMBO J.* **12,** 1681-1692

46. Seger, R., Seger, D., Lozeman, F. J., Ahn, N. G., Graves, L. M., Campbell, J. S., Ericsson, L., Harrylock, M., Jensen, A. M., and Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 25628-25631

47. Seger, R., N. G, A., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H., and Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 14373-14381

48. Ahn, N. G. (1992) *J. Neurochem.* **59(1),** 147-57

49. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K., and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 4220-4227

50. Sigurdson, W., Ruknudin, A., and Sachs, F. (1992) *Am. J. Physiol.* **262,** H1110-1115

51. Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K., and Yokoyama, M. (1992) *Circ. Res.* **71,** 620-630

52. Gotoh, Y., Nishida, E., Hoshi, M., Kawakami, M., and Sakai, H. (1990) *Eur. J. Biochem.* **193,** 661-669

53. Gregory, J. S., Boulton, T. G., Sang, B. C., and Cobb, M. H. (1989) *J. Biol. Chem.* **266,** 22003-22009

54. Maller, J. L. (1990) *Biochemistry* **29,** 3157-3166

55. Bonner, T. I., Kerby, S. B., Sutrave, R., Gunnell, M. A., Mark, G., and Rapp, U. R. (1985) *Mol. Cell. Biol.* **5,** 1400-1407

56. Bonner, T. I., Oppermann, H., Seeburg, P., Kerby, S. B., Gunnell, M. A., Young, A. C., and Rapp, U. R. (1986) *Nucleic Acids Res.* **14,** 1009-1015

57. Cleveland, J. L., Jansen, H. W., Bister, K., Frederickson, T. N., Morse, H. C., Ihie, J. N., Rapp, U. R., and Sander, T. (1986) *J. Cell. Biochem.* **30,** 195-218

58. Moelling, K., Heimann, B., Beimling, P., Rapp, U. R., and Sander, T. (1984) *Nature* **312,** 558-561

59. Kurabayashi, M., Komuro, I., Shibasaki, Y., Tsuchimochi, H., Takaku, F., and Yazaki, Y. (1990) *J. Biol. Chem.* **265,** 19271-19278

60. Nagai, R., Low, R. B., Stirewalt, W. S., Alpert, N. R., and Litten, R. Z. (1988) *Am. J. Physiol.* **255,** H325-H328

61. Tanaka, H., Obata, H., and Haneda, T. (1991) *Jpn. Circ. J.* **55,** 1233-1245

62. Cody, R. J., Tarazi, R. C., Bravo, E. L., and Fouad, F. M. (1978) *Clin. Sci.* **55,** 453-459

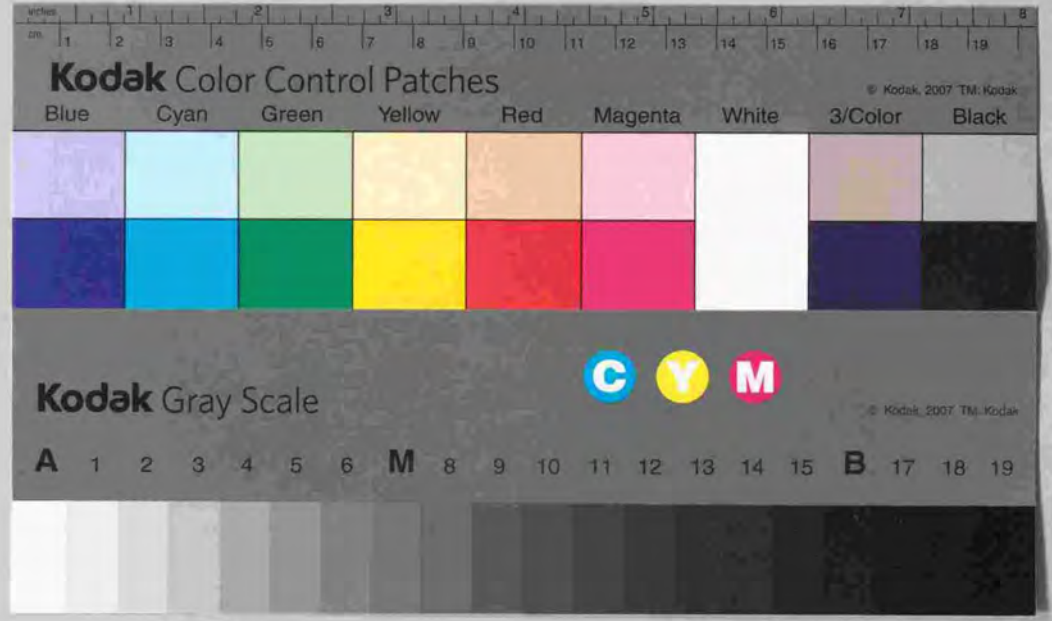
63. Fagard, R., Amery, A., Reybrouck, T., Lijnen, P., and Billiet, L. (1980) *Am. J. Cardiol.* **46,** 295-300

64. Gradi, A. M., Venco, A., Barzizza, F., Casadei, B., Marchesi, E., and Finardi, G. (1989) *Am. J. Cardiol.* **63,** 1093-1097

65. Nakashima, Y., Fouad, F. M., and Tarazi, R. C. (1984) *Am. J. Cardiol.* **53,** 1044-1049

66. Lavie, C. J., Ventura, H. O., and Messerli, F. H. (1991) *Drugs* **42,** 945-961

67. Leenen, F. H., Smith, D. L., Farkas, R. M., Reeves, R. A., and Marquez-Julio, A. (1987) *Am. J. Med.* **82,** 969-978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